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Effects of Metabolic Deprivation on Methotrexate Transport in L1210 Leukemia Cells: Further Evidence for Separate Influx and Efflux Systems with Different Energetic Requirements

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Summary. Measurements of methotrexate transport in L1210 cells in the presence and absence of D-glucose reveal that both influx and efflux are depressed in the absence of D-glucose, whereas the steady-state accumulation of drug is enhanced. The reason for the increase in steady state is that the relative decline in efflux is greater than the decline in influx. Analysis of the concentration dependence of steady-state methotrexate accumulation in D-glucose-deprived cells indicates a linear relationship consistent with a one-carrier active transport model. Similar data in nondeprived cells is highly nonlinear and strongly supports the postulate that under physiological conditions influx and efflux of methotrexate are mediated by separate carrier systems. These results indicate that the efflux system, preferentially transporting methotrexate under normal conditions, cannot operate in the absence of D-glucose, whereas the influx system is only partially inhibited under conditions of glucose deprivation.

Key Words methotrexate · transport · L1210 cells · nonidentical influx, efflux routes

Introduction

In an earlier report [3] from this laboratory, we demonstrated that several kinetic features of methotrexate transport in isolated L1210 cells could not be explained by conventional single-carrier transport models. As an alternative hypothesis we proposed that there are two distinct carrier systems involved in the mediation of methotrexate transport. According to our hypothesis, one of these carrier systems (the i-carrier) was solely responsible for mediating the inward flux of methotrexate. This is the high-affinity/low-capacity system shared by reduced folate coenzymes and methotrexate [reviewed in Ref. 5, 12]. The other carrier system (the e-carrier) was an actively driven efflux "pump." The natural substrate for this system remains unidentified.

The initial evidence for the two-carrier model of methotrexate transport was derived purely from kinetic studies of mediated flux in intact metabolically active L1210 cells. More recently, considerable pharmacological and genetic evidence has been obtained that also supports the two-carrier model. We have found that it is possible to select [14, 15] for methotrexate-resistant L1210 cells that demonstrate a sevenfold reduction in V_{max} for influx of methotrexate without any detectable change in the rate constant for efflux. Henderson et al. [9] found that it was possible to inhibit irreversibly and completely block influx of methotrexate by means of carbodiimide-activated substrates without producing similar blockage of efflux. The opposite kind of effect, in which efflux of methotrexate is greatly inhibited but influx is only slightly inhibited, has been obtained [16] by treating cells with low concentrations of probenecid.

Despite this supportive evidence, it may be possible to explain the accumulated data using models other than the two-carrier model. Furthermore, certain studies [6–8] have been interpreted as directly contradicting the two-carrier model. We will now present data comparing methotrexate transport in metabolically depleted and control cells. We find that the two-carrier model is consistent with our results although certain refinements related to the detailed coupling of energy to the e- and i-carriers are necessary. Our data also suggest that some of the evidence contradicting the two-carrier model could arise from the use of experimental conditions that inhibit the normal metabolic activity of L1210 cells.

Materials and Methods

Materials

[^{3}H]methotrexate at a specific activity of 20 Ci/mmol was purchased from Moravek Biochemicals, City of Industry, California. [Carboxy-^{14}C] inulin with a specific activity of 0.48 mCi/mg was obtained from New England Nuclear, Boston, Mass. [^{3}H]methotrexate was purified prior to use by paper chroma-

tography [13]. Radiochemical purity of the final preparation was >97%. Aliquots of purified material were stored at −70 °C. All other reagents had reagent grade.

Methods

Source of Cells

L1210/V cells were obtained by transplantation [12] in $BD2F_1$ mice. Ascites suspension was harvested in cold (0 °C) buffered isotonic saline (0.14 M NaCl + 0.01 M sodium phosphate, pH 7.4) from the peritoneal cavity 5–6 days after transplantation. Contaminating red blood cells were removed by washing of the tumor cell suspension with cold (0 °C) 0.17 M NH_4Cl [11] and then with 0.14 M NaCl.

Transport Measurements

A rapid sampling procedure modified [1] from Plagemann and coworkers [18] was employed during these studies for processing of samples for radioactive scintillation counting. In each experiment, cell suspensions were made in [carboxy-^{14}C] inulin and then centrifuged in order to determine the amount of aqueous medium carried down and trapped in the cell pellet. This value was used to determine a correction of the total radioactivity in the pellet to yield the intracellular radioactivity. Total water in the cell pellet (intracellular and extracellular) was determined by centrifugation with $[^3H]_2O$ [10] added to the cell suspension. Subtraction of extracellular water (determined from the labeled inulin value) from total water gave the intracellular water volume. Values for intracellular water derived in this manner were in agreement with values also determined from net and dry weight determinations [12].

Data derived during transport experiments were expressed as nmol/g dry wt in accordance with conventions established in our earlier [12] studies. Data for intracellular drug concentration are expressed as values for exchangeable drug, that is, unbound to dihydrofolate reductase.

