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ANTIFOLATE TRANSPORT IN L1210 LEUKEMIA CELLS

KINETIC EVIDENCE FOR THE NON-IDENTITY OF CARRIERS FOR INFLUX AND EFFLUX

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SUMMARY

The kinetics of methotrexate transport in L1210 cells are described. Data derived from the measurements of initial influx, the complete time-course of uptake, intracellular steady-state level and unidirectional efflux were found to be consistent with a simple empirical equation containing three constants. Properties of the system include the following: (1) saturability of initial influx; (2) approach to steady state during uptake is exponential; (3) the half-time for drug uptake is independent of external concentration and equal to half-time for efflux; and (4) transport is concentrative at low external concentrations, whereas the reverse is true at high external concentrations. These observations are incorporated into a kinetic model which quantitatively accounts for the data on the basis of the hypothesis that influx and efflux take place via different carriers.

INTRODUCTION

The transport of folate analogs, as well as natural folate derivatives by mammalian cells, has been studied [1–15] in several in vitro systems. In the case of L1210 cells, the accumulation of data is very extensive [3–6, 10–15] and strongly suggestive of a carrier-mediated transport process capable of supporting electrochemical gradients across the cell membrane. Although kinetic data has been derived [3, 6, 12–15] with this system, no comprehensive model, which successfully interrelates the various measurable parameters for influx, efflux and steady state, has been proposed. These studies were initiated in an attempt to establish such a model. The results show that, at least under the conditions used, the intracellular accumulation of freely exchangeable (unbound to dihydrofolate reductase) methotrexate is in essence described by a differential equation involving only three constants, the V and K_m for influx and a first-order rate constant (α) for efflux.

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Since the inhibitory 2,4-diamino folate analogs share [3, 6, 11, 14] the same transport system(s) for natural forms of folate in L1210 cells, the transport of methotrexate serves as a useful model for studies of this physiologically important system. The critical pharmacologic relevance of this transport system to the selective antitumor action of the antifolate class of drugs has also been established [16, 17]. In addition, methotrexate offers two unique advantages to the study of transport phenomena; (1) it is not metabolized by the type of cell system employed here, and (2) because binding by intracellular dihydrofolate reductase is many orders of magnitude greater than binding by carrier on the inside of the membrane, accurate measurements of unidirectional influx rates are possible.

EXPERIMENTAL PROCEDURE

Materials and general methodology. 4 days after transplantation as an ascites suspension, L1210 cells were harvested from the peritoneal cavity of BDF_1 (C57BL female/DBA2 male) mice and diluted in 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4). Only non-hemorrhagic ascites were used as a source of cells for experiments. The transplantation of the ascitic L1210 line (V) in vivo has been described [18]. Methotrexate was provided by Lederle Laboratories, Pearl River, N.Y. or obtained from Dr. Harry B. Wood, Jr., Drug Development Branch, Drug Research and Development, National Cancer Institute, Bethesda, Md. The final purity of drug samples had been evaluated bioautographically [19]. The dihydrofolate reductase content of L1210 cells was determined by titration inhibition with methotrexate [20] and the content of drug in cell extracts was determined by an enzyme assay [14] after cells were washed with 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4) and drug removed by heat extraction [13, 14]. Determinations for dry weight and intracellular water have been described [13].

Measurements of drug uptake. Cells were diluted to a concentration of $2.4 \cdot 10^7$/ml ($A_{600\,nm} = 3.0$) in an isomolar buffered salt solution [15]. The final pH was 7.4. Aliquots of 1 ml were incubated with drug at 37 °C. Experiments were terminated by dilution of cell suspensions in cold (0 °C) buffered isotonic saline, then cells were washed twice by centrifugation and resuspension in the same buffered saline. The uptake of drug at 37 °C corrected for drug rapidly associating with cells at 0 °C was used as a measure of intracellular drug. The component of drug rapidly associating with cells at 0 °C (about 1–2 % of the total) occurs within 1 min and represents drug adsorbed on the cell surface which is essentially temperature independent [3, 13, 15]. The amount of drug permeation which is attributed to passive diffusion during these experiments is essentially nil [15]. This fraction of uptake was determined by incubating cells with drug at 37 °C at a concentration of 500 μM which is far in excess of that required to saturate the carrier mechanism.

