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RETENTIVENESS OF METHOTREXATE POLYGLUTAMATES IN CULTURED L1210 CELLS

EVIDENCE AGAINST A ROLE FOR MEDIATED PLASMA MEMBRANE TRANSPORT OUTWARD

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Abstract—In the present report, studies are described examining the issue of methotrexate (MTX) polyglutamate retentiveness in cultured L1210 cells. Measurements made in intact L1210 cells showed that the rate of egress of [^{3}H]MTX and [^{3}H]MTX + G1 was different depending upon whether [^{3}H]-MTX and its polyglutamates were accumulated intracellularly following biosynthesis during growth (3 hr) in the presence of [^{3}H]MTX or when cells were pulse loaded (5 min) with [^{3}H]MTX or [^{3}H]-MTX + G1 just prior to measurement of egress. In the former case, [^{3}H]MTX egressed with a $T_{1/2}$ of 15 ± 2 min, while [^{3}H]MTX + G1 egressed with a $T_{1/2}$ of 50 ± 9 min. In pulse loaded cells, both [^{3}H]-MTX and [^{3}H]MTX + G1 egressed with a $T_{1/2}$ of 3.5 ± 0.5 min. The same rapid egress of [^{3}H]MTX and [^{3}H]MTX + G1 seen following pulse loading was also documented in cells grown for 3 hr in the presence of nonradioactive MTX to normalize conditions with respect to the intracellular accumulation of [^{3}H]MTX polyglutamates seen during exposure of cells to [^{3}H]MTX during growth. In light of these results, MTX, MTX + G1, MTX + G2 and MTX + G4 were examined directly as permeants for the outwardly-directed ATP-dependent pump (Schlemmer SR and Sirotnak FM, *J Biol Chem* **267**: 14746–14752, 1992) mediating most of MTX efflux in intact L1210 cells using inside-out plasma membrane vesicles isolated from these cells. ATP-dependent efflux of [^{3}H]MTX and [^{3}H]MTX + G1 exhibited values for K_m of 46–50 μM and values for V_{max} of 102–106 pmol/min/mg protein. As competitive inhibitors of [^{3}H]MTX and [^{3}H]MTX + G1 efflux, MTX + G1 and MTX, respectively, exhibited K_i values of 43–47 μM, that is, $K_m \approx K_i$ for both permeants. Also, values for K_i of 45–48 μM were obtained with MTX + G2 and MTX + G4 as competitive inhibitors of [^{3}H]MTX efflux. From these results, we conclude that MTX and its polyglutamates are equivalent as copermeants for ATP-dependent efflux through the plasma membrane and that retentiveness of MTX polyglutamates is not determined at this level in these cells.

Subsequent to the initial studies with methotrexate (MTX)‡ by Baugh and associates [1], it has been further shown [reviewed in Refs. 2–11] that classical folate analogues, in general, undergo metabolic conversion to γ-polyglutamates following their intracellular accumulation in mammalian tissues. The formation of these metabolites is catalyzed [reviewed in Refs. 2, 5, 12 and 13] by the cytosolic enzyme, folylpolyglutamate synthetase. It has also been shown [2–11] that the relative cytotoxicity of various folate analogues against normal proliferative and tumor tissues is at least partially determined by the extent of their polyglutamylation in these tissues. This increased cytotoxicity of 4-aminofolate analogues associated with the formation of polyglutamates in cultured tumor cells has been attributed to the "retentive" nature [2–10] of these polyglutamates as well as to the enhanced inhibition [14, 15] of these analogues as polyglutamates of folate-dependent biosynthetic reactions. Although it has been tacitly assumed that the increased retentiveness of these polyglutamates in comparison to the parent compound in tumor cells is determined at the level of mediated plasma membrane transport outward, studies specifically addressing this issue have not been carried out.

In the present report, studies are described examining the issue of the retentiveness of MTX polyglutamates in cultured L1210 cells. Data were derived during experiments measuring egress of MTX polyglutamates with intact L1210 cells and as permeants during measurements of efflux with inside-out plasma membrane vesicles derived from them. Our earlier studies [16] with the membrane vesicle system, identified an outwardly-directed plasma membrane ATPase as the major (bromosulfophthalein and probenecid sensitive) route for mediated efflux of MTX in ATP-replete L1210 cells [17, 18]. For these studies, we utilized a variant of the L1210 cell (L1210/R24) [19] with minimal mediated influx of MTX to eliminate the complication

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‡ Abbreviations: MTX, methotrexate; MTX + Gn, methotrexate polyglutamate containing *n* number of additional glutamate residues; PBS, phosphate-buffered salines and MES, 2-(*N*-morpholino) ethanesulfonic acid.

of influx or permeant in a smaller fraction of the membrane vesicle preparation that remains oriented rightside-out. Efflux of MTX in this variant is unaltered [19] compared to wild-type L1210 cells.

