J. Membrane Biol. 79, 285–292 (1984)

The Journal of
Membrane Biology

Interaction Between Anions and the Reduced Folate/Methotrexate Transport System in L1210 Cell Plasma Membrane Vesicles: Directional Symmetry and Anion Specificity for Differential Mobility of Loaded and Unloaded Carrier

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Summary. The effect of various anions on the mediated influx and efflux of [^{3}H]methotrexate by L1210 cell plasma membrane vesicles in a HEPES buffer system was studied. Our results show that flux is stimulated to the same extent in either direction when SO_4, P_i, or folate compounds (1,L5-CHO-folate-H_4, methotrexate), but not Cl^- was present in the opposite compartment. This implies the property of directional symmetry, a condition in which differential mobility of loaded and unloaded carriers occurs in both directions. We also observed a similarity in the specificity of the interaction between various anions and carrier in each orientation of the membrane, in the order, $Cl^- \ll P_i \cong SO_4^{2-} \ll$ methotrexate $<$ 1,L5-CHO-folate-H_4. Also, the absolute differential in mobility of loaded and unloaded carrier (assumed from the extent of transstimulation obtained) varied substantially among the anions examined. No stimulation was obtained with Cl^-, and stimulation was twofold with P_i, SO_4^{2-} and methotrexate and fourfold with 1,L5-CHO-folate-H_4. Transstimulation of flux from either external or internal compartment only occurred when a positive gradient of total anions was maintained in the opposite compartment. Also, no stimulation occurred when the same equivalence of two different anions are present in opposing compartments. The concentration of anions required to transstimulate [^{3}H]methotrexate influx was increased four- to 10-fold when vesicles were equilibrated in 145 mM NaCl. These results suggest that under physiological conditions, concentrative uptake of methotrexate in intact L1210 cells as a result of anion exchange would require a large positive gradient in the total concentration of internalized anions.

Key Words folate analog transport · anion stimulation · vesicles

Introduction

The folate analog, methotrexate, is accumulated in tumor cells by a system which normally transports 5-methyltetrahydrofolate [reviewed in references 4,17]. In L1210 cells transport of this analog is markedly influenced [4–9] by a variety of inorganic and organic anions. Since methotrexate influx was competitively inhibited [4] by these anions, it was postulated by Goldman [4] that a similar interaction at the inside orientation might occur and entry of this and other folate compounds could be mediated by an anion-exchange mechanism in which anion gradients might serve as an energy source for concentrative uptake. This observation was confirmed later by other workers [5–9] who also showed that efflux under certain conditions was enhanced by the addition of anions to the external compartment. Possible physiological exchange anions have been proposed [4–9] which include phosphate, bicarbonate and AMP or related compounds. However, direct exchange of intracellular phosphate or other anions with extracellular methotrexate has not been demonstrated and appreciable concentrative uptake of this analog was only observed [5–9] when anions were excluded from the suspending medium. Thus, a role, if any, for these interactions under physiological conditions existing *in vivo* is uncertain.

In a further attempt to examine this question we have studied the effects of various anions on the kinetics of mediated flux of methotrexate in plasma membrane vesicles isolated from L1210 cells. These membrane preparations were found in our earlier studies [19–21] to be devoid of any metabolic potential for folate compounds. In contrast to that seen with intact L1210 cells [2, 3] under physiological conditions, transport of this folate analog by the reduced folate system in these membrane vesicles [19–21] is equilibrating, bidirectional and shows characteristics of facilitated diffusion. Also, it is possible in this isolated membrane system to transload the internal compartment with individual anions and measure effects [19–21] on the mediated entry of methotrexate and other folate compounds. Our results show, that during transport of methotrexate by this system, flux is stimulated to the same extent when anions are present in either the internal or external compartment. This implies

the property of directional symmetry, a condition in which differential mobility of loaded and unloaded carrier occurs in both directions. We also observed a similarity in the specificity of the interaction between carrier and various anions in each orientation of the membrane. Also, the absolute differential in mobility of loaded and unloaded carrier varied substantially among the anions examined. Our results also suggest that concentrative uptake of methotrexate in intact L1210 cells as a result of anion exchange would appear to require a positive gradient in the total concentration of anions in the internal compartment.

