BBA 75355

CHARACTERISTICS OF FOLIC ACID TRANSPORT IN THE L1210 LEUKEMIA CELL

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(Received July 1st, 1969)

SUMMARY

Uptake of folic acid was studied in L1210 leukemia cells. Folic acid was rapidly metabolized in this cell system and the metabolites appeared to be exchangeable across the cell membrane. Metabolism was partially reduced when cells were pretreated with methotrexate (4-amino-4-deoxy-10-methylpteroylglutamic acid) to inhibit dihydrofolate reductase.

The energy dependence of folic acid transport was investigated in cells pretreated with methotrexate to minimize metabolism. These studies suggested an energy-dependent efflux mechanism for folic acid, since (1) the ratio of intracellular to extracellular folic acid at the steady state was far below that predicted for a passive equilibrating transport system and (2) inhibition of energy metabolism resulted in a decrease in the unidirectional efflux of folic acid and a marked rise in the net uptake velocity. Although interpretation of the data is complicated by residual metabolism of folic acid, many of the findings for folic acid are similar to those observed for methotrexate—a compound which shares, at least in part, the same transport mechanism as folic acid and is not metabolized under the conditions of these experiments.

Compatible with an active efflux mechanism was the observation that net uptake of [^{3}H]folic acid is a complex process in which back flux of label becomes significant within 30 sec after the addition of folic acid to the extracellular medium and at cell folate levels far below the steady state. Following this, the rate slows to an essentially constant velocity for the remainder of the period of observation and represents a very small difference between much larger unidirectional influx and efflux rates.

Net uptake is highly temperature dependent (Q_{10} of 5) and is markedly inhibited by methotrexate and folinic acid. Addition of these substances to the extracellular medium during uptake of folic acid results in net efflux of essentially all intracellular label.

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INTRODUCTION

Previous studies from this laboratory[1] indicated that transport of the folic acid analog, methotrexate (4-amino-4-deoxy-10-methylpteroylglutamic acid), in the L1210 leukemia cell is compatible with a carrier-mediated process capable of sustaining uphill transport of methotrexate into the cell. This and other studies[2] further suggested that the naturally occurring folates, folic and folinic acids, share at least in part the same carrier mechanism as methotrexate. Continued investigation of the energetics of methotrexate transport indicate that the energy requirements of this transport system are unusual in that energy-dependent transport of methotrexate appears to be enhanced in the presence of metabolic inhibitors. To account for this phenomenon, a model was proposed in which two different modes of energy coupling to this transport system result in either augmentation or depression of the intracellular methotrexate concentration[3].

The following studies were undertaken with folic acid to characterize the transport process for this naturally occurring folate and to compare these findings with the observations for methotrexate.

METHODS

L1210 murine leukemia cells were grown in cell culture as previously described[1]. In an individual experiment, the cells were separated by centrifugation at 600 $\times$ *g* for 2 min and resuspended into medium which consisted of the following: 117 mM NaCl; 5.3 mM KCl; 1.1 mM NaH_2PO_4; 26.2 mM $NaHCO_3$; 2 mM $CaCl_2$; 1 mM $MgCl_2$. The pH was adjusted to 7.4 by addition of HCl in the presence of O_2–CO_2 (95:5, v/v). The cytocrit of the final suspension ranged from 5–10 %. The cell suspension was incubated in a covered flask through which warmed, humidified O_2–CO_2 (95:5, v/v) was circulated. The suspension was stirred with a glass rod driven by a variable-speed motor and kept at constant temperature by immersion of the flask in a water bath at 37°. In the absence of energy substrate, the pH of the cell suspension did not fall more than 0.2 pH units during the course of a 1-h experiment.