A major advantage of the inside-out membrane vesicle technology in studies of mediated flux by this outwardly-directed system is that it allows a direct examination of the interaction of putative permeants with the permeant binding site on the cytoplasmic surface. The results from studies utilizing this system along with related data derived with intact L1210 cells strongly suggested that MTX and MTX polyglutamates are equivalent as copermeants for this ATP-dependent efflux route. Thus, the "retentiveness" of MTX polyglutamates observed in these cultured cells is not determined at the level of their mediated efflux through the plasma membrane.

MATERIALS AND METHODS

L1210 and L1210/R24 cells were maintained [20] in culture in RPMI-1640 medium supplemented with 10% fetal bovine serum. L1210/R24 cells were also transplanted in BD2F1(C57BL/DBA/2F$_1$) mice to provide adequate material for preparation [20] of inside-out plasma membrane vesicles. In experiments measuring MTX polyglutamate synthesis in L1210 cells during growth, culture medium was supplemented with 10 μM thymidine, 100 μM inosine and 2 mM glutamine. These cells were incubated for 3 hr with 2 μM [^{3}H]MTX in the supplemented RPMI-1640 medium. For analysis, cells were washed twice with phosphate-buffered saline (PBS), resuspended in PBS, and after a determination of cell number with a Coulter counter, the cell suspension was boiled for 15 min. Cell debris was removed by centrifugation and the supernatant frozen prior to analysis by HPLC. This was carried out by reverse-phase fractionation modified from Cashmore *et al.* [21] employing a linear gradient of 8–22% acetonitrile in 0.1 M ammonium acetate, pH 5.1.

Egress of [^{3}H]MTX and its polyglutamates was measured either in cells preloaded with [^{3}H]MTX or [^{3}H]MTX + G1 (methotrexate polyglutamate containing an additional glutamate residue) or in cells allowed to accumulate [^{3}H]MTX and [^{3}H]MTX polyglutamates during a 3-hr incubation with [^{3}H]-MTX. In the former case, L1210 cells (2–5 $\times$ 10^7/mL) grown for 3 hr in the presence or absence of 2 μM nonradioactive MTX were incubated for another 5 min at 37° in the presence of either 4 μM [^{3}H]MTX or 40 μM [^{3}H]MTX + G1. Following one PBS wash (0–4°), the cells were resuspended in transport medium [20] and incubated for various periods of time at 37°. At each time point, aliquots of cell suspension were removed and the cells processed [20] by rapid centrifugation through oil. Scintillation counting of the cell pellet was carried out after resuspending in cold PBS. Egress measurements with cells grown with [^{3}H]MTX for 3 hr in medium were done in the same way except that the final analysis of [^{3}H]MTX and its polyglutamates was carried out by HPLC [21].

The preparation of inside-out plasma membrane vesicles from L1210/R24 cells has been described in detail in our earlier report [16]. The method employed was a modification of that of Marin *et al.* [22] in which plasma membrane fragments obtained following homogenization of cells were precipitated in mannitol, fractionated by density-gradient centrifugation, and sealed during dialysis in a buffer without divalent cations. Approximately 1 mg of inside-out plasma membrane vesicles was obtained from 2.2 $\times$ 10^9 cells. Vesicle sideness and contamination by membranes from intracellular organelles were monitored by marker enzyme analysis described in detail in our earlier report [16]. The plasma membrane preparations used during these experiments were 94 $\pm$ 6% pure and 70–75% in the inside-out orientation.