Measurements of drug efflux. Efflux of methotrexate was measured by loading L1210 cells with drug at 37 °C for a period of 15–20 min and cooling the cells to 0 °C. The suspension was centrifuged, cells resuspended in cold drug-free medium and reincubated at 37 °C. During the preloading step enough drug was added to the external compartment (4.4 μM) to allow for the accumulation of exchangeable drug in intracellular water (total accumulation was 3–4-fold higher than the drug binding

equivalent of the intracellular dihydrofolate reductase). Efflux was determined by measurements of intracellular drug following rapid cooling of cells after varying periods of incubation to stop drug loss.

RESULTS

Preliminary considerations

Prior studies of antifolate transport in tumor cells from this laboratory [12–15] and elsewhere [3, 6, 8] have focused primarily on the kinetics of initial influx and to some extent on efflux. Measurements of initial influx are possible because of the nearly irreversible binding of drug ($K_i \cong 10^{-11}$ M) by the biochemical target, dihydrofolate reductase [20]. Therefore, until the dihydrofolate reductase is saturated, the concentration of drug in intracellular water is essentially zero and uptake is a unidirectional process. Unidirectional efflux rates are obtained by measuring drug loss from preloaded cells which have been resuspended in drug-free medium (see Experimental procedure). The external concentration during both influx and efflux experiments remains constant since the external compartment is large (1 ml during influx and 10 ml during efflux) in comparison to the internal compartment (0.005 ml for 10^7 cells, ref. 15). For reasons which will become apparent in the following Discussion section, an adequate kinetic analysis of transport also requires a measurement of the complete time-course for uptake as well as a study of the steady-state kinetics.

Uptake of methotrexate

The complete time-course of uptake of methotrexate by L1210 cells at 37 °C is shown in Fig. 1 for different external concentrations. Initial uptake under these conditions is linear and exhibits Michaelis-Menten saturation kinetics [12–15]. Influx of drug is described by the following equation for any particular external concentration (S).

$$v = \frac{VS}{K_m + S} \tag{1}$$

A double-reciprocal plot of data on initial influx obtained in this study gave a V value of 2.66 nmol per min per g dry weight and an apparent value for K_m of 4.85 μM.

During the initial linear phase of uptake, all of the dihydrofolate reductase is saturated. Consequently, net uptake begins to decrease because of the accumulation of free (exchangeable) drug and a steady state is attained. The accumulation of free drug is empirically described by a function of the form given in Eqn. 2.

$$C_t = C_\infty [1 - \exp(-\alpha_{in}[t - t_0])] \tag{2}$$

In this equation, C_∞ = steady state, α_{in} = rate constant for free drug accumulation and t_0 represents the time at which free drug starts to accumulate (above approx. 3.65 nmol per g dry weight in Fig. 1). Of course, Eqn. 2 only applies when $t \geqslant t_0$, since $C \cong 0$ before all of the dihydrofolate reductase is saturated. The validity of this equation is illustrated by the linearity of the semilogarithmic plots of the actual uptake data which are shown in Fig. 2. The fact that the plots in this figure are essentially parallel demonstrates that α_{in} is constant for external concentrations be-

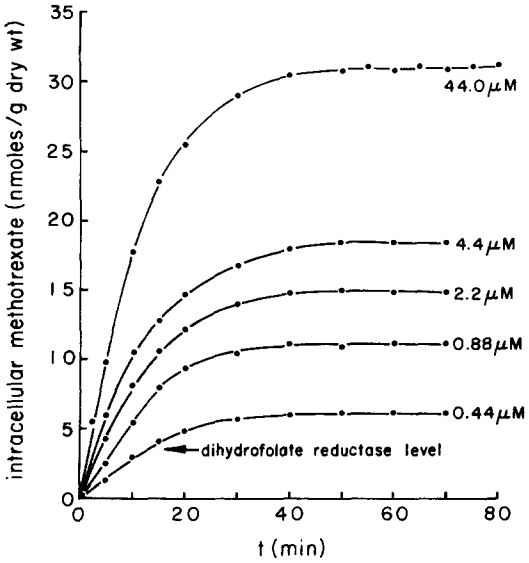

Fig. 1. Time-course of methotrexate uptake by L1210 cells at 37 °C at different external concentrations. The data are an average of 3–6 determinations at each concentration made on separate days. Standard deviation was ±15 %. The enzyme content (3.65 mmol/g dry wt.) was determined as indicated in ref. 12.