Cells were incubated with test substances, and at timed intervals small aliquots were removed and transferred to centrifuge tubes containing 10 vol. of ice-cold 0.85 % NaCl solution. The samples were centrifuged at 2000 $\times$ *g* for 20 sec, the supernatant was aspirated and the cell pellet was washed twice more by resuspending it with the ice-cold NaCl solution in order to remove extracellular folic acid. Three or more washes did not remove any additional folic acid. The cell pellet was then aspirated with a Pasteur pipet and transferred to a No. 3 glassine paper tare. Weighings were performed with a Cahn RG automatic electrobalance with output to a No. 3440A Hewlett Packard digital voltmeter. After overnight incubation at 65°, dry weights were obtained. The dried cellular material with tare was placed in small capped vials, 0.5 ml of 1 M KOH was added and the cells were digested in a 70° water bath for 1 h. Then 0.2-ml aliquots were added to 18 ml of a liquid scintillation mixture consisting of 70 % toluene, 30 % methanol, 3 g/100 ml 2,5-diphenyloxazole and 37.5 mg/100 ml 1,4-bis-(5-phenyloxazolyl-2)benzene. All samples were counted in a Packard Tri-carb 4000 liquid scintillation spectrometer.

Estimation of the concentration of folic acid and Cl^- in the intracellular water

of a cell pellet was performed by techniques previously described[1]. [^{14}C]Inulin was employed as an extracellular marker for these studies and scintillation counting was carried out with simultaneous recording of ^{3}H and ^{14}C. Cl^- was determined on the KOH digest with the Buchler-Cotlove chloridimeter.

In efflux experiments, cells were incubated with [^{3}H]folic acid then recovered by centrifugation and resuspended into a large volume of fresh folic acid-free medium. Samples were obtained rapidly at timed intervals and injected into centrifuge tubes containing 10 vol. of ice-cold NaCl solution. The cells were then processed as described above except for the omission of further washes.

MATERIALS

[^{3}H]Folic acid specifically labeled in the 3',5'-positions of the *p*-aminobenzoic acid moiety was obtained from Nuclear Chicago (Des Plaines, Ill.). It was purified by fractionation on a DEAE-cellulose column with elution by a 0.1–0.4 M NH_4HCO_3 (pH 8.3) linear gradient[4]. Optical transmittance was monitored at 254 nm. The fractions containing the pure folic acid were lyophilyzed until the NH_4HCO_3 was removed then stored in the dark at −70°. The final folic acid concentration was measured in the Beckman DU spectrophotometer in 0.1 M NH_4HCO_3 (pH 8.3) using molar absorbance coefficient (log ε) values of 3.9 at 360 nm and 4.4 at 280 nm. Final specific activities were approx. 500 mC/mmole.

The radiochemical purity of the [^{3}H]folic acid was confirmed by repeated column chromatography or by ascending chromatography using Whatman No. 1 paper with 0.5 % Na_2CO_3. By these techniques, more than 98 % of the radioactivity was recovered as folic acid. Unlabeled folic acid was obtained from Mann Research Laboratories (New York, N.Y.); methotrexate and 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (folinic acid) from Lederle Laboratories (Pearl River, N.Y.); [^{14}C]inulin was obtained from New England Nuclear (Boston, Mass.).

RESULTS

Metabolism of folic acid

Studies were done to determine whether folic acid was metabolized during the course of experiments. Cells incubated with 100–200 μM folic acid for 1 h were separated by centrifugation and washed twice with ice-cold NaCl solutions. NH_4HCO_3 buffer (0.1 M) was added and the cells were subjected to sonic oscillation for three 10-sec periods while maintaining the temperature at 0°. The sonicate was centrifuged at 10000 × *g* for 30 min. The sediment contained less than 0.5 % of the total radioactivity. 1 mg of nonlabeled folic acid was added to the supernatant, and it was fractionated on a DEAE-cellulose column as described above. In eight such experiments, 65 ± 8 % (S.E.) of the recoverable counts were identified as unchanged folic acid, while the remaining counts were in two separate unidentified peaks.

