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# Alterations in the Kinetics of Methotrexate Transport During Growth of L1210 Murine Leukemia Cells in Culture

PAUL L. CHELLO, FRANCIS M. SIROTNAK AND DIANE M. DORICK

*Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10021*

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SUMMARY

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Measurements of methotrexate transport during different phases of growth of L1210 murine leukemia cells in culture revealed no significant change in the $K_m$ for methotrexate influx (3.9-4.7 $\mu$M) during growth. However, the $V_{max}$ for influx into exponentially growing (midlog) L1210 cells was threefold greater (9.4 nmol/min/g dry wt) than the $V_{max}$ for influx into stationary cells (3.1 nmol/min/g dry wt), while the rate of methotrexate efflux from stationary phase cells ($K$, 0.06 $min^{-1}$) was twice that measured for midlog cells ($K$, 0.03 $min^{-1}$). These reciprocal changes in the kinetic parameters for influx and efflux accounted for a five- to sixfold greater steady-state level for exchangeable methotrexate in midlog cells than in stationary phase cells. These data may help to explain the increased sensitivity of exponentially growing versus stationary phase cells to methotrexate.

## INTRODUCTION

The transport of folate analogs by murine tumor cells is a concentrative, carrier-mediated process (1-5) which has been identified as a critical determinant of the cytotoxicity of these agents (5). Differences in kinetic parameters for transport appear to account for both the relative responsiveness of murine tumor models (5, 6) and the selective toxicity observed between tumor and normal proliferative tissue (6, 7).

Several laboratories (8-10) have reported alterations in the carrier-mediated accumulation of various substances which are related to the stage of growth of the cell in culture. Since knowledge of growth associated alterations in the kinetics of folate analog transport would be highly relevant to both the use and the design of such analogs, we investigated the transport of the folate analog, methotrexate,[1] during the exponential and the stationary phases of growth of L1210 murine leukemia cells in suspension culture. The results revealed that reciprocal and nonstoichiometric changes occurred in the relationship between the carrier-mediated influx and the efflux of methotrexate which were reflected in the steady state level of folate analog accumulation during growth. The findings may be important to understanding the increased sensitivity of exponentially growing versus resting cells to methotrexate. Considered with previous work reported from this laboratory (4, 11, 12), they also raise questions regarding the number of carrier systems involved in the transport of folate analogs.

## MATERIALS AND METHODS

L1210$\bar{V}$ (13) murine leukemia cells were grown in suspension culture with RPMI-1640 medium (Grand Island Biologicals, Grand Island, N.Y.), 10% (v/v) with respect to fetal bovine serum (Microbiological Associates, Walkersville, Md., or Flow Laboratories, Rockville, Md.). The population doubling time was 11-12 h. Twice a week, cells in the mid to late logarithmic stage of growth were diluted (v/v) 1:10- to 1:200-fold with fresh medium and serum in order to keep a portion of the cell stocks in the logarithmic stage of growth at all times. For experimental work, stationary phase cultures at a density of approximately $10^6$ cells/ml were diluted with fresh medium and serum to a starting density of $1.5-2.0 \times 10^4$ cells/ml and incubated at 37°C in 1- or 2-liter stoppered Erlenmeyer flasks (liquid volume/total volume = 40%). The flasks were swirled twice daily, and cell density was monitored at 24-h intervals with a Model ZBI Coulter counter (Coulter Electronics, Hialeah, Fla.). A representative growth profile is shown in Fig. 2a. After a lag period of 18-24 h, the cells divided logarithmically for 72 h and then entered a stationary phase. As indicated, the period of logarithmic growth was arbitrarily divided into the phases of early, mid, and late log growth.

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[1] Methotrexate: 4-amino-10-methylpteroylglutamic acid.

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In order to determine whether there were changes in cell viability during growth which could influence the measurements of methotrexate transport, cell viability was estimated by cloning in soft agar (14). In a series of five experiments, each during a separate week, only a slight difference in cloning efficiency was found for cells harvested at the midlog stage of growth (61.4 ± 5.5%) and for cells harvested during the stationary phase (57.4 ± 5.4%). The difference was not considered significant ($P > 0.1$) when analyzed by a nonpaired $t$ test. The ability of cell suspensions to exclude 0.2% nigrosin dye is also an indication of membrane integrity, although the exact significance of cell staining in terms of viability is not actually known. In determinations made at different times during this study, 93.9 ± 1.3% ($N = 5$) of the cells harvested at the midlog stage of growth excluded nigrosin dye, as compared to 88.3 ± 7.9% ($N = 5$) of the cells harvested during the stationary phase. This difference was not considered significant ($P > 0.1$) when analyzed by a nonpaired $t$ test. In some experiments, there was no difference in the exclusion of the dye by midlog versus stationary phase cells.