Materials and Methods

[^{3}H]Methotrexate at a specific activity of 20 Ci/mmol was purchased from Moravek Biochemicals, Brea, Calif. 3-O-[^{14}C]-Methyl-D-glucose at a specific activity of 58.9 mCi/mmol was purchased from Amersham/Searle, Chicago, Illinois. [^{3}H]Inulin with a specific activity of 0.48 nCi/mg was obtained from ICN Pharmaceutical Co., Irvine, Calif. 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, Md. [^{3}H]Methotrexate was purified prior to use by high-performance liquid chromatography [1]. Radiochemical purity of the final preparation was >97%. Aliquots of purified material were stored at −70°C. HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, obtained from Sigma Biochemicals, St. Louis, Mo. All other chemicals were reagent grade.

Source of Cells

L1210 cells (L1210/V/C_1) were obtained by transplantation [12] in $BD2F_1$ mice. Ascites suspension was harvested in cold (0°) 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°) 0.17 M NH_4Cl and then with 0.14 M NaCl [16].

Plasma Membrane Isolation

The method of Hochstadt et al. [11] was employed with some modifications [19–21] for the isolation at 0° 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 M hexylene glycol. The final washed membrane pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 150 mM sucrose or in 20 mM HEPES, pH 7.4, containing 2 mM $MgCl_2$ and 225 mM sucrose (HMS buffer) and stored at 70°C until used. At pH 7.4, the proportion of HEPES:HEPES-Na in 20 mM HMS buffer is 1.0 : 0.77. A determination of purity of membrane preparations by marker enzyme analysis and electron microscopy has been described [21].

These membrane preparations are devoid of any potential for metabolism of methotrexate or natural folate compounds as determined [19–21] by their chromatographic recovery following incubation with these preparations. They were also found in the current study to be devoid of any measurable dihydrofolate reductase activity using conventional assay methods [17]. This was also inferred from the results of our prior studies [19] showing that less than 2% added [^{3}H]methotrexate associating with vesicle preparations is bound to macromolecules.

Transport Assay

The membrane vesicle suspension was diluted and equilibrated in PBS buffer (50 mM potassium phosphate, 100 mM NaCl and 150 mM sucrose, pH 7.4) or HMS buffer (20 mM HEPES, 2 mM $MgCl_2$ and 225 mM sucrose) for 30 min at the required temperature and the experiment initiated by adding [^{3}H]methotrexate to a final volume of 0.1 ml. The membrane protein concentration in different experiments varied from 0.1 to 0.2 mg per tube. Incubation was terminated by 20-fold dilution with 0.8 M NaCl and then 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 Aquassure (N.E. Nuclear) 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 are expressed as the natural (1) diastereoisomer based upon previous studies [18] from our laboratory which have shown the high-affinity carrier system in intact L1210 cell exhibited, during influx, stereospecificity at carbon 6 of L-5-formyltetrahydrofolate. 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° which is presumed to represent surface adsorption. Measurements of effects on influx during exchange diffusion experiments were made by transloading of vesicles by preincubation for 30 min with the nonradioactive exchange anion in HMS buffer. All of the anions examined in this study were employed in the form of the sodium salt. Sodium phosphate solution at pH 7.4 was prepared as $NaH_2PO_4 : Na_2HPO_4$ in a proportion of 1.0 : 4.3.

Efflux of [^{3}H]methotrexate was studied by transloading vesicles with this analog for 30 min at 37°C and diluting in cold (0°) transport buffer. After centrifugation the vesicles were washed once with transport buffer and resuspended in transport buffer (extravesicular/intravesicular volume = 100 to 200) and aliquots removed at varying times after incubation at 37°C and processed as described above.

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 [10]. Values derived from an average of four determinations on different preparations was 3.22 ± 0.62 (PBS buffer) and 4.03 ± 0.58 (HMS buffer) per mg membrane protein. Protein concentration was measured by the method of Lowry et al. [13] using bovine serum albumin as a standard.

