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A MODEL SYSTEM FOR THE STUDY OF HETEROEXCHANGE DIFFUSION: METHOTREXATE–FOLATE INTERACTIONS IN L1210 LEUKEMIA AND EHRLICH ASCITES TUMOR CELLS

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SUMMARY

A unique interaction between the folate analog, methotrexate (4-amino-4-deoxy-10-methylpteroylglutamic acid), and the naturally occurring folates in L1210 leukemia and Ehrlich ascites tumor cells provides a useful model for the study of heteroexchange diffusion. The presence of intracellular binding sites with a high affinity for methotrexate but a low affinity for folic acid and its tetrahydrofolate derivatives permit the measurement of true unidirectional influx rates for methotrexate and assure that the trans-stimulation of methotrexate uptake by the intracellular presence of the other folates is due solely to a primary augmentation of this carrier influx mechanism. Further, since free methotrexate does not appear prior to saturation of the binding sites, the reaction between the folates and carrier at the inner cell membrane is undisturbed by methotrexate released from carrier as the complex enters the cell during heteroexchange, facilitating quantitation of the kinetic alterations which occur for methotrexate influx during trans-stimulation.

Trans-stimulation of methotrexate influx was observed in cells preloaded with 5-formyl-, or 5-methyl-tetrahydrofolate, or folic acid. The latter was observed even when intracellular reduction of folic acid to its tetrahydrofolate derivatives was inhibited. However, initial uptake rates for methotrexate were unaltered in cells preloaded with nonlabeled methotrexate.

Methotrexate influx conformed to Michaelis–Menten kinetics in L1210 cells preloaded to a single intracellular 5-formyltetrahydrofolate level and when compared to control cells, manifested an increase in the influx v_{max} without a significant change in the influx K_t.

The initial uptake rate for 5-methyltetrahydrofolate was increased in cells preloaded with either 5-formyltetrahydrofolate or folic acid.

These studies further support a carrier transport mechanism for methotrexate and the naturally occurring folates in L1210 leukemia cells and extend these findings to the Ehrlich ascites tumor cell as well.

Abbreviations: Methotrexate, 4-amino-4-deoxy-10-methylpteroylglutamic acid; folic acid, pteroyl glutamic acid.

INTRODUCTION

Previous studies from this laboratory characterized a carrier-mediated transport mechanism for the folate-analog, methotrexate (4-amino-4-deoxy-10-methylpteroylglutamic acid), in L1210 leukemia cells which is shared with the naturally occurring folates, folic acid, and 5-formyl-H_4folate[1]. Studies are now presented which (1) utilize the unique interaction between the folate compounds in these cells to quantitate unidirectional influx alterations for methotrexate which occur in heteroexchange diffusion, and (2) further demonstrate heteroexchange phenomena between methotrexate and the naturally occurring folates in both L1210 leukemia and Ehrlich ascites tumor cells to further verify that these compounds share at least in part the same transport carrier.

The properties of this system which facilitate a quantitative evaluation of unidirectional influx alterations for methotrexate during heteroexchange diffusion are as follows: (1) These cells contain dihydrofolate reductase, an enzyme which has an affinity for methotrexate which is several orders of magnitude greater than the K_t^{ef}* for methotrexate[1,2]. Thus, during uptake of methotrexate and prior to saturation of dihydrofolate reductase, essentially all methotrexate which enters the cell is tightly bound, free methotrexate is not present, and carrier from which methotrexate has dissociated at the inner cell membrane undergoes reorientation to the outer boundary of the cell membrane in the unloaded condition. Hence net methotrexate uptake is equal to the true unidirectional influx velocity and subsequent trans-stimulation of methotrexate uptake by the intracellular presence of other folates must represent a primary augmentation of the influx mechanism; (2) 5-formyl-H_4folate and 5-methyl-H_4folate competitively inhibit the influx of methotrexate with K_t^{in} within the same order of magnitude as the K_t^{in} for methotrexate[1,3,4], while the affinity of dihydrofolate reductase for these compounds is several orders of magnitude less than that for methotrexate[5]. As expected, 5-methyl-H_4folate and 5-formyl-H_4folate do not disturb the binding of methotrexate and unidirectional rates for methotrexate can be measured with these agents present within the intracellular compartment. Likewise, since free intracellular methotrexate does not exist during influx under these conditions, the reaction between the tetrahydrofolates and carrier at the inner cell membrane in preloaded cells should not be affected by the methotrexate released from carrier as the complex enters the cell. Dihydrofolate reductase has a very low affinity for folic acid relative to methotrexate and hence it too does not alter the methotrexate–dihydrofolate reductase interaction; however, the K_t^{in} for folic acid is 1 to 2 orders of magnitude higher than that of methotrexate[3] so that demonstration of competitive and exchange phenomena require the use of large relative concentrations[1,3].