Transport experiments were carried out at 37 °C with cell suspensions (2 to 3×10^7 cells/ml) prepared in buffer-salts solution containing (mM): 107 NaCl, 10 Tris-HCl, 26.2 $NaHCO_3$, 5.3 KCl, 1.9 $CaCl_2$, 1 $MgCl_2$ with or without D-glucose at pH 7.4. Influx was measured by incubating the cell suspension with [^{3}H]methotrexate under conditions [12] which assured unidirectional flux inward. During measurements of influx and net accumulation of [^{3}H]methotrexate a large ratio between the extracellular/intracellular volume was maintained [12] to insure that there was no significant change in extracellular concentration during the incubation period. Similar precautions were made during efflux to ensure measurement of unidirectional flux outward of [^{3}H]methotrexate in cells washed after preloading with this folate compound to intracellular concentrations representing a substantial exchangeable fraction in intracellular water. Experimental procedures used to derive values for influx V_{max}, influx K_m and the efflux rate constant have been described in detail [12].

Other Procedures

Assay of cellular ATP was by firefly luciferase assay using methods published elsewhere [17]. The procedure for chloride determination was also described elsewhere [2]. All data are expressed as mean ±SE (standard error of the mean). In the case of data in the Figures which show more than one mean the legend indicates the maximum value for SE obtained; that is, all of the measured values for SE are below this stated value.

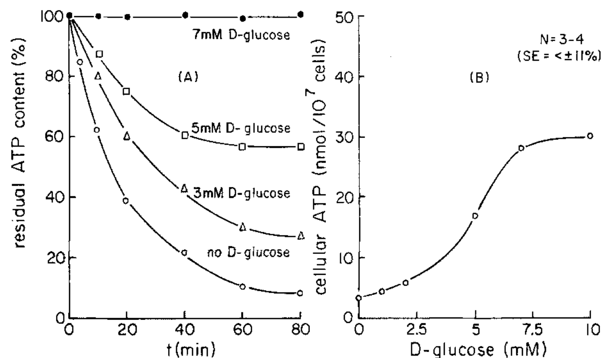

Fig. 1. Variation in cellular ATP content during incubation at 37 °C of L1210 cells in the absence or presence of different concentrations of D-glucose. Aliquots of cell suspension (0.3 ml of 3 to 7.5×10^7 cells/ml were removed at various times for processing. (*A*) Time-course for change in cellular ATP during incubation with no D-glucose and with varying concentrations of D-glucose. (*B*) Dependence of steady-state cellular ATP content on D-glucose concentration. Determinations were made after 80-min incubation. Average of 3 to 4 separate experiments (SE = < ±11%)

Results

Figure 1*A* shows measurements of the cellular ATP content when freshly isolated L1210 cells are incubated for various periods in the presence of different levels of D-glucose. As can be seen from this Figure, at D-glucose levels below the normal concentration found in mouse serum (i.e., 7 mM), the ATP levels in L1210 cells decrease and approach a new steady state with a half-time of 12 to 14 min. Data in Fig. 1B indicate how the final steady-state level of cellular ATP depends on the concentration of D-glucose in the external medium. Variations of external D-glucose between 0 and 10 mM resulted in a 10-fold difference in the cellular ATP content.

The effect of external D-glucose on the time course of accumulation of [^{3}H]methotrexate is shown in Fig. 2. In this experiment cells were suspended in D-glucose-free medium or in medium with 5 or 7 mM D-glucose and [^{3}H]methotrexate added at time zero. Thus, although the external concentration of [^{3}H]methotrexate (2 μM) was constant during the experiment, it can be seen from Fig. 1*A* that the metabolic state of the cells was changing at least for the first 30 or 40 min of incubation. As a consequence, the dynamic changes in intracellular [^{3}H]methotrexate shown in Fig. 2 could involve contributions from changes in the cells themselves. Despite this difficulty, the cell metabolism has stabilized by the time intracellular [^{3}H]methotrexate reaches its final steady-state level (*see* Fig. 1*A*). Thus, the steady-state levels of methotrexate (MTX) recorded in this kind of ex-

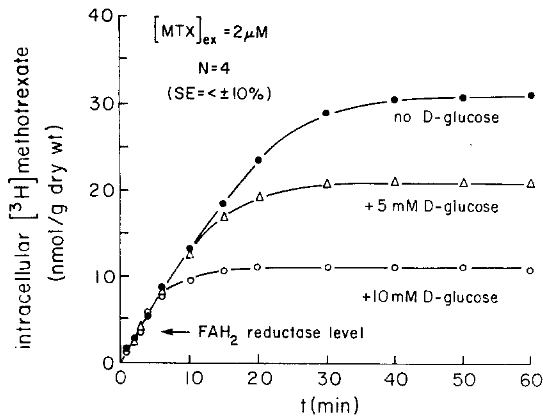

Fig. 2. Time-course for uptake of [^{3}H]methotrexate incubated at 37 °C in varying concentrations of D-glucose. L1210 cells (0.3 ml of 3 to 7.5×10^7/ml) were removed at various times following incubation with 2 μM [^{3}H]methotrexate and with or without D-glucose and processed as described in text. Total drug levels were corrected for drug bound to dihydrofolate reductase (SE = < ±10%)