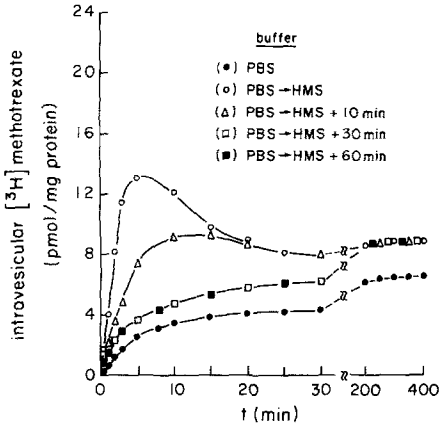

Fig. 1. Time-courses for intravesicular [^{3}H]methotrexate accumulation at 37°C in phosphate-saline (PBS) and HEPES (HMS) buffers. Aliquots of vesicle suspension were diluted in cold (0°) PBS or HMS and 2 μM [^{3}H]methotrexate added immediately or at varying times after (HMS) incubation at 37°C. Samples were removed at varying times after incubation with [^{3}H]methotrexate. Additional details are given in the text. SE of the mean did not exceed ± 14% (n = 3)

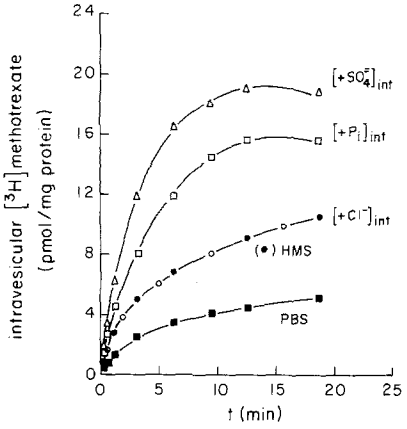

Fig. 2. Time courses for intravesicular [^{3}H]methotrexate accumulation at 37°C in PBS buffer or HMS buffer with or without various anions. Aliquots of vesicles were equilibrated for 30 min at 37°C in PBS, HMS or HMS plus 20 mM of each anion. Then, 2 μM [^{3}H]methotrexate was added and samples removed at varying times after incubation at 37°C. $P_i = H_2PO_4^- : HPO_4^{2-}$ (1 : 4.3). Additional details are provided in the text. SE of the mean did not exceed ± 16% (n = 4)

Results

General Characteristics of Intravesicular [^{3}H]Methotrexate Accumulation

As already documented in earlier studies [19–21] from this laboratory, folate compound accumulation in L1210 cell plasma membrane vesicles exhibits a complex time course. Uptake at 37°C is bi-exponential and characterized by an initial rapid phase followed within a few minutes by a slower phase until steady state is reached 4 to 5 hr later. For [^{3}H]methotrexate, values for $t_{1/2}$ obtained by the method of exponential stripping are approximately 1 min (rapid phase) and 36 to 38 min (slow phase). A typical time course for accumulation of this compound by vesicles suspended in PBS buffer is given in Fig. 1. The bi-exponential nature of this time course appears [19] to represent dual-compartment behavior in which two vesicular compartments of approximately equal volume in a parallel relationship show different accessibility to folate substrates. Similar compartmental behavior was also shown [19] for efflux by these substrates. From the results of these prior studies [19] 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.

As also seen in Fig. 1, resuspension of vesicles normally maintained in PBS buffer into HEPES buffer (HMS) prior to the addition of [^{3}H]methotrexate had a profound effect on the time course of accumulation. Initial accumulation of [^{3}H]methotrexate was more rapid and a high level of accumulation was obtained which was only transiently maintained. Preincubation of vesicles in HMS prior to the addition of [^{3}H]methotrexate resulted in a diminution of these effects. By 30 min of preincubation the system was apparently stabilized since further preincubation did not alter the time course in HMS. Preincubation at 0° did not eliminate (*data not shown*) the effect of [^{3}H]methotrexate accumulation observed in HMS buffer. The time course for intravesicular accumulation after 30 min of preincubation in HMS showed characteristics similar to those obtained in PBS. However, the rate of initial accumulation was higher and steady-state levels were somewhat greater. This latter difference can probably be accounted for by the fact that intravesicular volume per mg of membrane protein in HMS was larger (*see* Materials and Methods section) than in PBS.