The major pathway for folic acid metabolism involves reduction to its tetrahydrofolate derivatives. This process, which requires the presence of dihydrofolate reductase, is readily inhibited by methotrexate because of the intense affinity of methotrexate for this enzyme[5]. Because metabolism of folic acid complicates interpretation of the data with respect to the mechanism of folic acid transport, an

attempt was made to eliminate metabolism of folic acid by pretreating the cells with methotrexate. Cells were incubated with 0.5 μM methotrexate for 30 min to bind the dihydrofolate reductase[1] completely. The cells were then recovered by centrifugation and washed 3 times with 37° methotrexate-free medium. With each wash the cells were equilibrated with the medium for 3 min to facilitate efflux of methotrexate not bound to dihydrofolate reductase. After the last wash, free intracellular and extracellular methotrexate is absent while methotrexate binding to the enzyme is essentially irreversible under these conditions[1]. Following this, metabolism of [^{3}H]folic acid was evaluated, as described above, in these cells and control cells treated similarly except for the omission of methotrexate from the pre-incubation procedure. In three such experiments, of the total radioactivity taken up by the cells, the per cent which was not folic acid was reduced by half in the cells pretreated with methotrexate. Thus metabolism of folic acid can be reduced but not eliminated by this procedure.

In the studies to follow, cell radioactivity will be expressed as nmoles ^{3}H per g dry wt. 1 mole of ^{3}H will be considered equivalent to 1 mole of folic acid or 1 mole of its metabolites containing the labeled benzene moiety.

Time-course of uptake and efflux

The time-course of uptake of label was essentially linear when observed over an interval of 3–110 min (Fig. 1). Extrapolation of this line to zero time gave an intercept which is considerably above the point of origin; however, when measurements were taken at 10-sec intervals after the addition of folic acid, as shown in Fig. 2, the initial uptake velocity as determined from the slope through the first few points, 0.35 nmoles/min per g dry wt., greatly exceeded the later linear uptake rate by a factor of 7.5. Thus the net uptake velocity of label approximates the initial uptake rate only transiently. There is, in addition, a measurable amount of label which rapidly associates with the cells at 0° while the time-course indicates that transport

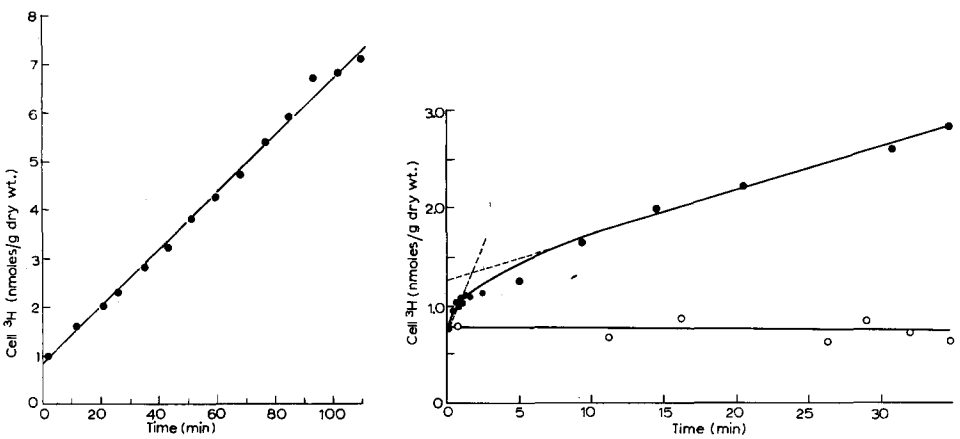


Fig. 1. Time-course of uptake. Extracellular folic acid concentration was 5.7 μM.

Fig. 2. Time-course of uptake with rapid initial points obtained. Extracellular folic acid concentration was 10.2 μM. ●, uptake at 37°; ○, uptake at 0°. The dashed lines compare the slope through the first seven points with the slope through the final five points. Both of these lines were drawn by the method of least squares.

at this temperature is negligible. This fraction of label probably represents an adsorption of folic acid to sites on or near the cell surface.

Fig. 3 suggests that the early fall in net uptake of label is related to a rapid build-up of a significant efflux component. It can be seen that net uptake is considerably slower than the unidirectional efflux of label into a large volume of folic acid-free medium. It is also apparent from this graph, as well as other efflux studies to follow, that virtually all intracellular label leaves the cell under these conditions except for the component adsorbed to the cell surface. Since a good part of intracellular label represents folic acid metabolites, this indicates that these compounds are in a form which can readily leave the cell.