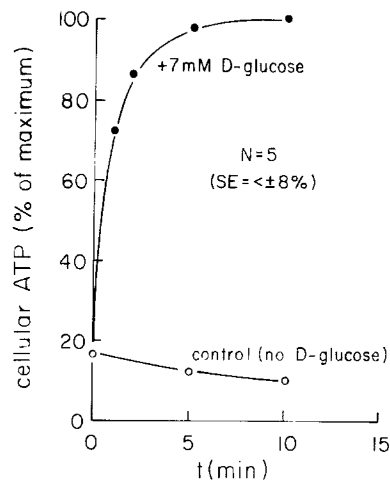

Fig. 4. Effect of D-glucose on cellular ATP levels in D-glucose-deprived cells. See legend of Fig. 3 for experimental details. Average of 5 experiments (SE = < ±8%)

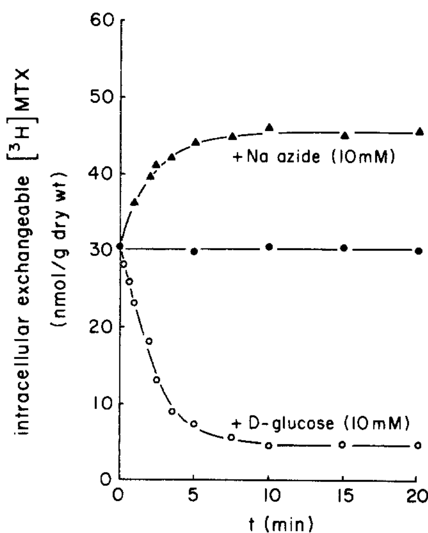

Fig. 3. Effect of D-glucose and sodium azide on steady-state levels of [^{3}H]methotrexate in D-glucose-deprived L1210 cells. The cell suspension (3 to 7.5×10^7/ml) was incubated with 2 μM [^{3}H]methotrexate and with or without 10 mM D-glucose or 10 mM sodium azide after 60-min incubation at 37 °C in the absence of D-glucose. 0.3 ml aliquots were removed for processing at various times. Data is expressed as exchangeable intracellular drug by subtracting a value for drug bound to dihydrofolate reductase from total drug levels. Average of 5 experiments (SE = < ±11%)

periment are fairly stable properties of the transport mechanism for a given level of extracellular D-glucose.

In accord with the effect of sodium azide obtained by Goldman [4], Fig. 2 shows that decreasing extracellular D-glucose and thus decreasing metabolic activity actually increases the accumulation of [^{3}H]methotrexate. In order to demonstrate that this effect of D-glucose deprivation does not involve irreversible changes in cell metabolism, D-glucose was restored to cells that had been deprived of this sugar for a prolonged period. As shown in Fig. 3, after addition of D-glucose, the elevated steady-state level of methotrexate characteristic of deprived cells rapidly drops to the lower level characteristic of nondeprived cells. Figure 3 also shows the rapid and contrasting effect produced when addition of sodium azide is substituted for addition of D-glucose, a result also consistent with an earlier observation by Goldman [4].

In order to see whether the effects of glucose restoration were related to underlying metabolic changes of a general nature, the effect of D-glucose restoration on cellular ATP content was studied in experiments parallel to those of Fig. 3. As indicated in Fig. 4, the rapid effect of D-glucose restoration is related to a similarly rapid restoration of cellular ATP level. Thus it would appear that the effects of D-glucose deprivation on cell metabolism and on transport of methotrexate are readily reversible under the conditions of these experiments. Furthermore, since metabolic poisons such as sodium azide produce effects similar to D-glucose deprivation [*see* Fig. 3 and Ref. 4], it would appear that the alterations of transport are in fact the result of a general metabolic change and not a specific consequence of D-glucose.