MATERIALS AND METHODS

Methodology employed in these studies has been described in detail previously[1,6] and will be discussed here only briefly except for instances in which there have been changes in experimental design and technique.

* The concentration of substrate at which the unidirectional efflux process is half-saturated is indicated by K_t^{ef}; half-saturation of the influx mechanism is K_t^{in}.

Cells and media

L1210 murine leukemia cells were grown as a cell suspension in tissue culture and were used for experimentation during exponential growth. Maintenance of these cells and methods of preparation have been described[1]. Some studies were performed on L1210/methotrexate cells maintained in ascitic form in CDF_1 mice. These cells contain high levels of dihydrofolate reductase. Ehrlich ascites tumor cells were grown in CF_1 mice by weekly intraperitoneal inoculation of 0.2 ml of undiluted ascitic fluid. Ehrlich ascites cells contain dihydrofolate reductase with a binding capacity for methotrexate in the range of 4 nmoles per g dry weight, a level about one-half that of the L1210 cells. Preliminary studies indicated that methodology employed with L1210 cells would be satisfactory for the Ehrlich ascites cells. Tumor bearing mice were sacrificed from day 6 through day 10 when 2–5 ml of ascitic fluid had accumulated. The small amounts of contaminating red blood cells were removed in the supernatant fluid after three washes with 0° buffer with separation of the cell pellet by centrifugation for 1 min at 250 × *g*. The cells were kept at 0° until experimentation.

The experimental buffer consisted of 135 mM NaCl, 16 mM $NaHCO_3$, 1 mM Na_2HPO_4, 4.4 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$. The pH was adjusted to 7.4 in the presence of 95 % O_2–5 % CO_2. The osmolality of the buffer was 290 mosm/l.

Experimental techniques

Cells were incubated with test substances or under control conditions for 45 min (unless otherwise indicated) and at 37° following which the cells were separated by centrifugation in a 40-ml round-bottomed tube, chilled to 0° and washed three times with 0° fresh buffer to remove the loading substances from the extracellular compartment. Prior to the final centrifugation, equal volumes of the cell suspension were distributed into 13-ml conical centrifuge tubes so that subsequent flux determinations could be made in 4 or 5 replicate measurements. After a final centrifugation at 250 × *g* for 1 min, the supernatant fluid was aspirated completely and the tubes gassed with 95 % O_2–5 % CO_2, capped, and the tip of the tube containing the cell pellet was kept in an ice-water bath. For measurement of unidirectional influx rates, the tube was removed from the ice bath and immersed in a 37° bath for 20 sec to warm the glass and pellet. Following this, 1 ml of buffer at 37° containing the isotopically labeled material was rapidly injected, the cell plug thoroughly dispersed by three vigorous manual shakes and a vortex mix, and the tube mechanically agitated at high speed in an Eberbach shaker-bath at 37°. The uptake reaction was stopped by injection of 10 ml of 0° isosmotic NaCl solution (pH 7.4)[1]. The final cytocrit was under 3 %.

Analytical procedures

The cells were washed twice with 5 ml of the 0° NaCl solution. After thorough aspiration of residual supernatant fluid, the pellets were drawn up into a pasteur pipet, extruded onto 1 cm × 1 cm strips of polyethylene, and then dried overnight at 75°. By the following morning the pellet was dried to constant weight and had formed a hard crust which was easily peeled off the polyethylene and could be weighed directly on a Cahn G-2 electrobalance. The average dry weight of the cell pellet was 1 to 3 mg and was weighed with an accuracy of better than ± 0.3 %. This technique eliminates the extra weighing of a tare and avoids the larger error incurred when cell

weight is calculated from the difference between cells plus tare and tare alone. After weighing, the cell crust was dropped directly into a scintillation vial, 0.2 ml of 1 M KOH was added, and the cells were completely digested by a 1-h incubation at 75°. After cooling, 18 ml of a scintillation cocktail[1] was added and the specimen was counted on the Beckman LS-133 liquid scintillation spectrometer.