Measurements of ATP-dependent intravesicular accumulation of [^{3}H]MTX or [^{3}H]MTX + G1 were carried out with a modification [16] of the procedure of Horio *et al.* [23]. The procedure utilizes the ATP-regenerating system described earlier by Kamimoto *et al.* [24]. Usually, a 20-μL aliquot of vesicles (50–70 μg of membrane protein) was incubated at 37° in the presence and absence of 5 mM ATP and an ATP-regenerating system, and accumulation of [^{3}H]MTX or [^{3}H]MTX + G1 was initiated by the addition of radiolabeled permeant. The final buffer content of the transport medium was adjusted to 25 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.1, with magnesium oxide and 225 mM sucrose with the final addition of permeants. The incubation was stopped after various times by the rapid dilution of the sample with 9 mL of cold (0–4°). Tris–HCl–sucrose (20 mM Tris–HCl, pH 7.4, and 250 mM sucrose), and vesicles were collected by filtration and dissolved in scintillation fluid for radioactive counting. Adsorption of radiolabeled permeant to the vesicle surface was determined by a 5-sec exposure to permeant at 0–4°. Alternatively, surface binding was determined by back-extrapolation of the 37° time course to the origin. Each time point was performed in duplicate and the data represent an average of three separate experiments. Kinetic constants (K_m, K_i and V_{max}) for initial intravesicular accumulation were determined by Lineweaver–Burk [25] or Dixon [26] plots of the time–course data at various external concentrations with the application of linear regression analysis. Verification that [^{3}H]MTX and [^{3}H]MTX + G1 were unchanged during their incubation with inside-out membrane vesicles was obtained by HPLC analysis [21] of the incubation mixture containing 1 mg of membrane protein.

[3′,5′,9-^{3}H]MTX and [3′,5′,9-^{3}H]MTX + G1 (sp. act. 10–15 Ci/mmol) were purchased from Moravek Biochemicals, City of Industry, CA. MTX was provided by the Division of Cancer Treatment, National Cancer Institute. MTX + G1, +G2 and +G4 were purchased from B. Schirks Laboratories, Jona, Switzerland. All of these materials were repurified by HPLC [21] prior to use. Purity levels were >98% based upon HPLC analysis. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

The retentiveness of MTX polyglutamates in cultured cells is reflected [3–11] in the decreased

egress of these polyglutamates from these cells when compared to MTX. This was demonstrated [3–11] by experiments in which tumor cells were first cultured for several hours in the presence of [^{3}H]-MTX. Following this, [^{3}H]MTX was removed by washing the cells and resuspending them in growth medium [3–10] or in a physiological salts solution with D-glucose [11] in the absence of [^{3}H]MTX. During incubation of these cell suspensions at 37° and periodic analysis of their intracellular contents by HPLC [21], a decreased rate of egress of [^{3}H]-MTX polyglutamates compared to [^{3}H]MTX, itself, was observed, which was approximately proportional to the polyglutamate chain length of these metabolites.

In our own studies [11] of these polyglutamates, that documented lower rates of egress of MTX polyglutamates compared to MTX as above, the rate of egress of [^{3}H]MTX from L1210 cells was also shown to be appreciably lower than that usually associated [11] with its mediated efflux from energetically competent tumor cells preloaded by a short (5 min) incubation with this folate analogue. Also, no difference in the rate of egress was observed [11] between MTX and MTX + G1 after "pulse" loading in this manner. However, these latter measurements were made [11] with drug-naive L1210 cells derived directly from mice rather than from cell culture and did not have MTX polyglutamates accumulated intracellularly. To control for these differences, we subsequently repeated these experiments in the following way. Cells were cultured with 2 μM [^{3}H]MTX for 3 hr. The cells were then washed and egress of [^{3}H]MTX and [^{3}H]MTX polyglutamates was measured. In parallel, aliquots of the same cells were cultured with and without a 2 μM concentration of nonradioactive MTX for 3 hr. These cells were then loaded by a 5-min incubation with 4 μM [^{3}H]-MTX or 40 μM [^{3}H]MTX + G1 added to the medium and egress of each compound was measured. The results of these experiments are given in Fig. 1. In Fig. 1A, the data show that [^{3}H]MTX and [^{3}H]-MTX + G1 formed *in situ* in cells cultured with [^{3}H]-MTX egress in an exponential manner. However, the rate of egress of [^{3}H]MTX + G1 was 3-fold lower ($T_{1/2} = 50 \pm 9$ min) than the rate of egress of [^{3}H]MTX ($T_{1/2} = 15 \pm 2$ min). During the same experiments, higher polyglutamates of [^{3}H]MTX were also detected (data not shown) by HPLC and shown as before [11] to egress at even lower rates than [^{3}H]MTX + G1. The data on egress were very different when obtained with cells cultured with or without nonradioactive MTX and then loaded separately with [^{3}H]MTX and [^{3}H]MTX + G1 (Fig. 1B). In either case, egress was also exponential but the rates of egress of [^{3}H]MTX and [^{3}H]MTX + G1 were the same. Also, the rate of egress for both compounds (shown only for cells grown in the presence of MTX in Fig. 1B) was much faster ($T_{1/2} = 3.5 \pm 0.5$ min) than that seen in Fig. 1A. The same "pulse" loading of cells used in the experiments shown in Fig. 1A with either 4 μM MTX or 40 μM MTX + G1 prior to measurement of egress of [^{3}H]-MTX and [^{3}H]MTX + G1 had no effect on the result. In other experiments, we also found (data not shown) that egress of [^{3}H]MTX and [^{3}H]-MTX + G1 in each experiment shown in Fig. 1A and 1B was similarly inhibited by probenecid, an inhibitor of the major ATP-dependent system effluxing [^{3}H]MTX [16, 18, 27, 28] in intact L1210 cells. This result would seem to suggest that there is some role for this outwardly-directed pump in the egress of [^{3}H]MTX and [^{3}H]MTX + G1 in each case.