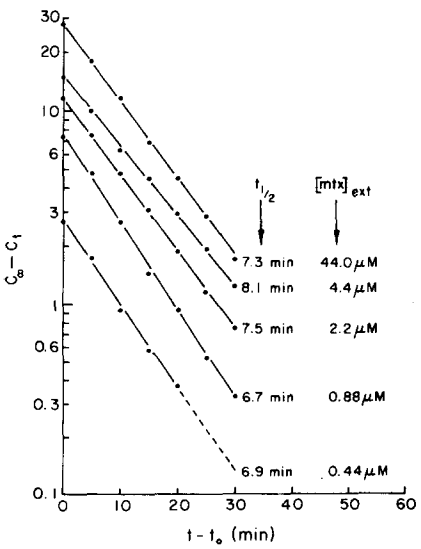

Fig. 2. Semilogarithmic plot of influx data during the accumulation phase for exchangeable intracellular methotrexate. See legend of Fig. 1 for experimental details; C_∞, steady-state level of intracellular exchangeable drug; C_t, level of exchangeable drug at various times. t_0, time at which dihydrofolate reductase is saturated.

tween 0.44 and 44.0 μM. This in our view is a critical observation, since it represents evidence (see Discussion) against the applicability of certain carrier models.

Steady-state levels of intracellular methotrexate

Experimental values for C_∞ at each external concentration (S) of drug were obtained after 60 min of incubation at 37 °C by subtracting the value for enzyme bound drug (3.65 nmol per g dry weight) from the total amount of drug accumulated. Different values for C_∞ are plotted in Fig. 3. The curve (solid line) drawn in the figure was not derived from the data points shown, but was based on the calculated values for C_∞ obtained from Eqn. 3,

$$C_\infty = \frac{VS}{\alpha_{in}[K_m+S]} \tag{3}$$

using values for K_m, V and α_{in} obtained independently during uptake experiments described above. One notes that this equation provides an excellent description of the data for values of S which are up to 10 times larger than the value for K_m and that no new parameters need be invoked to account for the experimental results.

It is of interest from the point of view of the energetics involved to observe that the gradient of methotrexate across the cell membrane differs from that expected for a passively distributed ion of similar charge. Assuming that methotrexate behaves as a negatively charged divalent ion, the expected distribution ratio may be calculated from the Nernst equation. Values obtained using a value for the chloride distribution ratio in L1210 cells reported elsewhere [3] and our own determination for intracellular free space (see legend of Fig. 3) are represented by the dashed line in the figure. The fact that the intracellular level of exchangeable drug at high concentrations is so much lower than the theoretical level calculated for an equilibrating process

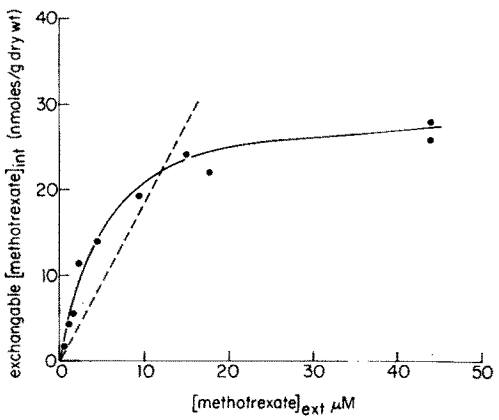

Fig. 3. Intracellular steady-state levels of methotrexate accumulated by L1210 cells at various external concentrations. Values shown represent total drug accumulation less that amount bound to dihydrofolate reductase (3.65 mmol/g dry wt.). The theoretical curve (solid line) was calculated from the equation $C_\infty = V\,S/\alpha\,(K_m+S)$ where $V = 2.7$, $K_m = 4.85$ and $\alpha = 0.09$. The relationship between drug accumulation and external concentration is also shown (broken line) for an equilibrating system. This was calculated from the Nernst equation using a value for the chloride distribution ratio reported elsewhere [3] and a value for intracellular free space of 3.2 ml/g dry wt. obtained during these studies.

is strong evidence that at least part of the efflux current is energy driven. Earlier evidence for a methotrexate efflux pump in mammalian cells has been presented [4–6, 7] by other workers. The smaller positive gradient at low external concentrations indicates that influx is also energy driven. However, errors in estimation of the chloride distribution ratio could also account for this apparent difference.