Effect of Internalized Anions on the Intravesicular Accumulation of [^{3}H]Methotrexate

Transloading of various inorganic anions was carried out by incubating vesicles prepared in HMS for 30 min in the presence of anion. This transloading period was chosen on the basis of data given in Fig.

1 which suggests that the system would be stabilized within 30 min. Both SO_4^{2-} and P_i transloaded at 10 mM in this fashion were found to stimulate (*see* Fig. 2) intravesicular accumulation of [^{3}H]methotrexate. However, Cl^- when transloaded at a concentration of 40 mM had no effect on accumulation when compared to HMS alone. Also, [^{3}H]methotrexate accumulation in vesicles equilibrated in HMS was greater than the same vesicles equilibrated in PBS.

The effect of internalized anion on the initial (0 to 30 sec) uptake of [^{3}H]methotrexate at varying concentrations in vesicles equilibrated in HMS was subjected to the analysis shown in Fig. 3. In vesicles transloaded with SO_4^{2-} or P_i (*data not shown*) the value for influx K_m was unaffected but influx V_{max} was increased twofold. Although we have reported [20] similar effects of transloaded folate compounds in a PBS buffer, it was of interest to derive similar data in HMS buffer. Transloading of 5-formyltetrahydrofolate (10 μM as 1,L5-formyltetrahydrofolate) increased influx V_{max} for [^{3}H]methotrexate almost fourfold (*see* Fig. 3).

We also derived related data on the relative potency of various anions following transloading for stimulating influx of [^{3}H]methotrexate. Again, all of these experiments were carried out in HMS following a transloading period of 30 min in the presence of varying concentrations of anion and followed by washing in cold (0°) HMS. These data are expressed as that concentration required for $\frac{1}{2}$ maximum stimulation (K^{in}_{stim}) and are summarized in Table 1 along with other data on V^{in}_{max} obtained with each anion. It can be seen that values for both kinetic parameters varied among the different ions. Values for K^{in}_{stim} for both SO_4^{2-} and P_i were similar and in the range of 1 mM. Corresponding values for V^{in}_{max} for vesicles transloaded with these anions were both approximately twofold higher than control values. Cl^- at the concentrations examined had no effect on [^{3}H]methotrexate influx. On the other hand, both folate compounds were the most potent anions examined for stimulating influx of this folate analog. Values obtained for K^{in}_{stim} were in the μM range and V^{in}_{max} was increased twofold and three- to fourfold, respectively, for transloaded methotrexate and 5-formyltetrahydrofolate.

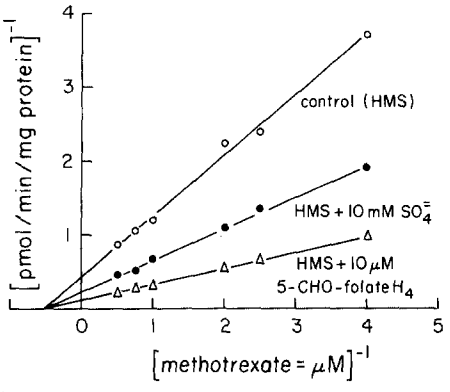

Fig. 3. Kinetic analysis of the effect of transloaded SO_4^{2-} and 1,L5-formyltetrahydrofolate on initial 30-sec influx of [^{3}H]methotrexate at 37°C in membrane vesicles. Aliquots of vesicle suspension were equilibrated for 30 min in HMS with and without 10 mM SO_4^{2-} or 10 μM 1,L5-formyltetrahydrofolate and then incubated for 30 sec at 37°C in various concentrations of [^{3}H]methotrexate. Additional details are provided in the text and legend of Fig. 2. SE of the mean did not exceed ± 13% (n = 5). Abbr: 5-CHO-folate H_4, 1,L5-formyltetrahydrofolate

Effects of External Anions on the Initial Rate of Entry of [^{3}H]Methotrexate in Vesicles

Inhibition of [^{3}H]methotrexate influx in HMS buffer was determined for the various anions and results expressed as values for K_i^{in} in Table 1. Of the inorganic anions, Cl^- was least potent as a competitive inhibitor of [^{3}H]methotrexate influx. Inhibition by SO_4^{2-} and P_i was much greater and values for K_i^{in} were in the range of 1 to 2 mM, similar to that derived for corresponding values of K^{in}_{stim}. Both folates were good inhibitors (μM range) of [^{3}H]methotrexate influx, with the natural folate compound being the more potent of the two. Also, values for K_i^{in} in each case were similar to values for K^{in}_{stim} (Table 1).