For transport measurements, 1.0–1.2 × $10^9$ cells were harvested by centrifugation at 650$g$ in Sorvall RC2-B refrigerated centrifuges. The cells were washed with cold 0.14 M sodium chloride–0.02 M sodium phosphate buffer, pH 7.4, and resuspended in a buffered salts transport medium (107 mM NaCl, 20 mM Tris–HCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$), pH 7.4. Each cell suspension was divided into two parts in order that measurements of labeled methotrexate influx and efflux could be made with the same cells. Tritium-labeled methotrexate was initially purchased from Amersham–Searle (Arlington Heights, Ill.) and later from Moravek Biochemicals (City of Industry, Calif.). The purity of the labeled methotrexate was checked before use and, when necessary, was repurified on DEAE-cellulose.

All transport measurements were made in the absence of glucose according to established methodology (3–5, 7). To summarize briefly, methotrexate influx was measured by incubating cells (4–5 × $10^7$ cells) in transport medium (see the preceding) with tritium-labeled drug at 37°C. Uptake was terminated by placing cells on ice and rapidly adding cold (0–4°C) 0.14 M NaCl–0.02 M potassium phosphate buffer, pH 7.4. The diluted cells were immediately centrifuged at 2000$g$ for 5 min, washed twice with cold 0.14 M NaCl–0.02 M potassium phosphate buffer, pH 7.4, and resuspended in a measured volume of the same buffered salt solution. Part of this final cell suspension was used to determine cell density according to the absorbance at 600 μM (see the following); the remainder was added to 10 ml of Scintisol (Isolab, Akron, Ohio) and counted in a Packard Model B3385 liquid scintillation spectrometer in order to determine the amount of labeled methotrexate present. Incubation at 0°C followed by identical dilution and processing was used to estimate cell surface absorption and passive diffusion (3).

Measurements used to calculate kinetic constants ($K_m$, $V_{max}$) were made at various external concentrations of labeled methotrexate. To ensure that only unidirectional influx was measured, the incubation time was adjusted for each concentration such that the intracellular accumulation did not exceed the dihydrofolate reductase drug-binding capacity. For efflux studies, cells were preloaded with labeled methotrexate for 40 min at 37°C. The cells were cooled to 4°C, centrifuged at 200$g$ for 5 min, resuspended in cold drug-free transport medium (see the preceding), and reincubated at 37°C. Aliquots were removed at intervals, diluted with cold 0.14 M NaCl–0.02 M potassium phosphate buffer, pH 7.4, and processed as before.

The cell density of experimental samples was determined from the absorbance at 600 μM of an aliquot of cells suspended in 0.14 M NaCl–0.02 M potassium phosphate buffer, pH 7.4. Cell number was calculated from a previously established correlation (5) between absorbance and cell number as measured with a Neubauer improved counting chamber. There was no significant difference ($P > 0.1$) in this correlation ($A_{600}$ of 3.0 equals 2.6 ± 0.3 × $10^7$ cells/ml) with cells from each stage of growth, suggesting no appreciable change in cell size. Microscopic estimates of cell size also revealed no differences during growth. The cell counts obtained with the Neubauer chamber were in excellent agreement (±10%) with those obtained on the same samples with a Model ZBI Coulter counter. The wet and dry weights of L1210 tumor cells were established from the weights of cell suspensions before and after drying in preweighed aluminum dishes. Extracellular water was determined on similar cell suspensions with [*carboxyl*-$^{14}$C]inulin purchased from New England Nuclear, Boston, Mass. Intracellular water was calculated by subtraction of the extracellular water from the total cell water (wet weight minus dry weight). Analyses by nonpaired $t$ tests showed no significant difference ($P > 0.1$) in the wet weight (0.0219 ± 0.0015 g/2.6 ± 0.3 × $10^7$ cells), dry weight (0.0031 ± 0.0005 g/2.6 ± 0.3 × $10^7$ cells), or intracellular water content (0.0113 ± 0.0017 ml/2.6 ± 0.3 × $10^7$ cells) of cells harvested at each stage of growth. All transport measurements were expressed as nanomoles methotrexate per gram dry weight of cells.