Efflux of methotrexate

Efflux of methotrexate from L1210 cells at 37 °C is shown in Fig. 4. In this experiment, cells were preloaded at an external concentration of 4.4 μM before centrifugation and resuspension in drug-free medium. Loss of exchangeable drug was rapid with an asymptotic approach to the binding equivalent of the cellular dihydrofolate reductase content. From a semilogarithmic plot of data on the level of intracellular free drug present at varying times during incubation, a first-order relationship with time is demonstrable with a decay time of 7.3 min. The rate constant (α_{ef}) calculated for unidirectional efflux is identical to the rate constant (α_{in}) derived for influx (Fig. 2). The demonstration of first-order kinetics for efflux indicates that any

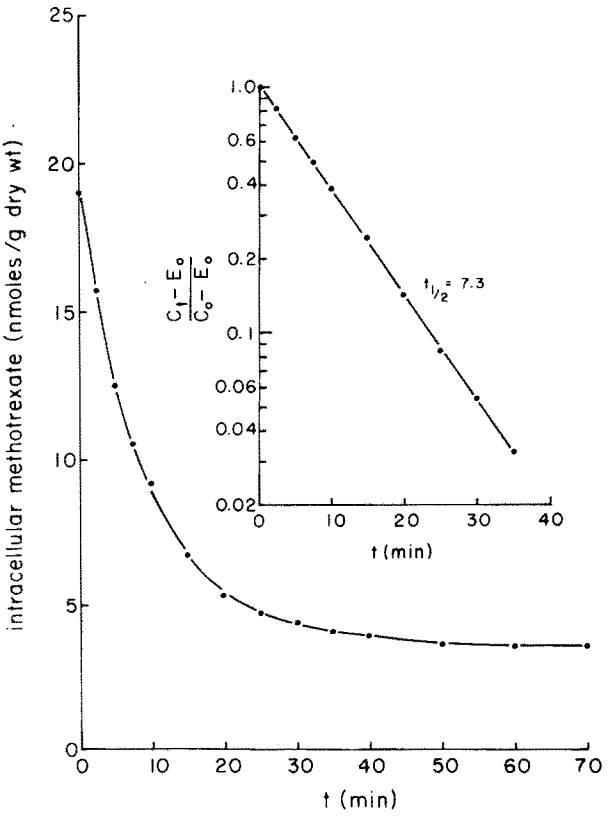

Fig. 4. Time-course of methotrexate efflux by L1210 cells at 37 °C. The data are an average of five experiments done on separate days. Standard deviation was ±17 %. In the insert: C_0, initial drug value; C_t, value at each time and E_0, drug bound to dihydrofolate reductase.

tendency toward saturation is negligible. It should be noted that although passive efflux is also assumed to occur, a first-order term describing this component is not used since it is kinetically indistinguishable from carrier-mediated efflux, thus the rate constant for efflux (α_{ef}) should be considered as the sum of both passive and carrier-mediated components.

DISCUSSION

The kinetics for methotrexate transport in L1210 cells may be summarized by the following equation (Eqn. 4a).

$$\frac{d}{dt}[E+C] = \frac{VS}{K_m+S} - \alpha C \qquad (4a^*)$$

$$E = \frac{E_0 C}{K_i+C} \qquad (4b)$$

This equation empirically accounts for the data on initial influx, half-time for uptake, steady state and efflux which have been described. Eqn. 4b is included in order to explicitly indicate the contribution of enzyme-bound drug (since $K_i = \approx 10^{-11}$ M, $E \cong E_0$, except during the initial period of uptake). The constancy of the $t_{\frac{1}{2}}$ for influx over a very wide range of S (Fig. 2) demonstrates that α is independent of S. Furthermore, since uptake of drug is a simple exponential process, any dependency of α, V or K_m on C must be very small. Energetically, C_∞/S is larger than expected for an equilibrating system when S is small, but C_∞/S approaches zero as S becomes large. A similar observation on steady-state levels of exchangeable methotrexate in L1210 cells has been reported [3] by others, but within a smaller range for S.