Effects of External Anions on Mediated Efflux of [^{3}H]Methotrexate from Vesicles

As already described for the time course for [^{3}H]methotrexate influx, efflux of this and other folate compounds from membrane vesicles also exhibits [19, 20] a complex time-course characteristic [19] of dual-compartmental behavior. Therefore, in order to accurately quantitate the effect of anions in the external compartment on the mediated component of this efflux time course, it is necessary to derive data in the form of individual rate constants. Efflux time courses were obtained for [^{3}H]methotrexate from vesicles held in HMS in the presence or absence of two anions (SO_4^{2-} and 5-formyltetrahydrofolate) in the external compartment. A decay-time analysis of this data is shown in Fig. 4. Values for the corresponding rate constants for mediated efflux (rapid initial component) were derived from this data by the method of "exponential stripping" and are given in Table 1 along with data for other anions. With the exception of Cl^- the addition of

anions in the external compartment stimulated [^{3}H]methotrexate efflux measured at 37°C from transloaded vesicles. No efflux from vesicles occurred in the presence or absence of anions at 0°. Values for ½ maximum stimulation (K^{ef}_{stim}) were found to be similar to values for K_i^{in}, that is, values for SO_4^{2-} and P_i were in the mM range and those for both folate compounds were in the μM range. Maximum stimulation (K^{ef}_{max}) was approximately twofold for P_i, SO_4^{2-} and methotrexate, but greater than threefold for 5-formyltetrahydrofolate. These values approximated that obtained for V^{in}_{max} for the same anions during [^{3}H]methotrexate influx (Table 1). No stimulation of efflux was observed when the same concentration of SO_4^{2-} or the equivalence in P_i was present in the internal compartment.

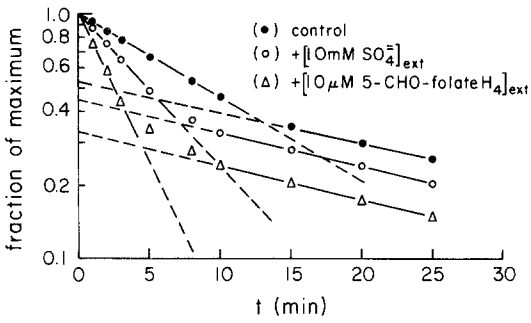

Fig. 4. Decay-time analysis of data obtained during efflux of [^{3}H]methotrexate in the presence and absence of anions in the external compartment. Vesicles were transloaded with 10 μM [^{3}H]methotrexate in HMS washed at 0°, resuspended in HMS ± 10 mM SO_4^{2-} or 10 μM 1,L5-formyltetrahydrofolate and aliquots removed after varying periods of incubation at 37°C. Additional details are given in the text. SE of the mean was less than ± 13%. Abbr: 5-CHO-folate H_4, 1,L5-formyltetrahydrofolate

Further Evidence for Directional Symmetry of Effects of Anions on Mediated Flux of [^{3}H]Methotrexate

The similarity of transstimulation of influx and efflux of [^{3}H]methotrexate by anions was verified in the following experiment in which the effect on intravesicular accumulation of saturating concentrations of P_i in each compartment alone or together was determined. From the results (Fig. 5) it is seen that stimulation of initial influx and intravesicular accumulation occurred when vesicles were transloaded with this anion. However, when the