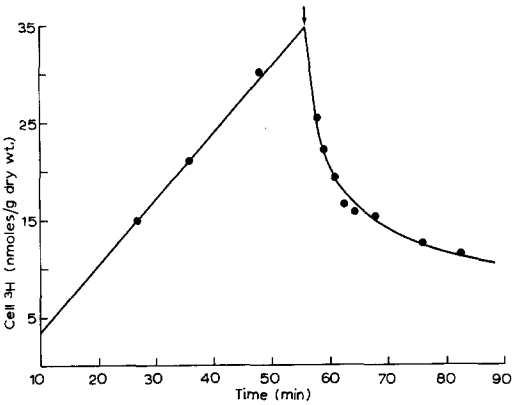


Fig. 3. Uptake and efflux. Cells were incubated for 55 min at an extracellular folic acid level of 70 μM, recovered by centrifugation and resuspended in folic-acid-free medium at the time indicated by the arrow.

The high unidirectional efflux rate as compared to the slower net uptake process is also observed in cells pretreated with methotrexate as described above, while the net uptake of label is reduced by 25 % in these cells. In the absence of significant free intracellular and extracellular methotrexate under these conditions, this inhibition of net uptake rate is not likely due to a primary alteration in the transport of folic acid and suggests that the metabolite(s), the production of which is inhibited by methotrexate, leave the cell at a slower rate than folic acid.

Uptake of folic acid is considerably slower than that of methotrexate. For instance, from the data of Fig. 2, the initial uptake rate for folic acid at 10 μM is less than 1/10th that observed for methotrexate at a similiar extracellular concentration and this represents about twice the extracellular level necessary to achieve half maximal influx velocity for methotrexate[1].

Unidirectional fluxes during net uptake of folic acid

The relationship between net uptake and the unidirectional fluxes was evaluated in another type of experiment. Two aliquots of cells were incubated in the presence of either labeled or unlabeled folic acid at 100 μM. The groups were then subdivided and tracer influx, efflux and net uptake were measured over a 30-sec interval as an approximation of the unidirectional fluxes. This is described in detail for a representative experiment in Table I. It can be seen that the net uptake of label represents a

TABLE I

Cells were incubated at 37° with either labeled or unlabeled 100 μM folic acid for 30 min. The cells were recovered by centrifugation, washed twice with fresh medium at 0°, then resuspended into medium at 37° which contained the test substances, and flux was measured over 30 sec. Correction was made for surface binding by measuring uptake at 0°. Velocities are expressed in nmoles per min per g dry wt.

Preincubated with 100 μM unlabeled folic acid ↓ *Resuspended with 100 μM [^{3}H]folic acid*	*Preincubated with 100 μM [^{3}H]folic acid* ↓ *Resuspended with 100 μM unlabeled folic acid*	*Preincubated with 100 μM [^{3}H]folic acid* ↓ *Resuspended with 100 μM [^{3}H]folic acid*
Approximate unidirectional influx	*Approximate unidirectional efflux*	*Net uptake*
4.0 ± 0.2	3.4 ± 0.4	0.8 ± 0.1

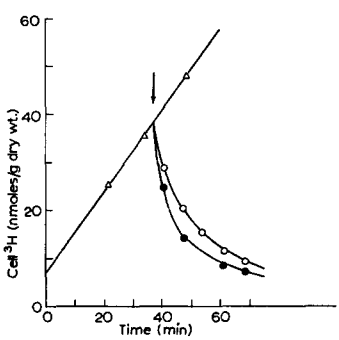
small difference between the much larger 30-sec uptake and efflux rates. Since this is only an approximation of initial rates the difference between the true unidirectional fluxes and the net uptake velocity is even greater.

The effect of folinic acid and methotrexate on exchangeable intracellular label

Cells were incubated with [^{3}H]folic acid following which two aliquots were removed and exposed to either methotrexate or folinic acid (Fig. 4). Under both conditions there was a rapid net efflux of essentially all intracellular label except for the fraction considered to be adsorbed to the cell surface.