The only difference between the experiments discussed in Fig. 1A and 1B is that in one case [^{3}H]-MTX and [^{3}H]MTX + G1 egress was measured after

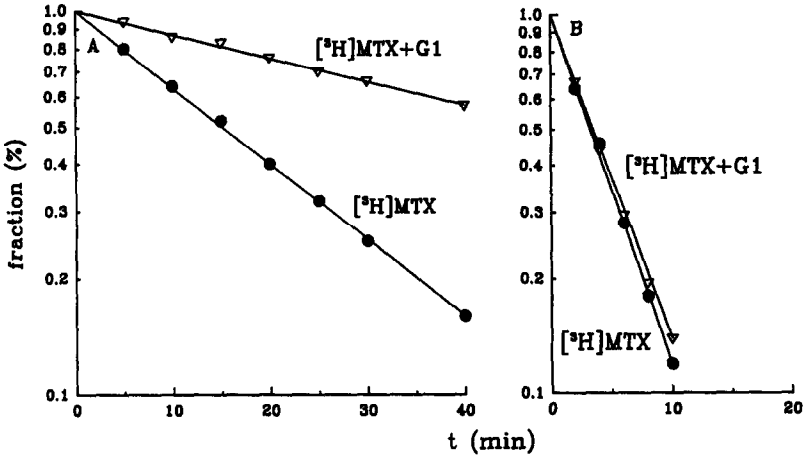

Fig. 1. Egress of [^{3}H]MTX and [^{3}H]MTX + G1 from L1210 cells. (A) Cells were grown for 3 hr in the presence of 2 μM [^{3}H]MTX. After washing of cells, [^{3}H]MTX and [^{3}H]MTX + G1 egress was measured after resuspending in transport medium at 37°. (B) Cells were grown for 3 hr in the presence of 2 μM MTX. Following this, the cells were preloaded with 4 μM [^{3}H]MTX or 40 μM [^{3}H]MTX + G1 by their addition to the culture medium for 5 min. After washing the cells, egress was measured in transport medium at 37°. Data represent an average of three separate experiments. The standard error of the mean was $< \pm 16\%$.

they and other polyglutamates had accumulated intracellularly over a period of 3 hr. In the other case, cells allowed to accumulate nonradioactive MTX and MTX polyglutamates intracellularly in the same way were loaded by a short-term incubation with [^{3}H]MTX and [^{3}H]MTX + G1 and then their egress was measured. Thus, the data in Fig. 1B appear to show the usual kinetics [20] for mediated efflux of [^{3}H]MTX from these cells grown under the same conditions as those cells utilized for the experiment in Fig. 1A. These data also suggest that [^{3}H]MTX and [^{3}H]MTX + G1 are equivalent as permeants during mediated efflux by the same probenecid-sensitive system and that this system is not in some way compromised by the intracellular accumulation of MTX polyglutamates. As a possible explanation for these results, we would suggest that the discrimination between [^{3}H]MTX and [^{3}H]-MTX + G1 observed during egress from cells grown in the presence of [^{3}H]MTX (Fig. 1A) did not occur at the level of mediated efflux through the plasma membrane but at some intracellular site (compartment?). We would also suggest that the interaction of MTX and MTX polyglutamates at this site for some unknown reason limits the rate of their egress when cells had accumulated substantial quantities of [^{3}H]MTX polyglutamates over an extended period of time. However, in either case, the same probenecid-sensitive system [16, 18, 27, 28] appears to be involved in ultimately mediating their outward flux through the plasma membrane.