Chemicals

[3′5′-^{3}H]Methotrexate and 5-methyl [^{14}C]H_4folate were obtained from Amersham/Searle (Des Plaines, Illinois). [^{3}H]Methotrexate was purified by DEAE-cellulose column chromatography as previously reported[1]. The purity of 5-methyl [^{14}C]H_4folate was verified by DEAE-cellulose column chromatography employing a modification of the separation technique of NORONHA AND SILVERMAN[7]. Using a 9 mm × 30 cm column, the material was eluted with a 0.05 to 0.5 M sodium phosphate linear gradient in 10-ml fractions. To prevent folate oxidation on the column, ascorbic acid was added to the two sodium phosphate eluent solutions to obtain a concentration of 1 mg/ml. The final pH of the eluent solution was 6.3. By this technique, the 5-methyl [^{14}C]H_4folate was recovered in Tubes 10 through 15 which represented 88 % of the activity added to the column. The additional 12 % was recovered in a single peak in Tubes 7 and 8. For experimentation, the barium salt of 5-methyl-H_4folate was employed as supplied by the manufacturer. Barium was removed by dissolving the crystals in 1 mM Na_2SO_4 containing 1 mg/ml ascorbic acid. The $BaSO_4$ precipitate was removed by centrifugation and the supernate was stored at −40°. Small aliquots of this solution were added to the experimental medium at the time of experimentation. Studies indicated that the amount of Na_2SO_4 and ascorbic acid which accompanies the 5-methyl[^{14}C]H_4folate under these experimental conditions does not affect the influx of methotrexate. 5-Formyl-H_4folate was obtained from Lederle Laboratories (Pearl River, N. Y.). Both tetrahydrofolates are racemic mixtures and concentrations expressed in this paper refer only to the "active" L-isomer.

Identification of accumulated material

Methotrexate is not metabolized in L1210 leukemia cells[1]. Similarly, 94 % of intracellular ^{3}H was recovered in the methotrexate peak with DEAE-cellulose column chromatography[1] following incubation of Ehrlich ascites cells with [^{3}H]methotrexate for 1 ½ h. Both folic acid[8], and 5-methyl-H_4folate[3] are rapidly metabolized in L1210 cells.

RESULTS

The effect of intracellular folates on the unidirectional influx of methotrexate

Incubation of L1210 cells with 5-formyl-H_4folate, 5-methyl-H_4folate, or folic acid results in the subsequent stimulation of the unidirectional influx of methotrexate (Fig. 1). The concentration of folic acid in the preincubation buffer exceeded the level of the tetrahydrofolates by 2 to 3 orders of magnitude while influx stimulation among these compounds was comparable. This must be due, at least in part, to the fact that folic acid uptake in these cells is an extremely slow process and requires very high extracellular levels to achieve adequate intracellular concentrations[8]. Folic acid is metabolized in L1210 cells and the tetrahydrofolates are likely derivatives synthesized

by a series of reactions which first involve reductions mediated by dihydrofolate reductase[9]. However, it seems likely that influx stimulation for methotrexate in cells preloaded with folic acid is due in part to a folic acid–methotrexate exchange rather than being related solely to a methotrexate-tetrahydrofolate exchange since a small, 10%, but significant, $P < 0.001$, stimulation of methotrexate influx occurs even in Ehrlich ascites tumor cells preloaded with 5 mM folic acid for 1 h after dihydrofolate reductase was inactivated by methotrexate utilizing a technique similar to that employed for the experiments of Figs. 5 and 6. However, the possibility cannot be excluded that this phenomenon is related to other metabolites of folic acid the production of which are not inhibited by this procedure.

The degree of methotrexate influx stimulation during heteroexchange increases as the concentration of 5-formyl-H_4folate in the preincubation buffer is increased and in general this appears to approach a maximum value (Fig. 2). However, in the absence of measurement of the intracellular folate level in these studies a precise definition of the relationship between intracellular folate concentration and influx stimulation is precluded. Fig. 3 illustrates the time course of methotrexate influx into control cells and cells preloaded with 5-formyl-H_4folate. As expected, in control cells methotrexate uptake is constant over the period of observation since the dihydrofolate reductase binding capacity of the cells is not exceeded. In cells preloaded with 5-formyl-H_4folate, the unidirectional influx of methotrexate is stimulated and the degree of stimulation is constant for the 200 sec period of observation in this experiment. The constancy of influx during heteroexchange is assumed to indicate that initial rates for 5-formyl-H_4folate efflux are sustained. It may also be seen in Fig. 3 that the extrapolated intercepts appear to pass through the point of origin. In other