It should also be noted that the further increase in steady state for [^{3}H]methotrexate obtained with sodium azide beyond that obtained with D-glucose alone implies that ATP in D-glucose-deprived cells is not completely eliminated. Cellular ATP levels in cells incubated with sodium azide were actually

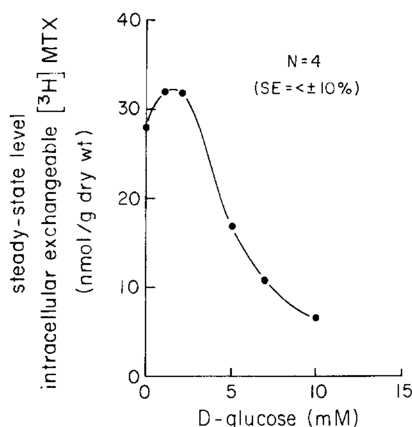

Fig. 5. Effect of different D-glucose concentrations on steady-state levels of [^{3}H]methotrexate accumulation. L1210 cells (3 to 7.5×10^7 cells/ml) were incubated at 37° C for 60 min in the presence of 2 μM [^{3}H]methotrexate and no D-glucose or various concentrations of D-glucose. Data expressed as exchangeable drug (unbound to dihydrofolate reductase). 0.3 ml aliquots removed for processing. Average of 4 experiments (SE = < ±10%)

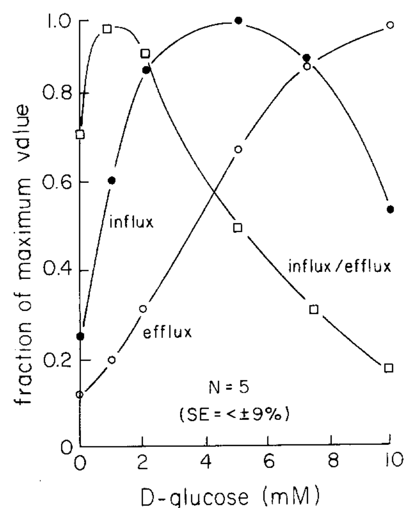

Fig. 6. Effects of different concentrations of D-glucose on unidirectional flux of [^{3}H]methotrexate. Determinations of V_{max} and K_m for initial influx were made after preincubation at 37 °C for 50 min with no D-glucose or various concentrations of D-glucose and incubation at various [^{3}H] methotrexate concentrations. For efflux determinations cells were preloaded at 2 μM [^{3}H]methotrexate during the 50-min preincubation period and efflux measured at the same concentration of D-glucose in drug-free medium. Initial time-courses for influx and efflux were obtained by removing 0.3 ml samples at intervals for processing. Average of 5 separate experiments (SE = < ±9%)

twofold lower than in cells only deprived of D-glucose (*data not shown*).

Figure 5 shows data indicating the detailed changes in steady-state [^{3H}]methotrexate levels as the D-glucose concentration is varied between 0 and 10 mM. Surprisingly, these data demonstrate

Table 1. Parameters of methotrexate influx and efflux in control and glucose-deprived cells[a]

Cells	Efflux	Influx	
	k (min^{-1})	K_m (μM)	V_{max} (nmol/min/g dry wt)
Control[b]	0.21 ±0.02	3.4±0.4	8.7±0.1
Deprived[c]	0.037±0.005	3.3±0.3	2.2±0.3

[a] Number of experiments = 5; errors indicate standard error of mean.
[b] Cells preincubated 40 min in 7 mM glucose.
[c] Cells preincubated 40 min in the absence of glucose.

that in the range of 0 to 2 mM glucose, there is a small increase in the [^{3}H]methotrexate level. Further increases in D-glucose above the 2 mM level result in progressively lower levels of [^{3}H]methotrexate accumulation.

As has been shown in a number of previous studies [3, 5, 12] the initial influx velocity of methotrexate is a saturable (Michaelis-Menten) function of the external concentration. In the present study we find that this description continued to be valid for cells that had undergone prolonged incubation at various levels of external glucose. In a similar way, the unidirectional efflux of methotrexate has been shown to be a well-behaved first-order decay process [3, 12]. Once again, we find that this description continued to give an extremely accurate representation of the efflux process at all levels of external glucose.

In order to understand the kinetic factors underlying the D-glucose dependence of [^{3}H]methotrexate accumulation, measurements of unidirectional influx velocity (V_{max} and K_m) and of the decay constant for efflux (k) were carried out in cells that had been equilibrated with various levels of D-glucose for 40 min. We found that the K_m for influx of methotrexate was independent of the D-glucose concentration (*data not shown*); the relative changes undergone by V_{max} and k are indicated in Fig. 6. Figure 6 also indicates the way that the ratio of influx to efflux rates (V_{max}/k) varies with glucose concentration. Absolute values of k, K_m and V_{max} in control and depleted cells are given in Table 1.

We have previously shown [3] that the steady-state levels of methotrexate in the presence of normal glucose are consistent with the independently measured values of the kinetic constants for influx and efflux. Using the data in Table 1 and Fig. 5 it can be readily verified that the steady-state level is also consistent with the kinetic parameters in glucose-deprived cells.