Because of the availability in our laboratory [16] of technology for preparing "inside-out" plasma membrane vesicles, we were able to address one aspect of this question in a more direct manner. A series of experiments was carried out with this methodology in which we examined various MTX polyglutamates as permeants for the ATP-dependent, outwardly-directed pump described in our earlier [16] studies that mediates [27, 28] most of MTX efflux. This is the same bromosulfophthalein- and probenecid-sensitive system [18] that mediates 90% of [^{3}H]MTX efflux in ATP replete L1210 cells. Data on the concentration–response for ATP-dependent intravesicular accumulation of [^{3}H]MTX + G1 are shown in Fig. 2. As seen in Fig. 2A, accumulation of [^{3}H]MTX + G1 was rapid and was constant with time during the 15-sec interval of the measurement at each concentration of [^{3}H]MTX + G1 examined. The concentration–response plot derived from these data is shown in Fig. 2B. The plot of these data in the *inset* to the figure delineates a single saturable component with a K_m of 45 ± 5 μM and a V_{max} of 102 pmol/min/mg protein. These data are essentially identical to that already derived for [^{3}H]MTX in the same experiment described in our earlier report [16].

Presented in Fig. 3 are data showing initial ATP-dependent accumulation in inside-out plasma membrane vesicles of [^{3}H]MTX and [^{3}H]MTX + G1 in the presence and absence of MTX + G1 or MTX, respectively. The initial rate of accumulation of [^{3}H]MTX (Fig. 3A) and [^{3}H]MTX + G1 (Fig. 3B) at an external concentration of 20 μM was 10- to 12-fold more rapid in the presence of 5 mM ATP than in its absence. Also, MTX + G1 and MTX at a concentration of 20 μM were equally effective as inhibitors of ATP-dependent [^{3}H]MTX + G1 and [^{3}H]MTX accumulation, respectively. Further evidence for the identity of MTX and MTX polyglutamates as equal copermeants for ATP-dependent efflux by the MTX efflux pump was

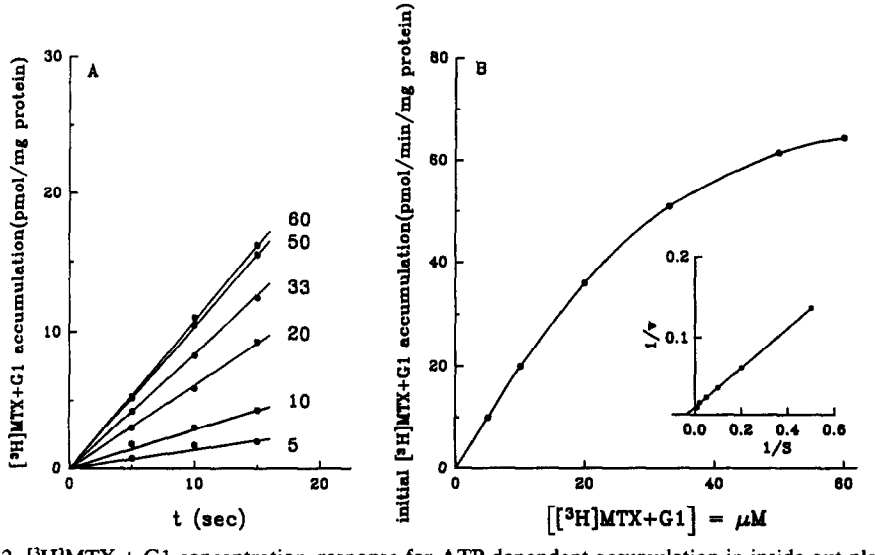

Fig. 2. [^{3}H]MTX + G1 concentration–response for ATP-dependent accumulation in inside-out plasma membrane vesicles. (A) Time–course for the initial accumulation at 37° of different micromolar concentrations of [^{3}H]MTX + G1. (B) Concentration–response plot for data derived in panel A. Inset: double-reciprocal plot of initial velocity of accumulation and concentration. Data represent an average of three separate experiments. The standard error of the mean was < ±13%.

