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K^+-INDUCED ALTERATIONS OF ENERGETICS AND EXCHANGE DIFFUSION IN THE CARRIER-MEDIATED TRANSPORT OF THE FOLIC ACID ANALOG, METHOTREXATE, IN EHRLICH ASCITES TUMOR CELLS

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Summary

The bidirectional fluxes and energetics of methotrexate transport in Ehrlich ascites tumor cells were profoundly altered in a high [K^+], low [Na^+] buffer (K^+ buffer). Incubation of cells for 30 min in K^+ buffer reduced influx by 27% and the efflux rate constant by 53%. This asymmetrical inhibition of bidirectional fluxes increased the net exchangeable intracellular methotrexate level per cell, but the actual intracellular methotrexate concentration at the steady state was similar to that in Na^+ buffer, since the high [K^+] caused an increase in intracellular water. Because cells exposed to K^+ buffer were depolarized, the apparent electrochemical potential difference for methotrexate was markedly reduced. However, the steady-state intracellular methotrexate level was still related to the extracellular concentration by an absorption isotherm, indicating asymmetry in the bidirectional fluxes similar to that observed in Na^+ buffer and thus predicting that transmembrane gradients would be produced at very low extracellular methotrexate concentrations. Glucose, which had little effect on bidirectional fluxes and reduced the steady-state level of methotrexate in Na^+ buffer, stimulated influx, inhibited efflux and rapidly increased the steady state in K^+ buffer similar to the effects of glucose in the presence of iodoacetate in Na^+ buffer. Finally, cells exposed to K^+ buffer exhibited *trans*-stimulation of [^{3}H]methotrexate influx when loaded with non-labeled methotrexate, a phenomenon not observed in Na^+ buffer.

The results indicate that although methotrexate transport is not affected by transient changes in the cationic composition of the extracellular compartment,

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

prolonged exposure of cells to a high [K^+], low [Na^+] environment markedly alters the physical properties of the cells and the transport parameters for methotrexate and reveals characteristics of the methotrexate carrier system that are not evident in other buffer systems.

Introduction

The carrier-mediated transport of methotrexate in Ehrlich ascites tumor cells is a high-affinity system exhibiting sulfhydryl and temperature dependence [1], heteroexchange diffusion [2] and inhibition by structural analogs [1,3]. The energetics of this system are complex. Both influx and uphill transport are inhibited by a wide variety of anions, especially organic phosphates [3—5]. Furthermore, inhibitors of energy metabolism stimulate rather than retard influx and the steady-state electrochemical potential for intracellular methotrexate [6]. Since these cells maintain an electrochemical potential difference for methotrexate even in the presence of metabolic poisons, we have proposed that the uphill transport of methotrexate into these cells might be linked to the large gradient of organic phosphates across the cell membrane by a countertransport mechanism which would not be immediately affected by metabolic inhibitors [3,5]. Translocation of methotrexate across the cell membrane does not appear to require extracellular Na^+, since exposure of cells to a medium in which Na^+ has been replaced with K^+ or Li^+ does not immediately affect methotrexate influx [3]. In this paper, however, we report profound alterations in the carrier-mediated transport of methotrexate in the Ehrlich ascites tumor when cells are exposed to a high [K^+], low [Na^+] medium for longer intervals: (i) There is a time-dependent inhibition of the bidirectional flows of methotrexate; (ii) although the chemical potential for intracellular methotrexate at the steady state does not change, when membrane depolarization is considered there is a fall in the transmembrane electrochemical potential difference for this bivalent anion; (iii) while glucose depresses the electrochemical potential difference for methotrexate in Na^+ buffer, this substrate augments the uphill transport of methotrexate into cells in K^+ buffer; (iv) finally, cells exposed to K^+ buffer demonstrate *trans*-stimulation of [^{3}H]methotrexate influx when loaded with non-labeled methotrexate, an interaction that is not observed in a high [Na^+], low [K^+] environment. These studies, therefore, indicate major qualitative and quantitative alterations in the transport of methotrexate induced by a high [K^+], low [Na^+] buffer. This buffer unmasks characteristics of the methotrexate carrier system not otherwise demonstrable and provides an experimental approach for further elucidating the unique kinetic and thermodynamic properties of the methotrexate-tetrahydrofolate cofactor carrier system.