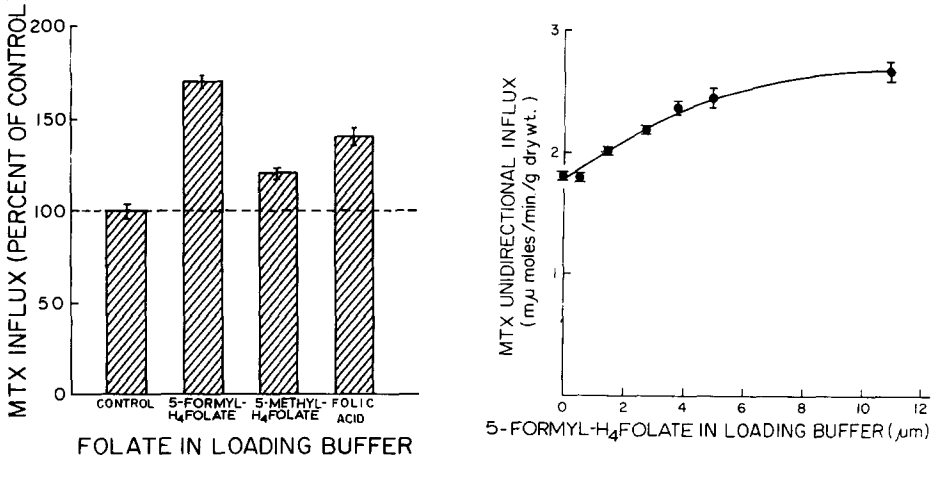


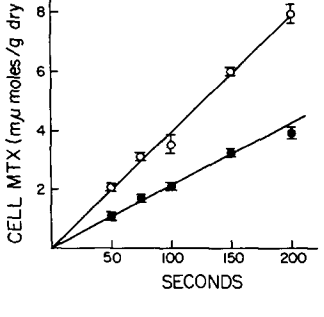
Fig. 1. The effect of intracellular folates on the 100 sec unidirectional influx of 1 μM methotrexate in L1210 cells. Cells were preincubated with 10 μM 5-formyl-H_4folate, 1 μM 5-methyl-H_4folate, or 1 mM folic acid following which extracellular folate was removed and methotrexate influx measured as described in MATERIALS AND METHODS. Results are mean ± S.E. of 5 measurements in a representative experiment. All differences are significant to $P < 0.001$. MTX, methotrexate.

Fig. 2. The effect of preincubation of cells with increasing levels of 5-formyl-H_4folate on the subsequent 50 sec unidirectional influx of 1 μM methotrexate in L1210 cells. Data is expressed as the mean ± S.E. of 4 measurements in a representative experiment. MTX, methotrexate.

experiments which permit a more accurate analysis of the zero-time intercept because cell methotrexate is measured more frequently and over a shorter time interval, the extrapolated intercepts are found to be within 3 sec of the point of origin. Hence there is only a small lag in the establishment of the final unidirectional influx velocity when a cell pellet is warmed for 20 sec, then dispersed at zero-time upon exposure to 37° buffer containing the labeled methotrexate.

The unidirectional influx kinetics for methotrexate during heteroexchange diffusion

The kinetics of 5-formyl-H_4folate stimulation of methotrexate influx was quantitated by measuring methotrexate influx over a 30- or 50-sec interval in control cells and cells preloaded with 5-formyl-H_4folate. During this interval and over the range of extracellular methotrexate concentrations studied at this cytocrit, the following limits were established for this system; (1) Uptake of methotrexate never exceeded the cell dihydrofolate reductase binding capacity so that unidirectional rates were always measured. (2) Appearance of 5-formyl-H_4folate in the extracellular buffer due to cell loss over the flux interval was negligible and could not exceed 2 % of the lowest extracellular methotrexate level, $[\text{methotrexate}]_e$, employed. (3) Stimulation of methotrexate influx during heteroexchange was constant so that initial efflux rates for the intracellular 5-formyl-H_4folate were considered to be sustained. (4) The short lag period that follows resuspension of the cell pellet is minimized. (5) Changes in $[\text{methotrexate}]_e$ due to cellular uptake are negligible. Fig. 4 is a double reciprocal plot of a representative experiment. Influx of methotrexate follows Michaelis–Menten kinetics in the preloaded as well as the control cells. The major kinetic alteration is an increase in the maximum influx velocity, v_{max}^{in}. The results of 10 such experiments are summarized in Table I. In some experiments there was an increase in the K_t^{in}, however, in no case was this change found to be statistically significant by two-tailed t-test analyses of the difference in the abscissa intercepts.