The dihydrofolate reductase content of cells at each stage of culture growth was measured by the following procedure. Cells were harvested (see the preceding) and resuspended in 0.05 M Tris–HCl buffer, pH 7.3, 0.001 M with respect to ethylenediaminetetraacetate. After determination of cell density (see the preceding), the dihydrofolate reductase enzyme was extracted into the Tris–HCl buffer by ultrasonic disruption with a Model W200R sonicator cell disruptor (Heat Systems–Ultrasonics, Inc., Plainview, N.Y.). Following centrifugation at 37,000$g$ for 20 min in a Sorvall RC2-B refrigerated centrifuge, the crude enzyme supernatants were titrated with unlabeled methotrexate (3, 15) (supplied by Dr. Harry B. Wood, Jr., Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.). Graphical analysis of enzyme activity versus the concentration of inhibitor allowed the dihydrofolate reductase content (as methotrexate equivalents) of each cell extract to be calculated and compared. To be consistent with the transport data, the dihydrofolate reductase binding equivalence was expressed as nanomoles per gram dry weight of cells.

## RESULTS

To initially determine whether there were alterations in methotrexate uptake with the stage of L1210 cell

growth, comparative measurements of the rate of uptake and the steady-state level were made with midlog and with stationary phase cells. The results are shown in Fig. 1. Cells harvested from either the midlog or the stationary phase of growth accumulated methotrexate in a similar fashion. The folate analog was taken up at a linear rate for approximately 15 min before the rate gradually decreased and an intracellular steady state was attained by 40 to 60 min. Both the initial rate of uptake and the steady-state level appeared approximately threefold greater for midlog than for stationary phase cells. Enzyme titration assays indicated that there was no significant difference in the dihydrofolate reductase content (indicated by the arrow) of cells from either phase of growth. Consequently, the steady-state level of exchangeable methotrexate (total drug corrected for drug bound to dihydrofolate reductase) was actually five- to sixfold greater in midlog cells than in stationary phase cells.

These results prompted a more detailed comparison of the initial rate of accumulation and the steady-state level of exchangeable methotrexate at all phases of L1210 cell growth. The results are shown in Fig. 2. Both curves in panel b were symmetrical, i.e., there was no significant difference ($P > 0.1$) in the parameters measured with the stationary phase cells which were diluted with fresh medium and serum to start the experiment (the 0-time value) and those measured with the stationary phase cells harvested after the 120 h of experimental growth. Broken lines have been used to connect the 0-time values with those determined for early log cells since the very low cell density during the lag period required harvesting amounts of the cell suspension which were not feasible in order to obtain enough cells for accurate measurements. At 48 h (early log) and at 72 h (midlog), both the initial rate of uptake and the steady state were increased significantly ($P < 0.01$) from the values measured at 0 time. After reaching maxima at midlog, both the initial rate and the steady state for exchangeable drug decreased as the cells entered the late log and the stationary phases of growth. The value for each parameter determined with late log cells was intermediate between and statistically different from that measured for either midlog ($P < 0.05$) or for stationary phase ($P < 0.05$) cells.

Although some individual differences in the pattern of fluctuation for the initial rate of methotrexate accumulation and the steady state for exchangeable drug might have been detected by more frequent sampling, the overall pattern, particularly with regard to the time interval for maximum differences in each parameter, would appear to be similar. As in the case of the results compared in Fig. 1, the fluctuation in the steady-state level for exchangeable drug was greater than that shown for the initial rate of uptake. In a comparison between midlog and stationary phase cells where the highest and the lowest values were observed, the difference in the steady-state level for exchangeable drug was almost sixfold ($P < 0.01$), while the difference in the initial rate of uptake was only threefold ($P < 0.01$).