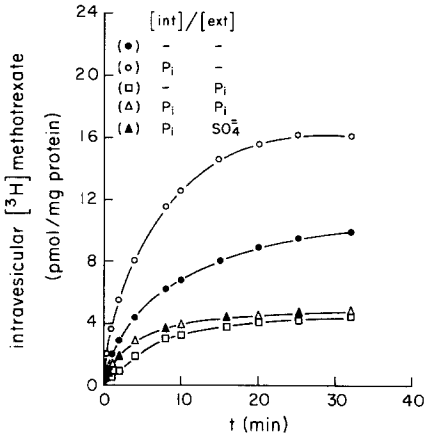

Fig. 5. The effect of anions in the intravesicular and extravesicular compartment on the time course of accumulation at 37°C of [^{3}H]methotrexate. Vesicles equilibrated in HMS or transloaded with 50 mM P_i[$H_2PO_4^-$ + HPO_4^{2-}(1 : 4.3)] were incubated at 37°C with 2 μM [^{3}H]methotrexate in HMS, HMS plus 50 mM SO_4^{2-} and aliquots removed at varying times thereafter. Additional details are given in the text. SE of the mean was less than ± 16% (n = 3)

Table 1. The effect of anions on various kinetic parameters of [^{3}H]methotrexate influx and efflux in L1210 cell plasma membrane vesicles[a]

Anion	K_i^{in} (μM)	K^{ef}_{stim} (μM)	K^{ef}_{max} (min^{-1})	K^{in}_{stim} (μM)	V^{in}_{max} (pmol/min/mg protein)
None	—	—	0.116 ± 0.2	—	3.33 ± 0.4
Chloride	28,230.0 ± 3900	—[c]	0.112 ± 0.03	—[c]	3.54 ± 0.4
Phosphate[b]	971.0 ± 136	1,235.0 ± 192	0.239 ± 0.04	1,320.0 ± 310	6.58 ± 0.9
Sulfate	1,520.0 ± 208	1,790.0 ± 135	0.228 ± 0.03	1,560.0 ± 232	5.65 ± 0.8
Methotrexate	2.63 ± 0.3	2.51 ± 0.4	0.233 ± 0.04	4.23 ± 0.4	6.85 ± 1.1
5-CHO-folate H_4	1.24 ± 0.2	1.38 ± 0.2	0.373 ± 0.05	0.89 ± 0.1	12.10 ± 1.9

[a] Influx and efflux of [^{3}H]methotrexate was measured at 37°C in aliquots of membrane vesicles suspended in HMS buffer (pH 7.4). Values for competitive inhibition of influx (K_i^{in}), ½ maximum stimulation of efflux (K^{ef}_{stim}) and maximum efflux (K^{ef}_{max}) from vesicles transloaded with [^{3}H]methotrexate were derived from data obtained in vesicles incubated in the presence or absence of anions in the external compartment. Values for ½ maximum stimulation of influx and maximum influx (V^{in}_{max}) were derived from data obtained in vesicles transloaded with [^{3}H]methotrexate with or without anions in the internal compartment. Average ± SE of the mean (n = 3 to 5). Abbr: 5-CHO-folate H_4, 1,L5-formyltetrahydrofolate.

[b] Phosphate exists as $H_2PO_4^-$ and HPO_4^{2-} in a proportion of 1 : 4.3 at pH 7.4.

[c] No stimulation was obtained at chloride concentrations as high as 100 mM.

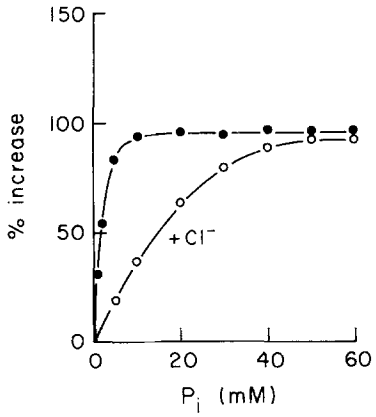

Fig. 6. The effect of intravesicular P_i on the initial influx of [^{3}H]methotrexate in the presence and absence of chloride. Vesicles were transloaded with varying concentrations of P_i in HMS or in chloride (total anion concentration was maintained isotonic). Vesicles in HMS + P_i were resuspended in HMS and vesicles in chloride + P_i were resuspended in isotonic chloride, 2 μM [^{3}H]methotrexate was added and samples removed after 30-sec incubation at 37°C. SE of the mean was less than ± 14% (n = 3)