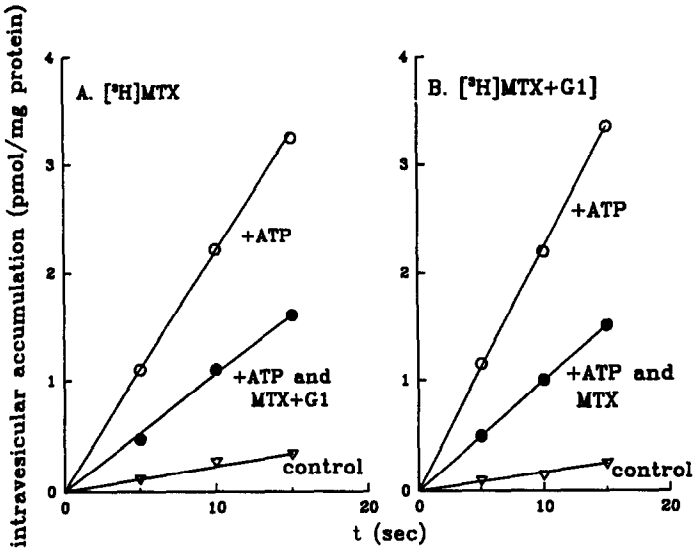

Fig. 3. Effect of MTX + G1 and MTX on initial ATP-dependent accumulation of [^{3}H]MTX (A) and [^{3}H]MTX + G1 (B), respectively, in inside-out plasma membrane vesicles. The concentration of each folate derivative used was 20 μM. Data represent an average of three separate experiments. The standard error of the mean was $< \pm 15\%$.

Table 1. Summary of experiments determining kinetic constants for initial intravesicular accumulation of [^{3}H]-MTX and [^{3}H]MTX + G1 and inhibition by MTX and MTX polyglutamates

Permeant	Initial intravesicular accumulation* K_m (μM)	K_i (μM)
MTX	46 ± 7	43 ± 5†
MTX + G1	50 ± 4	47 ± 8‡
MTX + G2		45 ± 9‡
MTX + G4		48 ± 8‡

Experimental details are provided in the text and in the legends of Figs 1–3.

* Values are means ± SEM of 3–5 separate experiments with [^{3}H]MTX + G1 as the permeant.

† With [^{3}H]MTX + G1 as the permeant.

‡ With [^{3}H]MTX as the permeant.

obtained in the following experiments. An analysis of the kinetics of inhibition of [^{3}H]MTX and [^{3}H]-MTX + G1 accumulation by MTX + G1 and MTX, respectively, was carried out by the method of Dixon [26]. These data are summarized in Table 1. Values for K_i derived by this method were essentially the same (K_i = 43–47 μM) for MTX and MTX + G1 and corresponded closely to the values for K_m obtained from experiments like those shown in Fig. 2. In other words, $K_m \approx K_i$ in each case. Values for K_i against [^{3}H]MTX derived for two other polyglutamates, MTX + G2 and MTX + G4, were also in close agreement (Table 1) with these other values.

From these results, we conclude that MTX and its polyglutamates interact equally as permeants for the same ATP-dependent, outwardly-directed mechanism shown earlier to mediate [16, 18, 27, 28] most of the efflux of MTX through the plasma membrane. The fact that the presence of additional glutamyl residues in a γ-linkage had no effect on the ability of this system to efflux MTX was not entirely unexpected in light of the results presented in Fig. 1B. However, in this regard, the lack of an effect of polyglutamylation is very different from that observed in the case of the one-carbon, reduced folate transport system [29, 30]. MTX polyglutamates compete very poorly as inward copermeants for this system as a result of its relatively low affinity for these polyglutamates compared to MTX. A similar differential in the interaction of these permeants with this system might also occur in the outward orientation. However, in ATP-replete cells [18], this system makes only a minor contribution to efflux of MTX. The results in Fig. 1B would suggest that the system also makes only a minor contribution to efflux of MTX polyglutamates.

Overall, the results obtained in these studies with inside-out plasma membrane vesicles support the notion offered above, namely, that interactions involving these permeants at a level other than mediated efflux through the plasma membrane determine retentiveness of MTX polyglutamates in these cells. The basis for this retentiveness is not revealed by the results derived herein but may relate to a form of intracellular compartmentation not as yet identified. Further studies will be necessary to address these remaining questions.

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