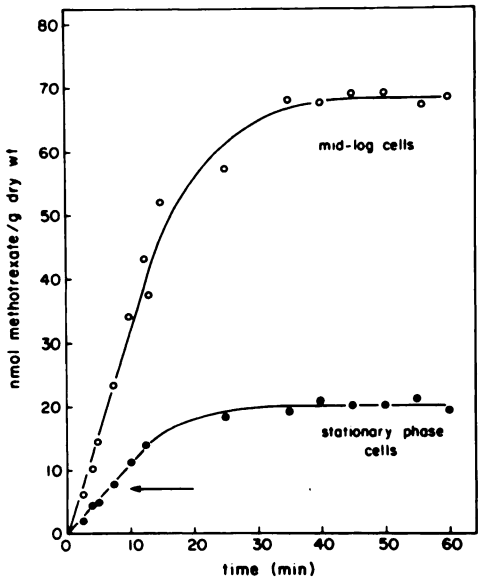


FIG. 1. *Time course for methotrexate uptake by L1210 murine leukemia cells*

L1210 cells harvested from the midlog (○) and the stationary phase (●) of growth (see Fig. 2a for representative growth profile) were incubated at 37°C with 1 μM methotrexate. Each curve represents a single experiment which has been repeated several times with similar results. The level of intracellular methotrexate bound to dihydrofolate reductase is indicated by the arrow.

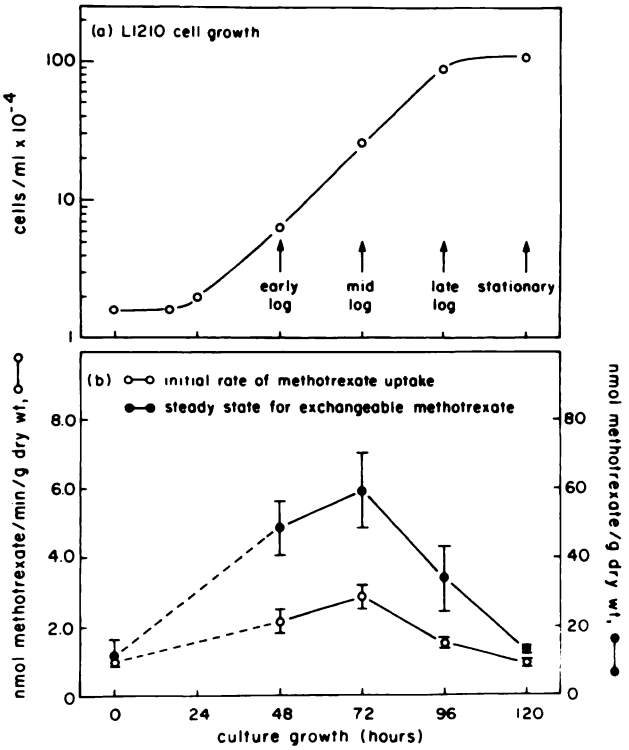


FIG. 2. *Initial rate of uptake and steady-state level for methotrexate during cell growth*

L1210 murine leukemia cells were harvested at each stage of growth and incubated at 37°C with 1.0 μM methotrexate. (a) Representative L1210 cell growth profile. (b) (○——○) Initial rate of methotrexate uptake calculated from the initial linear portion (0–10 min) of the uptake profile (see Fig. 1). (●——●) Steady-state level of exchangeable methotrexate calculated from the latter portion (40–60 min) of the uptake profile (see Fig. 1) and corrected for drug bound to dihydrofolate reductase (see text). Each point represents the mean ± standard deviation calculated from three or more separate experiments.

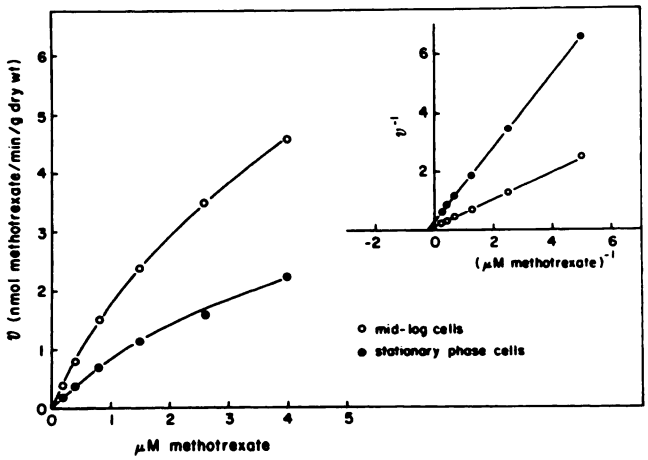


FIG. 3. *Determination of the kinetic constants for methotrexate influx at the midlog* (○) *and the stationary phase* (●) *of growth*