Transport of folic acid in cells pretreated with methotrexate

To minimize the complicating factor of folic acid metabolism in the interpretation of the following studies, experiments were performed on cells pretreated with methotrexate as described in *Metabolism of folic acid*. Again, under these conditions all free intracellular and extracellular methotrexate is removed.



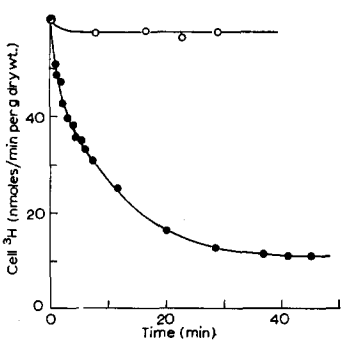


Fig. 4. Cells were incubated with folic acid and at the time indicated by the arrow aliquots of the cell suspension were removed and exposed to 0.9 mM methotrexate (●) or 1.0 mM folinic acid (○). The extracellular folic acid concentration was 0.1 mM (△).

Fig. 5. The effect of temperature on folic acid efflux. Cells were loaded with folic acid then separated by centrifugation and resuspended into folic acid-free medium at 0° (○) and 37° (●).

Temperature dependence of uptake and efflux

Net uptake is highly temperature dependent. The Q_{10} from 27 to 37° is 5. At 0° uptake of label is negligible (Fig. 2).

Efflux of folic acid and its metabolites is also temperature sensitive. At 0° loss of intracellular label is negligible (Fig. 5).

The effect of structural analogs on folic acid uptake

Folinic acid and methotrexate are potent inhibitors of folic acid uptake. When the extracellular folic acid concentration was 100 μM, methotrexate or folinic acid at 50 μM resulted in 75 and 70 % inhibition of net uptake velocity, respectively. At higher extracellular methotrexate levels, net uptake of folic acid was completely abolished.

Steady state

Studies were done to estimate the ratio of free intracellular to extracellular folic acid at the steady state. Although intracellular label will be referred to as folic acid, it is to be understood that a portion does represent folic acid metabolites not inhibited by methotrexate. Because of the slow rate of folic acid net uptake, a method was chosen in which the cells were loaded with folic acid for 45 min following which the extracellular folic acid concentration was reduced by dilution with fresh buffer and the cell folic acid level allowed to fall to equilibrium over an additional 45 min. One aliquot of cells was separated and resuspended into folic acid-free medium to determine the fraction of exchangeable cell folic acid.

Table II shows the results of two such experiments at four different extracellular folic acid concentrations ($[Fol]_e$). The concentration of exchangeable folic acid in the intracellular water ($[Fol]_i$) has been corrected for the total nonexchangeable portion. In addition, Cl^- measurements were made on these same cells. From the Cl^- distribution ratios and the Nernst equation, it was possible to predict the steady-state ratio for a passive equilibrating transport system, assuming folic acid to be present intracellularly as a divalent anion. From Table II it is apparent that at each concentration studied, the measured $[Fol]_i/[Fol]_e$ was less than the predicted value. This ratio appeared lower at high concentrations. There was no consistent relationship between the extracellular folic acid concentration and the Cl^- distribution ratio.

TABLE II

FOLIC ACID DISTRIBUTION RATIO AT THE STEADY STATE

Expt. No.	$[Fol]_e$ (μM)	$[Fol]_i$ (μM)	$[Fol]_i/[Fol]_e$ *ratio*	$[Cl^-]_e/[Cl^-]_i$ *ratio*	*Predicted* $[Fol]_i/[Fol]_e$ *ratio*
176	0.22	0.062	0.28	1.43 ± 0.07	0.49
	10.5	1.03	0.098	1.19 ± 0.01	0.71
177	0.35	0.16	0.46	1.28 ± 0.05	0.61
	205	15.5	0.075	1.45 ± 0.05	0.47

Abbreviations: $[Fol]_e$ and $[Fol]_i$, extra- and intracellular folic acid concn., respectively; $[Cl^-]_e$ and $[Cl^-]_i$, extra- and intracellular Cl^- concn., respectively.

* Mean ± S.E. of 3–5 measurements.