The data in Fig. 6 demonstrate a marked qualitative difference in the dependence of influx and efflux on D-glucose. The V_{max} for influx undergoes a steep rise between 1 and 2 mM glucose, goes through a broad maximum at approximately physiological D-glucose levels, and ultimately declines at very high D-glucose levels. In contrast, the efflux rate constant rises monotonically throughout the range of 0 to 10 mM D-glucose. We also note that the ratio of influx to efflux reproduces almost exactly the complex dependence of the steady-state MTX level on D-glucose. The rapid initial rise in influx is slightly more than is needed to counteract the rise in efflux, thus producing the small initial increase in steady-state levels of [^{3}H]methotrexate. Above 2 mM, where the influx rate no longer increases rapidly with D-glucose, the steadily increasing efflux rate is sufficient to cause the overlap drop in steady-state [^{3}H]methotrexate accumulation.

Henderson and Zevely [6–8] have suggested that the effect of D-glucose deprivation might be explained on the basis of an anion exchange transport mechanism. According to this idea the influx of a molecule of methotrexate is coupled in a more or less obligatory fashion with the efflux of one or more types of intracellular counter-ions. If D-glucose deprivation were somehow to elevate the intracellular level of these specific counter-ions, then influx of methotrexate would be stimulated and an increase in the steady-state level of methotrexate would result. In contradiction of this hypothesis, the results in Fig. 6 are difficult to reconcile with the idea that counter-ions are elevated in metabolically depleted cells. The data clearly indicate that inward transport is severely depressed in the absence of D-glucose, and that the elevation in intracellular [^{3}H]methotrexate is brought about only because of an even larger depression in efflux.

Since methotrexate is a divalent anion, the changes in the steady-state level of [^{3}H]methotrexate brought about by D-glucose deprivation and metabolic poisons might be directly related to a change in the membrane electric potential. Membrane potential could also influence methotrexate accumulation indirectly (e.g., through an effect on a potential-dependent channel or carrier protein). In order to test these hypotheses, measurements of the chloride distribution ratios were carried out in freshly isolated cells, cells that had been incubated 40 min in the presence of D-glucose, and cells incubated for 40 min in the absence of D-glucose. Parallel measurements of cellular water content were also made on each group of cells.

Assuming that all the intracellular chloride is in a freely exchangeable form, cell chloride and cell water measurements can be used to calculate the so-called chloride Nernst potential of the L1210 cell membrane according to the equation,

$$V_{Cl}=\frac{-RT}{Z_{Cl}F}\ln\frac{[Cl]_{in}}{[Cl]_{ex}}. \tag{1}$$

In this equation R is the gas constant, F the Faraday constant, T the absolute temperature, and Z_{Cl} is the charge of the chloride ion (i.e., -1). If some of the intracellular chloride is bound to various fixed charges in the cell, then the "true" membrane potential will be smaller (i.e., more negative) than the potential calculated using Eq. (1). In any event, it can be seen from the data summarized in Table 2 that the changes in V_{Cl} brought about by D-glucose deprivation are very close to the limit of experimental error. In addition, the small changes that are seen are in the wrong direction to bring about an increase in the intracelllular concentration of methotrexate.

Table 2. Measurement of the chloride Nernst potential[a]

Incubation	Cell H_2O (ml/g dry wt)	Cell chloride (μmol/g dry wt)	V_{Cl}[b] (mV)
None	3.3 ± 0.3	295 ± 27	-11.6
40 min without glucose	3.5 ± 0.3	303 ± 51	-12.8
40 min with glucose	2.9 ± 0.3	268 ± 22	-10.8

[a] Number of experiments = 5; errors indicate standard error of mean.
[b] Calculated using Eq. (1); $[Cl]_{ex}=138$ mM, $RT/Z_{Cl}F=-26.7$ mV.

From the Nernst equation, it can be seen that, if transport of methotrexate were an equilibrating process, the internal steady-state methotrexate concentration would be proportional to the external concentration.

$$[MTX]_{in}=[MTX]_{ex}\exp[-Z_{MTX}VF/RT]. \tag{2}$$

In this equation, Z_{MTX} is the charge of the methotrexate ion (i.e., $Z_{MTX}=-2$), and the membrane potential, V, is approximately equal to the chloride Nernst potential (*see* Table 2).

Equation (2) suggests a simple approach to distinguishing between the two-carrier and the one-carrier models of methotrexate transport. This approach is based on the common sense notion that a single type of carrier can pump methotrexate into cells or out of cells, but that a single carrier cannot actively pump methotrexate in both directions at once. If a hypothetical single carrier pumped methotrexate in the inward direction, then the steady-state intracellular MTX should exceed the predic-