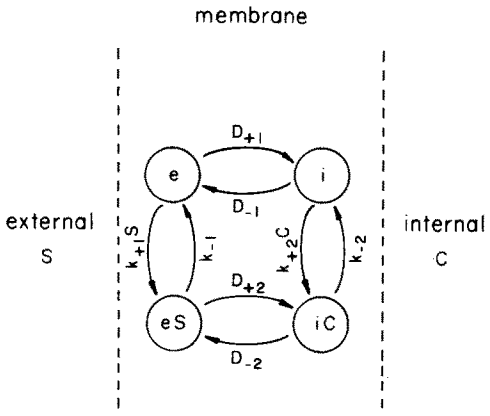

Fig. 5. Kinetic scheme for the antifolate transport mechanism in L1210 cells (Model I). Terms: S, external concentration (μM); C, internal concentration (nmol/g dry wt.); e, efflux carrier (nmol/g dry wt.); i, influx carrier (nmol/g dry wt.); iS, influx carrier-drug complex; eS, efflux carrier-drug complex. The designation on the directed line segments in the scheme denote the elementary rate constants for the associated transitions.

* α denotes the common value of α_{in} and α_{ef}.

A detailed kinetic scheme, commonly used to describe carrier-mediated transport (see ref. 21 for a review of literature on this subject) is shown in Fig. 5. The analysis of this model (referred to as Model I) yields an explicit formula for net flux of drug into the cell (Eqn. 5).

$$\frac{d}{dt}[E+C] = \frac{J_1 S - J_2 C}{1+K_1 S+K_2 C+K_3 SC} \tag{5}$$

This equation (formulas for the various constants are given in Appendix) has a number of consequences which serve as a test of the validity of Model I. First of all, in the limit $C \to 0$, the unidirectional influx is given by a simple Michaelis-Menten equation. Thus, Model I predicts saturable initial uptake with K_m and V given by Eqn. 6.

$$V = J_1/K_1, \text{ and } K_m = 1/K_1 \tag{6}$$

Model I also predicts saturable efflux kinetics when the external concentration is zero. Thus, the slope of a semilog plot like that shown in Fig. 4 should increase as C approaches zero unless $K_2 C \ll 1$. For similar reasons, the approach to steady state during influx will depart from a simple exponential unless $K_2 C$ and $K_3 SC$ are negligible compared to $1+K_1 S$. Since an exponential approach to steady state was observed (Fig. 2) and the slope of the semilogarithmic plots of drug loss during efflux did not increase (Fig. 4), we conclude that in terms of this model K_2 and K_3 cannot be of appreciable magnitude. Under these circumstances, the half-time for approach to steady state at constant external concentration (S) according to Model I is given by Eqn. 7,

$$t_{\frac{1}{2}} = \frac{0.693}{J_2}\left(1+\frac{S}{K_m}\right) \tag{7}$$

where $K_m = 1/K_1$. Even if K_2 and K_3 are not negligible, Eqn. 7 represents a lower limit on the half-time to steady state. Thus over the concentration range studied here (Figs. 1, 2 and 3), Model I predicts a 10-fold increase in the $t_{\frac{1}{2}}$ value. Finally, we note that the steady-state level of intracellular drug according to Model I is given by Eqn. 8.

$$C_\infty = \frac{J_1}{J_2} S \tag{8}$$

Therefore, we would expect a simple linear relationship between C_∞ and S (Fig. 3) regardless of the gradient (positive or negative) across the membrane. Clearly then, Model I is grossly inconsistent with the experimental results.