Effect of anaerobiosis on uptake and efflux of folic acid

When O_2 is replaced by N_2, the net uptake of folic acid is markedly increased (Fig. 6), in this experiment by a factor of 7. Often uptake stimulation was transient although in this experiment the late fall in net uptake velocity might also be related to the cells approaching the steady state. In no case was the intracellular concentration observed to reach the level of the extracellular fluid or the value expected for a passive equilibrating system. Uptake stimulation is reversible as readdition of O_2 returns the uptake velocity to the control rate. Addition of glucose alone does not significantly affect net folic acid uptake but glucose abolishes the stimulatory effect of anaerobiosis; interpretation of this effect is complicated by a concurrent fall in pH. NaN_3 (10 mM) also enhanced net folic acid uptake, although in a few experiments this effect was not observed; the reason for this is not clear.

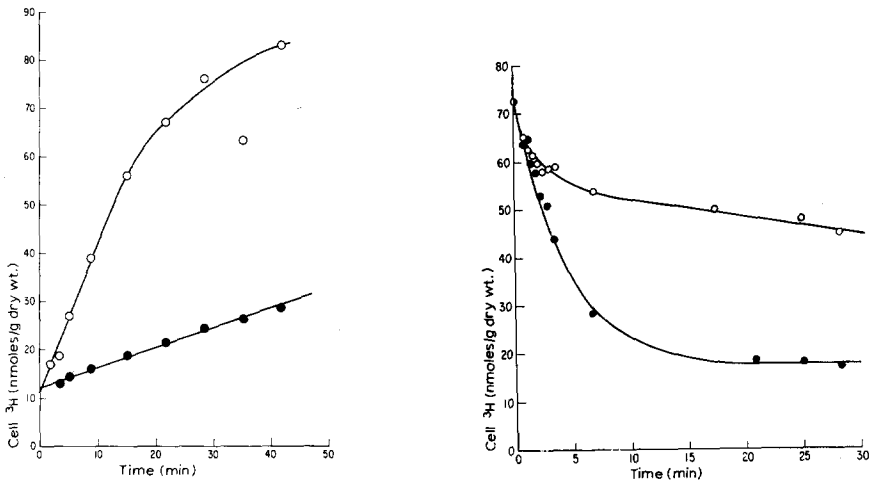


Fig. 6. The uptake of folic acid in the presence of O_2 (●) or N_2 (○). Extracellular folic acid concentration is 250 μM. The cells were pretreated with methotrexate as described in the text (*Metabolism of folic acid*).

Fig. 7. Cells were incubated with 250 μM folic acid for 30 min in the presence of O_2 then exposed to N_2 for an additional 15 min. The cells were then recovered by centrifugation and resuspended into folic acid-free medium in the presence of O_2 (●) or N_2 (○). The cells were pretreated with methotrexate as described in the text (*Metabolism of folic acid*).

Fig. 7 illustrates the effect of anaerobiosis on folic acid efflux. Efflux in the presence of O_2 was rapid with the loss of all exchangeable label within 25 min. In the absence of O_2 there is a small rapid initial efflux of folic acid following which the rate of efflux is markedly reduced. In experiments continued over a longer interval, the bulk of exchangeable intracellular label leaves the cell at a low, essentially constant, velocity. It is clear that the overall time-course of efflux with anaerobiosis cannot be characterized by a single exponential, and this suggests the presence of two efflux components similar to that described for methotrexate[3].

DISCUSSION

Previous studies indicated that transport of the nonmetabolized folic acid analog, methotrexate, is compatible with a carrier mediated process[1]. Transport of

methotrexate into the L1210 cell appeared to be uphill since a small electrochemical potential difference across the cell membrane could be demonstrated at low extracellular levels and the relationship between the free intracellular methotrexate concentration and the extracellular methotrexate concentration at the steady state approximated an adsorption isotherm. The energy dependence of this system was unusual, however, in that a variety of metabolic inhibitors were found to enhance the unidirectional influx of methotrexate, markedly depress its unidirectional efflux, with the net effect reflected in a large increase in the intracellular electrochemical potential for methotrexate at the steady state[3]. These studies suggested two types of energy coupling for methotrexate transport. It was proposed that there were forces which tended to depress uphill transport into the cell which were selectively inhibited by metabolic poisons while a second energy pathway or energy reserve not immediately susceptible to the effects of the metabolic inhibitors was required to account for the energy-dependent uphill transport of methotrexate into the cell in the presence of these agents.