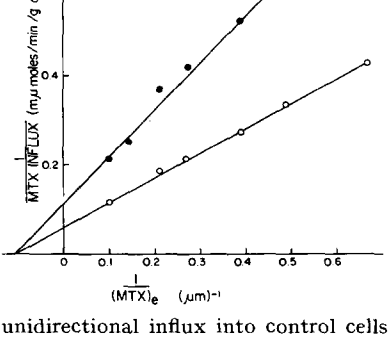


Fig. 3. The time course of 1 μM methotrexate unidirectional influx into control cells (●) and cells preloaded with 5-formyl-H_4folate (○). Each point represents the average ± S.E. of four determinations from a representative experiment with L1210 cells. MTX, methotrexate.

Fig. 4. A double reciprocal plot of the unidirectional influx kinetics for methotrexate in control L1210 cells (●) and cells preloaded with 5-formyl-H_4folate (○) from Expt. 3, Table I. This data as well as all other linear graphs in this paper are plotted by the method of least squares. MTX, methotrexate.

Also, the average increase in the K_t^{in} for the 10 experiments was 7.9%, a value not significantly different from zero ($P > 0.1$), while the increase in v_{max}^{in} differs from zero with a P value of < 0.001. Because there is considerable variation in the degree of influx stimulation during heteroexchange from day to day even when the preincubation level of 5-formyl-H_4folate is unchanged, the data does not clarify whether there is a relationship between the level of 5-formyl-H_4folate in the preincubation buffer and alterations in the K_t^{in}.

The effect of intracellular nonlabeled methotrexate on the unidirectional influx of labeled methotrexate

Although the system as described is of particular value in the study of heteroexchange diffusion, it was of interest to study the effect of intracellular nonlabeled methotrexate on the unidirectional influx of labeled methotrexate. Experimentally, this interferes with the technique for measuring the unidirectional influx of methotrexate since the exchange of labeled for nonlabeled methotrexate bound to dihydrofolate reductase is slow, free labeled methotrexate accumulates within the intracellular water early in the uptake process and back-flux of label leads to an early deviation from initial rates in the cells preloaded with nonlabeled methotrexate (Fig. 5), tending to mask a possible stimulatory effect in these cells. Fig. 6 illustrates the data from several experiments in which unidirectional influx alterations for methotrexate were evaluated for heteroexchange and autoexchange phenomena in Ehrlich ascites tumor cells. Cells were initially divided into two groups, one of which was exposed to nonlabeled methotrexate for 5 min to occupy all the available dihydrofolate reductase binding sites (Group II), while the other cells were similarly incubated but as a control without the nonlabeled methotrexate (Group I). Following this, both groups were washed twice with a large volume of fresh buffer at 37° to remove extracellular methotrexate and any excess intracellular methotrexate that had accumulated

TABLE I

ALTERATIONS IN THE UNIDIRECTIONAL INFLUX K_t AND v_{max}^{in} FOR METHOTREXATE DURING HETEROEXCHANGE WITH 5-FORMYL-H_4FOLATE

Each experiment was performed on a different day. K_t^{in} and v_{max}^{in} were determined from a least squares analysis of a double-reciprocal plot consisting of measurements at 6 to 7 extracellular methotrexate concentrations.

Expt. No.	K_t^{in} *(fraction of control)*	v_{max}^{in} *(fraction of control)*	*Flux interval (sec)*	*5-Formyl-H_4folate level in the preincubation buffer (μM)*
1	0.857	1.502	50	11.3
2	0.875	1.369	50	11.3
3	1.011	1.889	50	22.5
4	1.041	1.458	30	8.4
5	1.158	1.807	30	8.4
6	1.385	1.879	30	6.2
7	1.063	1.628	30	5.1
8	1.201	1.494	30	5.6
9	1.376	2.077	30	25.0
10	0.826	2.077	30	25.0
Average ± S.E.	1.079 ± 0.064	1.664 ± 0.074		