same concentration of P_i was maintained in both external and internal compartments, no stimulation of initial influx occurred and the time course for accumulation resembled that obtained in PBS (Fig. 2). A similar result was obtained (Fig. 5) when the same ionic equivalence of SO_4^{2-} was present in the extracellular compartment of vesicles transloaded with P_i or *vice versa* (*data not shown*). P_i in the external compartment alone at a concentration substantially higher than the K_i^{in} did not completely eliminate entry of [^{3}H]methotrexate. A similar result is observed when time courses for [^{3}H]methotrexate entry were measured (Fig. 2) in PBS *versus* HMS. The smaller than expected effect of high concentrations of anion on initial influx and intravesicular accumulation of [^{3}H]methotrexate can be partially explained by the fact that in vesicles equilibrated in the presence of anion, stimulation of influx will offset the inhibitory effect of external anion. It is also probable that some entry of [^{3}H]methotrexate is mediated by a poorly saturable, high V_{max} system also transporting folate compounds which was delinated [20] in our earlier studies with these vesicle preparations. If this system, which has low affinity for all folate compounds, also has a relatively lower affinity for other anions, then a small, but measurable, fraction of [^{3}H]methotrexate influx would be unaffected by these concentrations of anions.

Table 2. The effect on [^{3}H]methotrexate influx of anions transloaded in vesicles with and without chloride

Transloaded anion	No chloride[a] K_{stim}^{in} (μM)	With chloride[b] K_{stim}^{in} (μM)
Phosphate	1,348.0 ± 231	14,900.0 ± 2230
Sulfate	1,620.0 ± 289	16,200.0 ± 2890
Methotrexate	3.19 ± 0.5	24.6 ± 5.5
5-CHO-folate-H_4[c]	1.04 ± 0.2	4.34 ± 0.7

[a] Influx was measured by incubating vesicles with 2 μM [^{3}H]methotrexate when in HMS after transloading with test anion. Average ± SE of the mean (n = 3 to 5).
[b] Influx was measured in 145 mM NaCl in vesicles transloaded with 145 mM NaCl at a concentration which maintained ionic equivalence. Average ± SE of the mean (n = 3 to 5).
[c] Abbr: 5-CHO-folate H_4, 1,L5-formyltetrahydrofolate.

Transstimulation of [^{3}H]Methotrexate Influx by P_i in the Presence of a Physiological Cl^- Concentration

Although low concentrations of either SO_4^{2-} or P_i are stimulatory to [^{3}H]methotrexate influx in vesicles equilibrated in HMS buffer, it was also of interest to determine the stimulatory potency of these anions in the presence of Cl^- which is the anion predominating under physiological conditions. Vesicles were transloaded with isotonic Cl^- or isotonic mixtures of Cl^- and PO_4^{2-} and resuspended in isotonic Cl^- with 2 μM [^{3}H]methotrexate. From the data shown in Fig. 6 it can be seen that, although the maximum stimulation obtained was the same, much higher concentrations of P_i were required for stimulation of [^{3}H]methotrexate influx in the presence of internalized Cl^-. Values for K_{stim}^{in} in this case were approximately 10 times greater than the same value derived in the absence of Cl^-. Similar results were also obtained with SO_4^{2-}, methotrexate and 5-formyltetrahydrofolate when transloaded with isotonic Cl^-. Data for K_{stim}^{in} from this group of experiments are summarized in Table 2. The difference in stimulation obtained in the presence *versus* absence of Cl^- was greatest in the case of both inorganic anions and less in the case of the folate compounds.