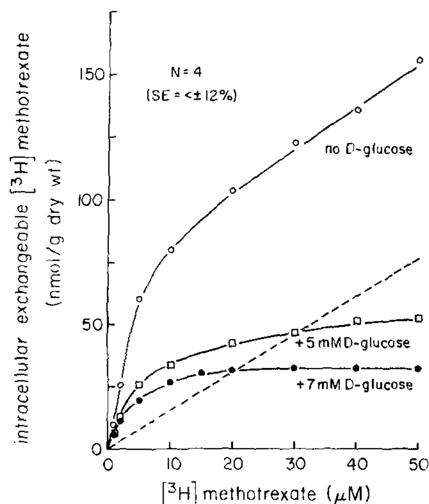

Fig. 7. Steady-state levels of [^{3}H]methotrexate at different concentrations of [^{3}H]methotrexate in the presence and absence of D-glucose. L1210 cells (3 to 7.5×10^7/ml) were incubated at 37 °C for 80 min in the presence of various [^{3}H]methotrexate concentrations and aliquots of cell suspension removed for processing. Average of 4 separate experiments (SE = $< \pm 12\%$)

tion of Eq. (2) for all possible values of $[MTX]_{ex}$. Similarly, if the hypothetical carrier were an outward-directed pump, then the observed steady state should be less than the prediction of Eq. (2) for all values of $[MTX]_{ex}$. In the case of a two-carrier model, it is possible for the drug to be pumped inward under some conditions and outward under other conditions, depending on which of the two carriers has the greater capacity.

Figure 7 shows the actual dependence of the steadystate on $[MTX]_{in}$ and $[MTX]_{ex}$ in control cells [7 mM D-glucose], in cells incubated in 5 mM D-glucose, and in cells deprived of D-glucose. The dashed line in Fig. 8 shows the linear relationship expected on the assumption of an equilibrating transport mechanism. This line was calculated using the value of the chloride Nernst potential from Table 2 as an approximation for the true membrane potential. Even if this estimate of the potential is in error by two- or threefold, we could still conclude from Fig. 7 that control cells pump methotrexate inward when the external concentration is sufficiently low, whereas they pump methotrexate outward when the external concentration is sufficiently high. In distinction to this behavior, D-glucose-deprived cells actively pump methotrexate inward at all external concentrations of drug.

In accord with the results of our previous study [3] we conclude from Fig. 7 that standard models for single-carrier systems cannot explain the transport of methotrexate by L1210 cells under normal physiological conditions. In contrast, the behavior

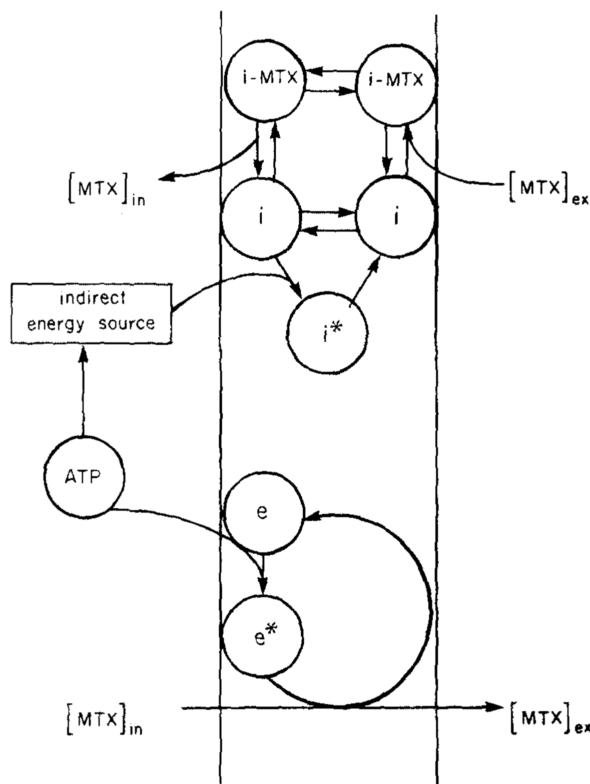

Fig. 8. A model for the mechanism of energy coupling to separate influx and efflux carrier systems postulated for methotrexate transport. The upper part of the Figure represents the proposed mechanism for operation of the influx or i-carrier. Coupling of energy to this carrier is indirect, and if uncoupled, the carrier continues to operate in a "facilitated" diffusion mode. Under normal conditions transport by the i-carrier is biased in the inward direction, but reversal of the carrier is possible at least to some degree. The lower part of the Figure represents the proposed mechanism for the efflux or e-carrier. Direct coupling to metabolic energy is obligatory for functioning of this carrier. Furthermore, this carrier is unable to mediate transport of methotrexate in the inward direction, i.e., reversal of the carrier is negligible

of D-glucose-deprived cells can be reconciled with a single-carrier model. Furthermore, the data indicate that the single active carrier remaining in D-glucose-deprived cells is an inward-directed pump.

Discussion

Figure 8 illustrates a hypothetical model involving two distinct carrier systems that can account for the differential effects of D-glucose deprivation on influx and efflux of methotrexate. As illustrated in the upper part of Fig. 8, one of the carriers (the i-carrier) is the only route by which influx of methotrexate can occur. Active transport in the inward direction is made possible by an asymmetry or bias in the cyclic movement of the loaded and unloaded forms of the i-carrier between the outer and the

inner surfaces of the plasma membrane. Thermodynamically, this transmembrane asymmetry in the cyclic operation of the i-carrier requires expenditure of some form of metabolic energy in order to be maintained.