Materials and Methods

Chemicals

[3′,5′,9-^{3}H(n)]Methotrexate was obtained from Moravek (City of Industry, CA) and purified by diethylaminoethyl cellulose chromatography [1]. (±)-5-Formyltetrahydrofolate was from Lederle Laboratories (Pearle River, NY),

[*carboxy*-^{14}C]inulin from New England Nuclear Corp. (Boston, MA), NaN_3 from Fisher Chemical Co. (Fairlawn, NJ) and iodoacetate from Eastman (Rochester, NY).

Cells, media and incubation techniques

Ehrlich ascites tumor cells were grown in CF1 mice (Sprague Dawley, Madison, WI) and passed weekly by intraperitoneal inoculation of 0.2 ml of undiluted ascitic fluid. Cells were harvested after 7—10 days and washed twice in 0.85% NaCl solution to remove erythrocytes and once with buffers in which transport studies were performed. Two buffers were employed. The first, designated Na^+ buffer, was composed of 136 mM NaCl, 4.4 mM KCl, 16 mM $NaHCO_3$, 1.1 mM KH_2PO_4, 1.0 mM $MgCl_2$ and 1.9 mM $CaCl_2$. The pH of this buffer was maintained at 7.4 by passing warmed and humidified 95% O_2/5% CO_2 over the cell suspension. The second buffer, designated K^+ buffer, was composed of 140 mM KCl and 20 mM Na^+-Hepes at pH 7.4. Both buffers were of equal osmolality. The cell suspension was stirred with a Teflon paddle in specially designed flasks inserted into a 37°C water bath. Unidirectional fluxes, net uptake and intracellular binding of [^{3}H]methotrexate were measured as previously described [1,6]. Transport fluxes were stopped by injection of the cell suspension into 10 vol. of 0°C 0.85% NaCl solution. The cell fraction was separated by centrifugation (500 × *g* for 2 min) and washed twice with the 0°C NaCl solution. The washed pellet was aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene tare and dried overnight at 70°C. The dried pellets were weighed on a Cahn electrobalance (Cahn Instruments, Paramount, CA), placed in a scintillation vial and dissolved in 0.2 ml of 1 M KOH for 1 h at 70°C. The digest was neutralized with 0.2 ml of 1 M HCl and the solution was incorporated into 3 ml of Ready-solv (Beckman, Irvine, CA). Radioactivity was determined in a Beckman LS-230 scintillation spectrometer and counting efficiencies were determined employing [^{3}H]- or [^{14}C]toluene internal standards.

Determination of intracellular water and chloride distribution ratio

Intracellular water was determined from the difference between wet and dry weights of a cell pellet less the [^{14}C]inulin space as described in detail [1,7]. The chloride distribution ratio was measured as an indication of changes in membrane potential [1,7]. Na^+ and K^+ were determined by flame photometry as previously described [8].

Results

Effects of K^+ buffer on methotrexate influx; interaction with glucose and iodoacetate

Exposure of Ehrlich ascites tumor cells to a medium in which K^+ has largely replaced Na^+ has been shown to have no immediate effect on methotrexate influx [3]. This was substantiated in these studies by the observation that influx over a 100 s interval in K^+ buffer was identical to that in Na^+ buffer if cells were exposed to [^{3}H]methotrexate and the buffer simultaneously. If, however, cells were incubated in the buffers for 30 min and then exposed to

TABLE I

EFFECTS OF K^+ BUFFER, GLUCOSE AND/OR IODOACETATE ON METHOTREXATE INFLUX

Cells were incubated in either Na^+ or K^+ buffer in the presence or absence of 10 mM glucose, 1 mM iodoacetate, or both for 30 min at 37°C and then exposed to 1 μM [^{3}H]methotrexate. Influx was measured over 100 s and is shown as a percentage of the control in Na^+ buffer. The data are expressed as the mean ± S.E. of (*n*) separate experiments performed on different days.

	Control	10 mM glucose	1 mM iodoacetate	Glucose + iodoacetate
Na^+ buffer	100.0 ± 5.4 (16)	96.4 ± 5.8 (8)	93.2 ± 10.8 (3)	144.2 ± 6.0 (3)
K^+ buffer	72.9 ± 3.5 (16)	115.6 ± 6.0 (8)	66.3 ± 0.2 (3)	125.4 ± 11.7 (3)

[^{3}H]methotrexate, influx in K^+ buffer was reduced (Table I). In 16 experiments, influx in K^+ buffer was 72.9 ± 3.5% of the control ($P < 0.001$). As indicated further in Table I, while exposure of the cells to 10 mM glucose had little effect on influx in Na^+ buffer, (96.4 ± 5.8% of control, $P < 0.200$), glucose stimulated influx in K^+ buffer (158.6 ± 15% of control, $P < 0.001$). Iodoacetate had little effect on methotrexate influx in either buffer. However, addition of both iodoacetate and glucose caused a 44.2 ± 6.0% stimulation of influx in Na^+ buffer but produced no further stimulation above that induced by glucose alone in K^+ buffer. The effects of K^+ buffer must be attributed to the K^+ alone rather than the Hepes or the absence of Na^+, since neither addition of 20 mM Hepes to Na^+ buffer nor replacement of Na^+ with Li^+ had any significant effect on influx or the steady-state level of methotrexate. Substitution of KCl for NaCl in Na^+ buffer, however, produced effects on methotrexate transport similar to those observed with K^+ buffer.