Each curve shows the change in initial velocity of methotrexate uptake with increasing external concentrations of folate analog. Each curve represents a single experiment which has been repeated several times with similar results (see Table 1). Inset: Double-reciprocal (Lineweaver-Burk) plots of these data. Midlog cells: $K_m$ = 4.0 μM; $V_{max}$ = 10 nmol/min/g dry wt of cells. Stationary phase cells: $K_m$ = 4.0 μM; $V_{max}$ = 3.3 nmol/min/g dry wt of cells.

In order to determine the basis for the fluctuation in the initial rate of methotrexate uptake, comparative kinetic analyses of the initial uptake at different external concentrations of methotrexate were carried out with cells harvested at each growth phase. The period of uptake at each concentration was adjusted so that the intracellular accumulation of drug never exceeded the dihydrofolate reductase binding capacity of the cells. In this way, any appreciable back flux of drug was avoided and the values derived at each concentration were true measurements of influx. A representative experiment with midlog cells and with stationary phase cells is shown in Fig. 3. Influx exhibited saturation kinetics in each case. As shown in the inset, double-reciprocal plots of velocity versus concentration revealed a threefold higher $V_{max}$ for midlog cells than for stationary phase cells, but no difference in the apparent $K_m$. Mean values calculated from at least three separate determinations with cells harvested at each stage of growth are tabulated in Table 1. There was no significant difference ($P > 0.1$) in the $K_m$ for methotrexate influx at each stage of growth, while the value for the maximum velocity of influx ($V_{max}$) fluctuated in a manner similar to that found for the initial velocity of uptake shown in Figs. 1 and 2.

Although the threefold difference in the $V_{max}$ for methotrexate influx into midlog as compared to stationary phase cells explained the observed threefold variation in the initial rate of influx, it did not account totally for the greater fluctuation in values for the steady state level of exchangeable drug. Consequently, measurements of the efflux rate of labeled methotrexate were also made with cells harvested at each growth phase. A representative experiment with midlog cells and with stationary phase cells is shown in Fig. 4. The decay-time analysis in the inset showed that efflux was first order in both cases, but

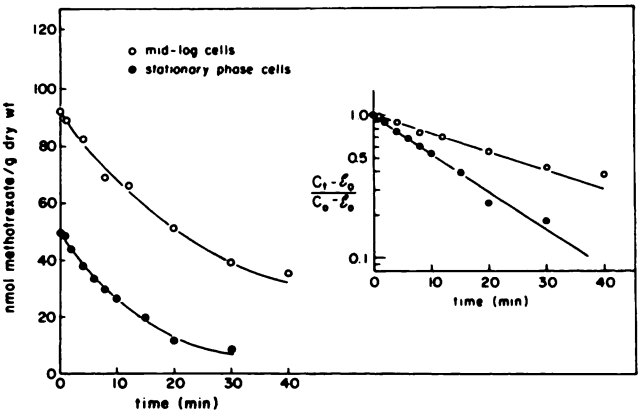


FIG. 4. *Determination of the kinetic constants for methotrexate efflux at the midlog* (○) *and the stationary phase* (●) *of growth*

Each curve shows the efflux of methotrexate from cells preloaded at 37°C for 40 min with 5.5 μM folate analog. All values have been corrected for nonexchangeable drug bound to dihydrofolate reductase. Inset: Decay time analysis of methotrexate efflux. $C_0$, total intracellular drug at 0 time; $C_t$, total intracellular drug at time $t$; $E_\infty$, nonexchangeable drug. Midlog cells, $T_{1/2}$ = 23 min; stationary phase cells, $T_{1/2}$ = 11 min.

was half as fast from midlog cells ($T_{1/2}$ = 23 min) as from stationary phase cells ($T_{1/2}$ = 11 min). Mean values for the efflux of methotrexate calculated from at least three separate determinations with cells at each stage of