An essential feature of the i-carrier, according to Fig. 8, is the "indirect" mechanism of metabolic coupling. In essence, an indirect coupling mechanism implies that the transmembrane asymmetry of the i-carrier is powered by an underlying asymmetry of a completely different sort. For example, return of the unloaded i-carrier from the inner to the outer side of the membrane could be accelerated by an elevated intracellular concentration of a specific competitive counter-ion (perhaps one of the naturally occurring folates or even a simple organic anion of some sort). Accelerated cycling of free carrier from the interior to the exterior could also be mediated by a mechanism based on nonspecific differences in the pH or ionic strength of the interior and exterior compartments. Whatever the detailed mechanism the hypothesis of indirect coupling implies that the inward-directed bias of the i-carrier will remain only as long as the underlying asymmetry between the interior and exterior compartments is maintained. This feature of the model is needed to explain methotrexate transport in systems where the underlying transmembrane asymmetry has been experimentally eliminated, such as in membrane vesicles [19, 21]. Such systems still display some carrier activity because the i-carrier continues to operate in a simple "facilitated diffusion" mode. Naturally, membrane vesicles do not demonstrate any ability for active transport unless appropriate conditions of transmembrane asymmetry are recreated (work in progress in this laboratory).

Since the i-carrier is indirectly coupled to metabolic activity, Fig. 8 can account for the decrease of the unidirectional influx velocity when the D-glucose concentration is increased or decreased from its physiological level (*see* Fig. 6). The postulated properties of the i-carrier can also explain the more or less linear concentration dependence of steady-state methotrexate accumulation under conditions of complete D-glucose deprivation (*see* Fig. 7). However, as pointed out previously (*see* discussion of Fig. 7), a theory based on a single carrier has great difficulty explaining the highly nonlinear concentration dependence of steady-state methotrexate accumulation, under physiological conditions.

The properties of the i-carrier as postulated by Fig. 8 are also unable to explain the single most striking feature of the data we have presented, namely, the inverse correlation between methotrexate accumulation and the level of metabolic activity. Clearly, if the i-carrier is an inward-directed pump that operates best at physiological D-glucose concentrations, then accumulation should be maximum at physiological D-glucose and minimal at suboptimal D-glucose, and not the other way around. As we have shown previously [3], the difficulties of explaining methotrexate transport with a one-carrier model can be overcome by invoking the existence of a completely separate carrier system, the efflux or e-carrier (lower portion of Fig. 8).

In contrast to the characteristics of the i-carrier, we propose that the e-carrier is an obligate unidirectional pump that carries methotrexate out of the cell at the direct expense of metabolic energy of some form. This means that the e-carrier will not operate unless a high-energy source (probably ATP) is available in sufficient quantity. The model also implies that the e-carrier can transport methotrexate in only one direction, from the inside towards the outside of the cell membrane.

If sufficient energy is available, the e-carrier is a much faster route for efflux of methotrexate than is the i-carrier. This competitive preference for the e-carrier on the inner surface of the membrane implies that, to a good approximation, methotrexate enters cells solely by means of the i-carrier and leaves solely by means of the e-carrier; the small amount of drug that leaves by "reversal" of the i-carrier is negligible. Thus, under physiological conditions, Fig. 8 reduces to the previously proposed "simple" two-carrier model, with distinct carriers exclusively devoted to influx and efflux, respectively [3]. We have shown that the simple two-carrier model can easily explain the highly nonlinear concentration dependence of methotrexate accumulation by metabolically active cells. By extension, Fig. 8 can also explain these results since it is equivalent to the previous model under physiological conditions. Similarly, both models can readily explain previous kinetic data [3] on the detailed time dependence of uptake and loss of methotrexate under physiological conditions [3].

Figure 8 begins to depart from the previous model under conditions of metabolic deprivation. Under mildly depleting conditions, the efflux route utilizing the e-carrier, though not completely gone, will be greatly reduced. Because of reduced competition from the e-carrier, the efflux route utilizing the i-carrier is no longer negligible; thus, significant efflux occurs by both of the two-carrier systems. Under conditions of complete metabolic depletion, the activity of the e-carrier will eventually

be completely eliminated, whereas the i-carrier will retain some level of function. Under such conditions, the i-carrier must mediate both the influx and efflux routes; in essence, Fig. 8 becomes equivalent to a one-carrier active transport model.

The major refinement of the previous two-carrier model, necessitated by the present data, is the elimination of the "absolute" distinction between influx and efflux carrier systems. Both models propose only one route for influx; however, in the generalized version of the two-carrier model, methotrexate has two alternative routes for efflux: return by the "reversal" of the i-carrier or utilization of the e-carrier. Under normal conditions, the latter of the two routes is greatly preferred; in depleted cells, the former is predominant.