after the dihydrofolate reductase binding sites were completely saturated. The cells were then further subdivided as indicated in Fig. 6 and incubated under a number of conditions following which uptake of methotrexate was measured. It may be seen that the unidirectional influx of methotrexate was stimulated when cells were loaded with 5-formyl-H_4folate (IIc) but there was no change in the cells which contained free intracellular methotrexate (IIb). Pre-exposure of the cells to nonlabeled methotrexate did not significantly alter the stimulatory effect of 5-formyl-H_4folate ($P > 0.2$), hence the absence of transstimulation in cells preloaded with nonlabeled methotrexate (IIb) cannot be attributed to some nonspecific inhibitory effect of the methotrexate itself over a 5-min exposure. The unidirectional influx of methotrexate into the control cells (Ia) was not different from the uptake of methotrexate into cells which have no free intracellular methotrexate but in which all dihydrofolate reductase was bound (IIa) indicating that net uptake has approximated the unidirectional influx rate over this flux interval in the Group II cells. In another 5 experiments with L1210 cells consisting of a total of 20 measurements, the 10-sec uptake rate for methotrexate

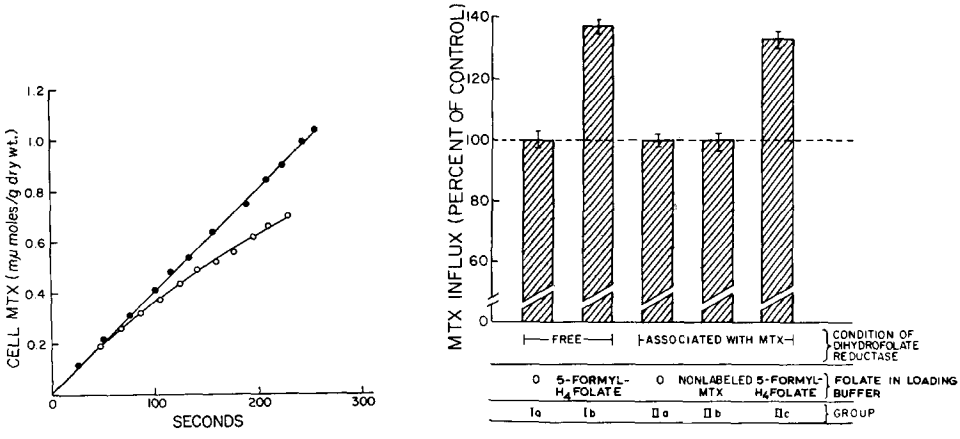


Fig. 5. Uptake of methotrexate in control cells (●), and cells in which dihydrofolate reductase was inactivated with nonlabeled methotrexate by the method described in the text (○). MTX, methotrexate.

Fig. 6. The 50-sec uptake rate for 1 μM methotrexate in Ehrlich ascites cells under several conditions described above and in the text. Each bar represents the mean ± S.E. of 15 measurements obtained in 3 separate experiments. MTX, methotrexate.

TABLE II

THE EFFECT OF INTRACELLULAR FOLATES ON THE INITIAL UPTAKE RATE FOR 5-METHYL-H_4FOLATE

Results are expressed as mean ± S.E. of 4 measurements in a single representative experiment. All differences are significant, $P < 0.001$.

Addition to preincubation buffer	*5-Methyl-H_4folate (% of control)*
Control	100 ± 2.0
6 μM 5-formyl-H_4folate	211 ± 8.4
500 μM folic acid	188 ± 2.1

was not significantly affected by the presence of nonlabeled methotrexate within the intracellular compartment. In these studies, the cells were preincubated with 50 μM nonlabeled methotrexate for 1 h and the concentration of labeled methotrexate during influx measurements was at the K_t^{in} of 5 μM. The significance of this preloading technique is discussed below.

The effect of intracellular folates on the initial uptake rate for 5-methyl-H_4folate

At an extracellular level of 1 μM, uptake of 5-methyl-H_4folate is linear for 50 sec; hence over this interval net uptake approximates the unidirectional influx rate. From Table II it is apparent that the initial uptake rate for 5-methyl-H_4folate is stimulated in cells preincubated with either folic acid or 5-formyl-H_4folate.