TABLE 1

*Kinetic constants for methotrexate influx and efflux*[a]

| | Influx | | | | | | Efflux | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ | | | $V_{max}$ | | | $T_{\frac{1}{2}}$ | | | $K$[b] | | |
| | μM | | | nmol/min/g dry wt | | | min | | | $min^{-1}$ | | |
| Early log | 4.7 ± 1.8 | $P > 0.1$[c] | | 8.5 ± 1.1 | $P > 0.1$ | | 20.0 ± 3.1 | $P > 0.1$ | | 0.035 ± 0.22 | | |
| Midlog | 4.0 ± 0.7 | $P > 0.1$ | $P > 0.1$ | 9.4 ± 1.2 | $P < 0.01$ | $P < 0.001$ | 22.8 ± 2.9 | $P = 0.01$ | $P < 0.001$ | 0.030 ± 0.24 | | |
| Late log | 4.7 ± 1.2 | $P > 0.1$ | | 6.9 ± 0.8 | $P < 0.001$ | | 16.8 ± 2.9 | $P < 0.05$ | | 0.041 ± 0.24 | | |
| Stationary | 3.9 ± 0.6 | | | 3.1 ± 0.6 | | | 11.4 ± 3.3 | | | 0.060 ± 0.21 | | |

[a] Each value represents the mean ± standard deviation calculated from three or more separate experiments.
[b] $K = \ln 2/T_{\frac{1}{2}}$.
[c] Nonpaired $t$ test.

growth are also shown in Table 1. Efflux was slowest during the early and midlog stages and fastest during the stationary phase, the reverse of that shown for influx. However, in contrast to the threefold change ($P < 0.001$) observed in the influx rate, the maximum difference observed in the efflux rate was only twofold ($P < 0.001$).

Graphical representation of these data are shown in Fig. 5, together with a comparison of the experimentally derived (see Fig. 1) versus the theoretically calculated values for the steady-state level of exchangeable methotrexate measured or expected at an external folate analog concentration of 1 μM. The inverse relationship in the fluctuation of the $V_{max}$ for influx and of the first-order rate constant ($K$) for efflux is quite apparent. When these values were used to calculate the expected or theoretical steady state by the previously derived equation (see Ref. 11) $C_{ss} = V_{max}S/K(K_m + S)$, where $C_{ss}$ is the steady-state level for exchangeable drug, and $S$ is the extracellular concentration of folate analog (1 μM), the curve shown by the broken line in panel c was obtained. It can be seen that the calculated values were in excellent agreement with these derived experimentally. This agreement verifies further that the fluctuation in the steady state during growth was in fact due to the reciprocal change in influx and efflux.

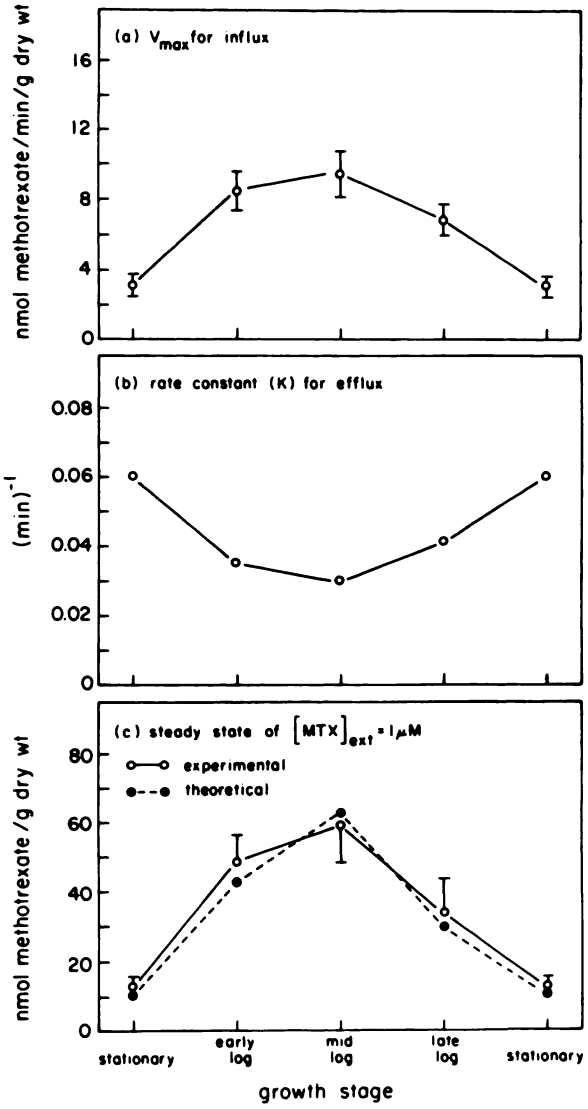


FIG. 5. *Change in the kinetic constants for methotrexate influx and efflux with L1210 cell growth*