The energetics of folic acid transport is similar to that of methotrexate since net folic acid uptake is markedly enhanced and its unidirectional efflux retarded by inhibition of energy metabolism. The data suggest, in addition, that an energy-dependent process tending to depress cell folate levels is dominant for folic acid under these experimental conditions and results in its uphill transport out of the cell. Thus, over the concentration range studied, the steady-state distribution ratio for folic acid was far below that expected for a passive equilibrating transport mechanism. Indeed, considering that a significant fraction of intracellular label appears to represent folic acid metabolites, the distribution ratio for unchanged folic acid must be even lower. Also compatible with energy-dependent efflux of folic acid is the observation that an efflux component is a major part of the net uptake of folic acid within 30 sec after the addition of label to the medium and at intracellular levels far below the steady state.

There are, however, other considerations in the evaluation of the low distribution ratios for folic acid at the steady state (1). The folic acid distribution ratio at which the electrochemical potential difference should be zero was estimated on the basis of the Cl^- distribution ratio and assuming folic acid to be transported across the cell membrane as a divalent ion. It is possible that the measured Cl^- distribution ratio predicted an erroneously high value. On the other hand, if the diffusional leak for folic acid is negligible at the concentrations employed in these studies and if the mediated translocation of folic acid were not energy dependent and occurred with neutralization of the folic acid charge so that a net charge transfer across the cell membrane does not occur, the membrane potential may have no role in determining the final steady-state distribution ratio for folic acid and this ratio would be expected to be unity when no electrochemical potential difference for folic acid exists across the cell membrane. If this were the case, the difference between the folic acid distribution ratio observed and that expected is even greater. Indeed, if this consideration is pertinent for folic acid then the data for methotrexate would also be compatible with energy-dependent efflux of this compound in the absence of metabolic inhibitors since steady-state distribution ratios significantly less than unity were observed for methotrexate at concentrations above 1–2 μM (ref. 1). (2) An apparently low concentration for intracellular folic acid at the steady state could be due to its exclusion

from an intracellular compartment so that its actual space of distribution is less than that of the total intracellular water. (3) Interpretation of this data is complicated by residual folic acid metabolism not abolished when the cells are pretreated with methotrexate. In view of this it is possible that steady-state levels for intracellular folic acid could be depressed due to a complex equilibrium in which efflux of rapidly exchanging metabolites maintain intracellular folic acid at a low level; augmentation of net folic acid uptake by anaerobiosis might be related to an inhibition of this metabolic pathway. However, considering the similarity between the energetics of folic acid transport and that of its nonmetabolized analog, methotrexate, is seems likely at this point that the major findings for this substance are related to its transport characteristics rather than its metabolism.

A number of other observations suggest that folic acid, folinic acid and methotrexate share, at least in part, the same carrier mechanism: (1) Folic and folinic acids competitively inhibit the unidirectional influx of methotrexate[1]. At sufficiently high concentrations these substances can completely abolish methotrexate influx (I.D. GOLDMAN, unpublished observation). (2) Folic acid influx is markedly inhibited by methotrexate and folinic acid, and the former was shown to completely inhibit folic acid uptake. (3) Addition of folic or folinic acids to the extracellular medium of cells at the steady state with methotrexate can induce an uphill flow of methotrexate out of the cell[1], while addition of methotrexate or folinic acid during folic acid uptake results in net efflux of virtually all label out of the cell. Even though some intracellular label represents exchangeable folic acid metabolites, since virtually all label leaves the cell, some unchanged folic acid must exit against an electrochemical potential gradient. Net uphill efflux of a substance which occurs upon addition of a competing analog to the extracellular medium may be related to a coupled carrier-mediated downhill influx of the competing substance from the extracellular fluid, hence, countertransport; but in view of the possibility of an energy-dependent efflux mechanism, the phenomenon observed might be due to inhibition of the influx mechanism by the competing substance while an independent uninhibited efflux pump might be responsible for uphill efflux. However, that a carrier mechanism must be involved in the transport of these substances is indicated by the observation that the true unidirectional influx of methotrexate is stimulated in cells preloaded with folic or folinic acids[2]. It has not been established, though, that this phenomenon is due to the presence of intracellular folic acid rather than one of its metabolites.