There is a large category of possible variations of Model I in which a single conserved carrier species is responsible for both influx and efflux. Within the framework of Model I, it is possible to account for the saturable influx kinetics, the first-order efflux kinetics and the exponential approach to steady state. By postulating the existence of two interconvertible conformations of the carrier, it is possible to obtain a non-linear relationship between C_∞ and S. By including the possibility of feedback regulation of the interconversion between carrier forms (such as that proposed by Kotyk and Janacek [21]), it is even possible to obtain both positive and negative con-

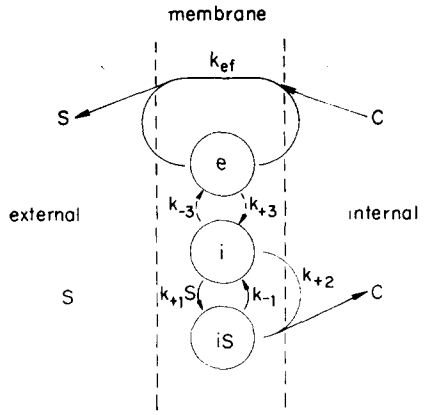

Fig. 6. Kinetic scheme for the antifolate transport mechanism in L1210 cells (Model II). Terms: S, external concentration (μM); C, internal concentration (nmol/g dry wt.); e, efflux carrier (nmol/g dry wt.); i, influx carrier (nmol/g dry wt.); iS, influx carrier-drug complex; k_{ef}, second-order rate constant for reaction between efflux carrier and internal drug ($min^{-1} \cdot$ (nmol/g dry wt.)$^{-1}$); the designations on the directed line segments in the scheme denote the elementary rate constants for the associated transitions.

centration gradients with this type of model. However, all models in which both influx and efflux occur via a single conserved carrier species have the property that unidirectional fluxes depend on both internal and external concentrations. This means that the half-time to steady state will depend on S for this class of models. Since we find this dependency notably lacking, we conclude that this entire category of models can be eliminated.

In Fig. 6, we present a simple kinetic scheme (Model II) which can account for the observations presented here. In this model the presence of two non-identical carriers (i and e) is postulated; where i is involved only with influx and e only with efflux. The i species can only combine with drug which is in the external compartment. The initial product of the reaction is an intermediate complex (iS). This product may dissociate reforming S or proceed via an irreversible transition to produce C and regenerate i. The intermediates in this "translocation "step are viewed as transients which have no kinetic importance. The e species reacts only with drug in the intracellular compartment and translocation is irreversible and rapid when compared to the binding step. Thus, no intermediate complexes of kinetic importance are formed.

If we assume that significant interchange between carriers can occur with rate constants k_{+3} and k_{-3} (shown by the dashed arrows in Fig. 6), then the net flux of drug is given by Eqn. 9.

$$\frac{d}{dt}[C+E] = \frac{[e_0+i_0]\left[k_{+2}S - \frac{(k_{-1}+k_{+2})}{k_{+1}k_{+3}}k_{-3}k_e C\right]}{\left[\frac{k_{-1}+k_{+2}}{k_{+1}}\right]\left[\frac{k_{+3}+k_{-3}}{k_{+3}}\right]+S} \tag{9}$$

In this form the model is in the same category as Model I, since it really involves only a single conserved carrier. The forms for influx and efflux are due merely to a rapidly

reversible change in conformation. An examination of Eqn. 9 reveals that under these circumstances C_∞ is proportional to S and $t_{\frac{1}{2}}$ is proportional to $[K_m+S]$. Therefore, as discussed above, this model fails to explain the experimental data. If it is now assumed that k_{+3} and k_{-3} are zero, then Model II corresponds to a situation in which the two carrier species are at constant levels within the time-scale of these kinetic experiments. The net flux for this case is represented by Eqn. 10.

$$\frac{d}{dt}[C+E] = \frac{k_{+2}i_0 S}{\left[\frac{k_{+2}+k_{-1}}{k_{+1}}+S\right]} - k_{ef}e_0 C \tag{10}$$

This extremely simple model accounts for all of the experimental observations. $V = k_{+2}i_0$, $K_m = (k_{+2}+k_{+1})/k_{+1}$ and $\alpha = k_{ef}e_0$ are the interpretations of the measured kinetic constants in terms of the elementary rate constants.