L1210 leukemia cells were harvested at the indicated stages of growth and utilized for transport measurements as shown in Figs. 2–4. Each value represents the mean ± standard deviation calculated from three or more separate experiments. (a) $V_{max}$ for methotrexate influx. (b) Rate constant ($K$) for efflux calculated from the relationship $K = \ln 2/T_{1/2}$. (c) Experimental steady state for exchangeable methotrexate (○——○) after incubation at 37°C with 1.0 μM methotrexate and correction for drug bound to dihydrofolate reductase. Theoretical steady state of exchangeable methotrexate (●- -●) calculated from the equation describing free extracellular drug $C_{ss} = V_{max}S/K(K_m + S)$ (see Ref. 11), the kinetic constants from Table 1, and $S = 1$ μM.

## DISCUSSION

These studies showed a variation in the carrier-mediated transport of the folate analog, methotrexate, by L1210 murine leukemia cells during growth in culture. This in itself is not a unique finding since a growth phase dependence for membrane transport is common to a variety of substances in mammalian cell systems (8–10). Also, the variation in intracellular folate analog accumulation during the growth of L1210 cells in culture is not unique to this cell type, since others have reported (16, 17) an increased accumulation of folate analogs in log versus stationary phase HeLa and 3T6 cells. However, this study is the first to provide evidence that the specific kinetic parameters for the influx and for the efflux of methotrexate are both inverse and different in magnitude depending upon the stage of L1210 culture growth. These differences in the kinetic parameters for influx and for efflux during growth result in large changes in the intracellular steady-state level for methotrexate, a factor which may have therapeutic relevance.

Differences in the sensitivity of log versus resting cells to the cytotoxic effects of methotrexate have been reported (17–19). Hyrniuk *et al.* (18) showed that the difference in the sensitivity of rapidly growing versus resting populations of L5178Y murine leukemia cells to methotrexate was not simply due to a difference in the percentage of cells in the S phase at each growth stage, but rather that the S-phase cells from the logarithmic phase of population growth were actually more sensitive to methotrexate cytotoxicity than the S-phase cells in a resting culture. One explanation suggested for this phenomenon was that an increased transport of methotrexate into rapidly growing versus resting cells might result in a more profound inhibition of dihydrofolate reductase.

It is now known that free intracellular (exchangeable) methotrexate (20, 21) in excess of that calculated to stoichiometrically bind all cellular dihydrofolate reductase is necessary to completely inhibit the enzyme. This requirement for free intracellular drug is due to competition (21) for the active site of the enzyme between the folate analog and intracellular dihydrofolate generated by thymidylate synthetase. Since the cellular content of dihydrofolate reductase is in excess in most cell types (20, 22), even a small fraction of active enzyme will prevent methotrexate cytotoxicity. Therefore, the higher the steady state for exchangeable drug, the greater the

probability for cytotoxicity since competition by dihydrofolate will be less effective.

In the present study, the reciprocal and different changes in magnitude in the kinetic parameters for influx and for efflux resulted in a five- to sixfold difference in the steady-state level for exchangeable methotrexate in log versus stationary phase cells. Since the dihydrofolate reductase activity in L1210 cells did not change during growth, the higher level of exchangeable drug may explain the increased sensitivity of rapidly dividing cell populations such as these to methotrexate.