It should also be mentioned that the notion of an active efflux pump was proposed earlier by Goldman [5] on the basis of the differential effects of sodium azide on influx and efflux. However, as these workers had indicated [5], the data presented at that time did not distinguish between different energy processes coupled to the same system or to entirely separate systems. In addition to making this distinction, our own studies document conditions under which this efflux pump can operate.

Our present model of methotrexate transport implicitly assumes that metabolism has a direct effect on the activities of the e- and/or i-carrier systems. An alternative point of view is that metabolic changes do not influence the e- or i-carrier systems but rather acts by affecting the intracellular substrate of these systems (i.e., intracellular methotrexate ions). A simple example of this kind of alternative model is the proposal that metabolic deprivation somehow lowers the intracellular pH causing changes in the ionization of intracellular methotrexate. It must then be further assumed that, for some reason, the new ionic species have a very much reduced affinity for the efflux carrier; whereas the affinity for the i-carrier is not greatly affected.

We cannot claim to have definitely eliminated all alternative "substrate effect" models; however, we believe that they are very unlikely in view of the fact that significant changes in intracellular pH would significantly affect membrane potential and thus the chloride distribution ratio. Furthermore, we note that this uncertainty concerning the site of action of metabolic changes has no bearing on our more general conclusion that methotrexate uses separate carriers for influx and efflux under normal cicumstances, and only a single carrier under conditions of metabolic deprivation; the uncertainty surrounds only the reason for this switch.

Caution must be exercised in extending the present model of methotrexate transport to transport of other folate compounds, particularly the naturally occurring folates. Competition studies [reviewed in Ref. 5, 12] make it quite clear that many folate compounds utilize the same influx route as methotrexate (i.e., the i-carrier). An important exception to this generalization is folic acid itself. Not only does folic acid not utilize the i-carrier, but this substrate appears to have a separate influx carrier of its own [20]. Although the i-carrier is utilized by many different folate compounds, there is great variability in the affinity (as measured by the K_m for influx) of different compounds. Similar variations in the affinity of the i-carrier for different folates must also occur on the inner surface of the plasma membrane. Likewise, different folates doubtless have different affinities for the e-carrier, and, by analogy with the situation for folic acid, some compounds may not utilize the e-carrier at all.

If we consider the extension of the two-carrier model to transport of methotrexate or some other given folate in a variety of cell types or mutant lines, then the various cell types should generally fall into one of three categories according to their mechanism of transport. Group I cells will behave like L1210 cells; i.e., under normal metabolic conditions methotrexate will enter by the i-carrier and be pumped out almost exclusively by the e-carrier. Steady-state accumulation of methotrexate will be inversely related to the level of metabolic activity. Group II cells will lack the e-carrier or at least have very greatly reduced e-carrier activity. In such cells, both influx and efflux of methotrexate will occur by means of the i-carrier. Experimentally, such cells should actively accumulate methotrexate under all experimental conditions, and there should be a direct correlation between level of metabolic activity and steady-state accumulation. Finally, Group III cells will consist of cell types that completely lack the i-carrier system or at least have very greatly reduced i-carrier activity. Experimentally, such cells should actively exclude methotrexate except under conditions of very strong metabolic deprivation. Cells of Group III should be very resistant to methotrexate toxicity, and we have recently used this approach to select and clone a type III cell [14, 15]. As we would expect, this cell line exhibited a greatly reduced V_{max} for influx of methotrexate without any significant change in the rate constant for efflux.

Recently, Henderson and Zevely [6–8] reported evidence that appeared to contradict the notion of a two-carrier model for methotrexate transport. These workers observed from experiments with L1210 cells carried out in a Hepes-Mg-sucrose

buffer, that efflux of methotrexate could be stimulated by the presence of reduced folates in the external compartment. They also reported that a diverse group of anions, including phosphate, sulfate and chloride, which are weak competitive inhibitors of methotrexate influx, could also stimulate efflux of methotrexate when present in the external compartment. Since these experiments were carried out in nonphysiological buffers and in the absence of an exogenous energy source, the results should reflect the properties of nonenergized cells. The present study indicates that such cells catalyze both influx and efflux of methotrexate by means of the i-carrier; thus, the ability to transstimulate efflux of methotrexate in nonenergized cells is understandable. The present model predicts that such results would not be obtained under physiological conditions.

The idea that methotrexate influx occurs by an anion exchange mechanism originated with Goldman [5] and has been recently expressed by Henderson and Zevely [6–8]. Whether or not this idea is really correct, its essential feature from the viewpoint of the present data is that the influx carrier can act either as an inward-directed MTX pump or as an equilibrating carrier depending on whether or not an energy source (i.e., anion gradient) is available. Thus our present data are consistent with the hypothesis that the i-carrier system operates by an anion exchange mechanism, although other models of the i-carrier could be equally acceptable.

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