The possibility exists that more than one transport mechanism is involved in energy-dependent transport of the folates. Although a second low-affinity influx route for methotrexate was described[3], it was unaffected by azide, and, aside from this, influx of methotrexate appears to occur by a single high-affinity mechanism. However, transport heterogeneity may be obscured when the kinetic constants of the transport mechanisms do not readily permit their discrimination by usual kinetic plots. In addition, the absence of multiple influx routes does not exclude the possibility of multiple efflux routes. Considering that two modes of energy coupling may be involved in folate transport, it is possible that they are coupled to different transport mechanisms rather than a single carrier unit. Thus one transport route which could effect uphill transport of folate out of the cell might have a higher affinity for folic acid than methotrexate, while another route which could effect uphill transport of folate into the cell might have a higher affinity for methotrexate than folic acid.

If present, the latter transport mechanism would appear to play a major role for 5-[^{14}C]methyltetrahydrofolic acid, the major naturally occurring tetrahydrofolate in man[6], since initial studies (I. D. GOLDMAN, unpublished observation) suggest that uphill transport of this substance into the cell is characterized by steady-state concentration gradients far greater than that observed for methotrexate. Studies are currently under way to evaluate the possibility that more than one high-affinity transport route for methotrexate is present in L1210 cells.

The rapid and slow efflux components for folic acid observed with anaerobiosis are not considered to be due to parallel efflux routes nor is it likely that this can be attributed to rapid efflux of folic acid metabolites since this was also observed for methotrexate efflux in the presence of azide[3]. In both situations the rapid efflux component represents too small a fraction of the total exchangeable intracellular folate and its half-time too short for this to be related to parallel efflux routes in a two compartment system. This better fits a three-compartment analysis in which the rapid efflux component is related to a population of cells unable to maintain a high concentration gradient for intracellular folate under these conditions, as suggested for methotrexate[3].

It is clear from this and previous studies[1] that the influx process for folic acid in this cell system is much slower than that of methotrexate and in all likelihood, considerably less than that of the naturally occurring tetrahydrofolates. Thus initial uptake rates for folic acid are much lower than those observed for methotrexate, the K_i for folic acid inhibition of methotrexate influx is about 50 times that of the methotrexate influx K_t (extracellular concentration at which influx is half-maximal), while the K_i for folinic acid inhibition of methotrexate influx is comparable to the methotrexate influx K_t[1]. Other studies, (I. D. GOLDMAN, unpublished observations) suggest that the influx K_t for 5-[^{14}C]methyltetrahydrofolic acid is less than that of methotrexate with higher influx rates at comparable extracellular concentrations.

There have been few previous reports on the transport of folic acid. JACQUEZ[7] concluded that folic acid uptake in Ehrlich ascites cells was due to passive diffusion, but those experiments were performed at very high extracellular folic acid concentrations (2 mM) at which a higher affinity transport mechanism might be saturated and passive diffusion could account for the bulk of uptake. In Yoshida sarcoma cells[8], folic acid was found to inhibit uptake of aminopterin, (4-amino-4-deoxypteroylglutamic acid) an analog of methotrexate. Studies in rats using an intestinal perfusion technique *in vitro*[9,10], have shown that folic acid absorption exhibits saturation and is uphill. Folic acid reabsorption by the dog kidney, *in vivo*, was saturable and could be inhibited by methotrexate[11].

ACKNOWLEDGMENTS

We are grateful to Dr. Clifford Patlak for his many helpful suggestions in these studies and his critical evaluation of this manuscript. The excellent technical assistance of Mrs. Irene Clark and Mrs. Frances Francis is acknowledged.

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