DISCUSSION

True unidirectional influx rates are often elusive measurements because of the departure from linear uptake due to the appearance of a significant efflux component early in the uptake process. Thus, in the first report of uptake stimulation of isotopically labeled glycine in Ehrlich ascites tumor cells preloaded with nonlabeled glycine, a significant departure from initial uptake rates was present by 1 min in the control nonloaded cells. The conclusion that there was a primary stimulation of the influx process rather than a competitive inhibition of the efflux component by the intracellular nonlabeled glycine could be made only after it was shown that the efflux mechanism was too far from saturation for significant competitive inhibition to have occurred at the inner cell membrane[10]. On the other hand net uptake stimulation in exchange diffusion has been so great in other studies that primary stimulation of the influx process must have occurred[11,12]. More recently, accurate initial uptake rates have been measured in rabbit ileum and unidirectional influx stimulation has been clearly demonstrated in heteroexchange diffusion[13].

The unique interactions between the tetrahydrofolate derivatives of folic acid and methotrexate with a mutually high affinity for the transport mechanism but a marked difference in affinity for the intracellular binding site affords a useful experimental model for the accurate quantitation of alterations in unidirectional influx kinetics during heteroexchange diffusion in L1210 and Ehrlich ascites tumor cells. The unidirectional influx of methotrexate during heteroexchange follows Michaelis–Menten kinetics. From the carrier model proposed by Jacquez (Eqn. 9, ref. 14), it may be shown that Michaelis–Menten kinetics are expected when the heteroexchange flux is measured under conditions in which the unidirectional rates of the exchanging species are constant over the flux interval. In the experiments reported here methotrexate influx was determined after cells were loaded to a single intracellular 5-formyl-H_4folate level which resulted in a rise in the v_{max}^{in} without a significant change in the K_t^{in}. This change in influx kinetics indicates that the percent increase in methotrexate influx during heteroexchange is independent of the degree of saturation of the carrier at the outer cell membrane by the extracellular methotrexate.

These studies indicate that the unidirectional influx for methotrexate is stimulated during heteroexchange and it is presumed that within the framework of existing carrier models that this is related to the proposition that during influx of methotrexate

the rate at which the unloaded carrier reorients from the inner to the outer cell membrane is rate-limiting to the carrier cycle and that the rate at which the carrier complexed with either folic acid or its tetrahydrofolate derivatives reorients to the outer cell membrane is greater than that of the unloaded carrier. From this it follows that since unidirectional influx stimulation for methotrexate does occur during heteroexchange, the rate of reorientation of the unloaded carrier to the outer cell membrane must be slow relative to the rate of translocation of the methotrexate–carrier complex toward the inner cell membrane. In view of this it was surprising to find that influx stimulation for methotrexate did not occur when methotrexate was present within the intracellular compartment and when carrier reorientation to the outer cell membrane should have occurred complexed in part with methotrexate. The absence of unidirectional flux alterations in autoexchange while present during heteroexchange is unusual but has been reported previously. MUNCK AND SCHULTZ[13] noted that the initial uptake rate for lysine is increased in rabbit ileal cells preloaded with leucine but no significant change occurred in cells preloaded with nonlabeled lysine. The reason for the absence of autoexchange for methotrexate in both Ehrlich ascites and L1210 tumor cells is uncertain. It is possible that insufficient intracellular methotrexate is accumulated over the period of preincubation and its initial efflux rate too slow to result in a measurable increase in the influx of labeled methotrexate. The relationship between free intracellular methotrexate levels at the steady-state and the extracellular methotrexate concentration follows an adsorption isotherm in L1210 cells with a maximum intracellular concentration due to the carrier system[1]. In these studies, the cells were loaded in the presence of extracellular methotrexate levels ten times greater than the K_t^{in} and to an intracellular concentration which approximates the theoretical maximum. Although it is possible that autoexchange might be demonstrable at higher intracellular methotrexate levels, it seems fair to assume that autoexchange is of no consequence in the transport of methotrexate within the operational range of this carrier system. This is compatible with earlier data from this laboratory[1] in which it was shown that the K_t^{ef} and v_{max}^{ef} predicted from the measured steady-state adsorption isotherm and measured K_t^{in} and v_{max}^{in} closely approximated the measured K_t^{ef} and v_{max}^{ef}.

Previous studies from this laboratory suggested that the transport of methotrexate and the naturally occurring folates in L1210 cells is related to at least one carrier-mediated process shared by all these substances[1,3,8]. This report further supports a carrier transport mechanism for these compounds in L1210 cells and indicates that a similar mechanism is operative in the Ehrlich ascites tumor cell line.

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