Discussion

The results presented here which were derived with L1210 cell plasma membrane vesicles confirm many

of the results of earlier [4–9] studies with intact L1210 cells reported elsewhere. That is, under certain conditions, anions can be shown to be competitively inhibit methotrexate influx and stimulate the efflux of this folate analog. Also, the specificity and relative potency of various anions appear to be similar [5–9] for both interactions. Although, a larger number of anionic species were employed in the prior [4–9] studies, our own results extend these earlier studies substantially, particularly with regard to data pertaining to anionic interactions with this transport system on the inside orientation. Both SO_4^{2-} and P_i interact with this system and exchange with [^{3}H]methotrexate during both influx and efflux. Similar effects were also shown for methotrexate and 5-formyltetrahydrofolate, but these interacted with much higher potency than the inorganic anions. Of the anions examined, Cl^- was the least potent inhibitor of [^{3}H]methotrexate influx and did not stimulate flux in either direction, a result in contrast to earlier findings [5–9] with intact L1210 cells. It was also of interest to note, that the stimulation of flux exhibited the property of directional symmetry. That is, flux was stimulated in each direction and to the same extent. Further evidence that the extent of transstimulation of flux was anion specific was also obtained in the form of data on the differential in stimulation between methotrexate and 5-formyltetrahydrofolate. These results could relate to differences in net charge and (or) lipophilicity of these anions. However, it is clear that the absolute differential in mobility of loaded *versus* unloaded carrier can vary substantially with the loading anion. Although the extent of these interactions in affecting carrier mobility most likely reflects some physical property as yet unidentified of this system, or a putative carrier, it is not yet possible to interpret these results mechanistically in the context of other carrier-mediated membrane transport processes. Although, differential mobility of loaded and unloaded carrier is a property common to many transport systems [14, 15], apparently, it is not [14, 15] an inherent property of facilitated transport in general.

The directional symmetry observed during these studies with this isolated membrane system is compatible with the notion of a simple model for methotrexate transport in which a single carrier accounts for flux of this analog in each direction. Both fluxes are limited by the return of the same empty carrier from the opposite orientation. These results are also consistent with prior studies [19, 20] from our laboratory with this isolated membrane system. In contrast, evidence for the operating of a second carrier system, which preferentially mediates methotrexate efflux in physiologically competent intact L1210 cells, was presented in earlier [2, 3] reports from our laboratory.

Although a physiological role of these anionic interactions on the transport of folate compounds in L1210 cells has not been excluded by the results of these studies, it is difficult to envision a role for an exchange anion in concentrative uptake of methotrexate. Such a role has been proposed for P_i. However, in view of our results which are suggestive of a much weaker interaction of specific anions with this transport system in a normal physiological environment where other anions predominate, this would appear unlikely. The concentration of this anion normally found [6] in intracellular water is only in the range of 3 mM, a concentration we have found to be well below that necessary to significantly stimulate folate compound influx in the presence of physiological levels of Cl^-. Alternatively, organic mono-phosphates have also been suggested [4–9] as possible physiological exchange anions. Although, this transport system in intact L1210 cells displays relatively high affinity [4–9] for these organic anions, at least in the outside orientation, the same limitations as to a possible effect similar to that described for inorganic anions would also apply under physiological conditions.

Finally, although it is probable that the effects of anions on methotrexate transport observed in this and earlier studies by others [4–9] are due to competitive binding of both categories of anions at the same or overlapping sites on a putative carrier, it should be pointed out that other explanations for some of these effects have not been entirely ruled out. In particular, the fact that very large concentrations of anions do not block mediated influx of [^{3}H]methotrexate to the extent predicted by a purely competitive model argues that other explanations should also be considered. For example, one might hypothesize that inorganic anions do not interact directly with the carrier at all, but rather that the ionization or solvation of the substrate (i.e. methotrexate) on the carrier is affected by the presence of various anions. Also, anions may cause changes in electrochemical potential of the substrate which would alter transport by increasing or decreasing the avidity of the binding of substrate to carrier on the inside *versus* the outside of the membrane. We do not suggest that the more conventional view is necessarily incorrect, but we do feel that additional and careful kinetic studies will be needed to eventually eliminate alternative possibilities.

The studies described were supported in part by grants CA 08748 from the National Cancer Institute, DHHR, CH-26 from the American Cancer Society and a grant from the Elsa U. Pardee Foundation. M. Dembo was supported by the U.S. Department of Energy.

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Received 18 October 1983; revised 6 December 1983