Under the conditions of the proposed model (Fig. 6), it would still be possible to demonstrate a form of "countertransport" [21] as expected in the case of Model I. Recent reports from other laboratories [4, 11], in fact, provide convincing evidence for a countertransport phenomenon in the case of methotrexate transport by L1210 cells. On the other hand, according to Model II, true "trans-stimulation" (exchange diffusion) effects [21] would not be possible. However, an apparent heteroexchange with naturally occurring folates has been demonstrated [5, 7] and similar observations suggesting "heteroexchange" have also been made in our laboratory (Sirotnak, F. M., unpublished results). Nevertheless, the question as to whether or not this is true "trans-stimulation" remains in doubt since "homoexchange", i.e. exchange of labeled and unlabeled methotrexate could not be demonstrated [5, 7]. Therefore, it is possible that the apparent "heteroexchange" reported is artifactual. On the other hand, it is also possible that complexities not considered in Model II are needed to account for the transport of normal folate derivatives. An interesting modification of Model II, which accounts for the apparent "heteroexchange" between methotrexate and natural folates, is shown in Fig. 7. Model II assumed that the translocation step for influx was irreversible. In Model III we specify that only the return of unloaded carrier (rate

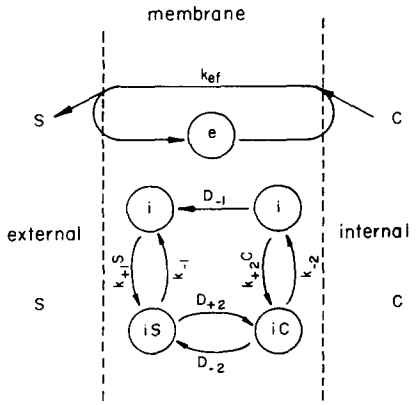

Fig. 7. Kinetic scheme for the antifolate transport mechanism in L1210 cells (Model III). See legends of Figs. 1 and 2 for the meaning of the various symbols.

constant D_{-1} in Fig. 7) from the inside of the membrane is irreversible. If the binding constant for carrier on the inside (k_{+2}/k_{-2}) is small, then Model III reverts to Model II as in the case of methotrexate, which explains the lack of homoexchange. However, if the binding constant of the influx carrier for natural folates on the inside of the membrane is large, then trans-stimulation is possible, since an alternate route for return of influx carrier to the outside of the membrane is opened up. A number of experimental approaches can be devised to test this aspect of the proposed hypothesis.

The notion that non-identical carriers are involved in methotrexate transport by L1210 cells is supported by biochemical evidence [14] showing a drastically different stereochemical specificity for influx versus efflux. This would be difficult to explain as a random property of the same carrier species. On the other hand, the notion of non-identical carrier does not necessarily imply the existence of two entirely different carrier proteins. Alternatively, two very slowly interconverting species of the same protein could account for the experimental observations. Non-identical carriers for influx and efflux have already been postulated for unrelated systems by other workers [23, 27]. Extensive evidence for such a notion has recently been derived [28] in the case of the glutamate transport system in *Escherichia coli*.

APPENDIX

The net flux associated with Model I is given by Eqn. 2. The parameters which appear in this equation J_1, J_2 and K_1, K_2 and K_3 are functions of the elementary rate constants shown in Fig. 5. The precise definition of these parameters is as follows:

$$K_1 = k_{+1}[D_{+2}[k_{-2}+D_{-1}]+D_{-1}[k_{-2}+D_{-2}]]/A$$
$$K_2 = k_{+2}[D_{-2}[k_{-1}+D_{+1}]+D_{+1}[k_{-1}+D_{+2}]]/A$$
$$K_3 = [D_{+2}+D_{-2}]k_{+1}k_{+2}/A$$
$$J_1 = D_{-1}D_{+2}k_{+1}k_{-2}/A$$
$$J_2 = D_{+1}D_{-2}k_{-1}k_{+2}/A$$

where $A = [D_{+1}+D_{-1}][D_{-2}k_{-1}+D_2k_{-2}+k_{-1}k_{-2}]$

The net flux according to Model II is given in Eqn. 10, where the dependence on the elementary rate constants shown in Fig. 6 have been explicitly indicated.

The net flux according to Model III (Fig. 7) is

$$\frac{d}{dt}[E+C] = \frac{i_0 J_1 S}{[1+K_1S+K_2C+K_3SC]} - e_0 k_{ef} C$$

In this equation, the various K values and J values have been obtained from those given above by adjusting $D_{+1} = 0$. k_{ef} is the rate constant for the efflux step as shown in Fig. 7.

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