Growth-related changes in methotrexate transport, however, may not provide the answer in each instance to differences in the cytotoxicity observed between rapidly growing and resting cell cultures. Serum-deprived resting cultures of 3T6 mouse fibroblasts are believed (17) to be resistant to methotrexate because they are in the "$G_0$" state and do not traverse the cell cycle. Hence, even though methotrexate uptake and dihydrofolate reductase inhibition could be demonstrated for these cells, it had no apparent effect on their viability. Also, the relevance of transport alterations observed with cells in culture to the tumor cell population *in vivo* must be determined. Hyrniuk and Bertino (19) did observe that the older the L1210 ascitic population isolated from the mouse peritoneal cavity, the less effective was the blockade of labeled deoxyuridine incorporation into DNA produced by a 1-h exposure of $10^{-6}$ M methotrexate *in vitro*. However, their findings may reflect metabolic differences as readily as they may reflect transport alterations.

Turning from the therapeutic considerations of these data, the differences in the transport parameters for influx and for efflux measured during this study are also relevant to membrane carrier function. If it is assumed that there is a single membrane carrier system responsible for methotrexate transport, the reciprocal and nonstoichiometric changes in the influx and the efflux of the folate analog cannot be explained solely on the basis of a variation in the amount of carrier during growth. Other factors such as alterations in carrier distribution across the cell membrane, alterations in carrier mobility or carrier binding, alterations in the functional integration of carrier into the membrane, or a combination of the preceding must also play a role. Any of these possibilities may or may not be attendant to a change in some physical property of the cell during growth or to a change in cellular metabolism.

Conversely, until evidence is provided that one or more of these factors is responsible for the differences observed in influx and efflux, these data may also be explained in terms of separate carriers mediating each flux. A consideration of the possibility is appropriate in view of experimental data already reported from this laboratory (4, 11, 12, 23–25) and elsewhere (2). By using preparations of plasma membrane vesicles, the membrane transport of natural folates may be studied without attendant intracellular metabolism. Studies in this laboratory (23–25) have shown that there is accelerated influx of labeled methotrexate into L1210 plasma membrane vesicles preloaded (1–20 μM) with either 5-formyltetrahydrofolate or 5-methyltetrahydrofolate (heteroexchange diffusion), but not into those preloaded with similar concentrations of nonlabeled analog (homoexchange diffusion). Only after loading with much higher concentrations (>100 μM) of nonlabeled methotrexate could accelerated uptake of labeled analog be demonstrated. Exchange diffusion studies (2) with intact L1210 cells, although complicated by intracellular metabolism, have provided similar results. These data suggest that although there is a membrane transport system for the influx and efflux of reduced natural folates, only the influx portion is utilized by methotrexate. A mathematical model formulated by Dembo and Sirotnak (11) to characterize folate analog transport by L1210 cells also postulated the existence of two separate carrier systems mediating folate analog influx and efflux in order to account for the observed steady state kinetics and related data on exchange diffusion. Finally, the structural specificities for folate analog influx and efflux by L1210 cells are drastically different (4, 12), suggesting a difference in carrier recognition which is difficult to explain as a random property of the same carrier system. For influx, the affinity of the carrier system for 2,4-diaminoheterocycles is pteridine > quinazoline ≫ pyrimidine; for efflux, the affinity of the carrier system is pteridine > pyrimidine ≫ quinazoline.

The identity of the carrier system which might be utilized preferentially by methotrexate for efflux from L1210 cells is at present unknown. Although folic acid shares the transport system for reduced folates (1, 3, 5), evidence has been presented that it also enters the L1210 cell by a second system (26, 27). A recent report (28) suggests that this alternate system is the adenine transport system. It is possible that methotrexate uses this carrier transport system during efflux. However, preliminary investigations in this laboratory (unpublished observations) have found that the activity of the carrier system mediating folic acid influx is five- to sixfold higher at midlog than during the stationary phase of growth. Assuming that this carrier transport system for folic acid functions for both influx and efflux, the difference between midlog and stationary cells appears too large and in the opposite direction to account for the twofold change in methotrexate efflux observed between these two growth stages.

The resolution of the question of single or multiple carrier systems mediating the influx and the efflux of methotrexate must await further experimental work. At present, these data provide basic tools to study regulatory mechanisms responsible for carrier system synthesis and function. If the changes in transport kinetics observed with cultured cells eventually prove to be representative of tumor cells *in vivo*, the differences in the structural specificity and the kinetics for efflux may suggest better therapeutic regimens through the use of different folate analogs to treat older tumor cell populations.

## ACKNOWLEDGMENT

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**Send reprint requests to:** Paul L. Chello, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, N.Y. 10021.