Other characteristics of influx in K^+ buffer remained identical to those observed in Na^+ buffer. Influx in both buffers was stimulated by 10 mM NaN_3 and inhibited by adenosine 3′-monophosphate. Furthermore, (±)-5-formyltetrahydrofolate and *p*-chloromercuribenzenesulfonate abolished methotrexate influx in both buffers, indicating that transport in K^+ buffer as well as in the Na^+ buffer was mediated by the high-affinity tetrahydrofolate-methotrexate carrier system.

Effects of K^+ buffer on trans-stimulation of [^{3}H]methotrexate influx by non-labeled methotrexate

In previous studies from this laboratory [2] and elsewhere [9], no *trans*-stimulation of [^{3}H]methotrexate influx was observed when cells were preloaded with non-labeled methotrexate in Na^+ buffer even when the intracellular concentration was at its theoretical maximum for the carrier system. *Trans*-stimulation of methotrexate influx could be demonstrated only in cells preloaded with the naturally occurring folates such as (±)-5-formyltetrahydrofolate or (±)-5-methyltetrahydrofolate [2,9]. However, cells in K^+ buffer exhibited a *trans*-stimulation of [^{3}H]methotrexate influx when preloaded with non-labeled methotrexate. In the present experiments, cells in either Na^+ buffer or K^+ buffer were loaded with 50 μM non-labeled methotrexate or 100 μM (±)-5-formyltetrahydrofolate for 45 min at 37°C. The cells were then washed twice in folate-free buffer at 0°C and resuspended into buffer containing 1 μM [^{3}H]-

TABLE II

EFFECT OF K^+ BUFFER ON THE *TRANS*-STIMULATED INFLUX OF [^{3}H]METHOTREXATE BY NON-LABELED METHOTREXATE OR (±)-5-FORMYLTETRAHYDROFOLATE

Cells either in Na^+ buffer or K^+ buffer were loaded with 50 μM non-labeled methotrexate or 100 μM (±)-5-formyltetrahydrofolate for 45 min at 37°C, washed twice in 0°C buffer, and resuspended into buffer containing 1 μM [^{3}H]methotrexate. Influx was measured over 100 s and is shown as a percentage of the control. The data are expressed as mean ± S.E. of five separate experiments.

	Control	±(5)-Fornyltetrahydrofolate	Methotrexate
Na^+ buffer	100.0 ± 6.5	202.3 ± 24.6	100.1 ± 12.5
K^+ buffer	100.0 ± 4.8	230.0 ± 14.3	166.2 ± 13.7

methotrexate. Table II shows the results of five such experiments. Influx in cells loaded with methotrexate was compared to the control values obtained in their respective buffers. In Na^+ buffer, influx in cells loaded with (±)-5-formyltetrahydrofolate was 202.3 ± 24.6% that of control, however, in cells loaded with non-labeled methotrexate, influx was 100.1 ± 12.5% of the control, again showing no homo-exchange as previously reported [2]. In K^+ buffer, *trans*-stimulation in cells preloaded with (±)-5-formyltetrahydrofolate was comparable to that in Na^+ buffer with an influx of 230.0 ± 14.3% of the control. However, influx in cells preloaded with methotrexate was 166.2 ± 13.7% of its control.

Effects of K^+ buffer on methotrexate efflux

To evaluate the effects of K^+ buffer on methotrexate efflux, cells were incubated with 2 μM [^{3}H]methotrexate for 50 min in either Na^+ or K^+ buffer with or without 10 mM glucose, washed twice at 0°C, and then resuspended into their respective methotrexate-free buffers. The steady-state levels expressed as methotrexate per g dry wt. of cells in Fig. 1 were different due to cell swelling as discussed in the next section. Intracellular methotrexate consists of an exchangeable fraction that rapidly leaves the cells in the absence of extracellular methotrexate and a fraction that is tightly bound and comparable to the dihydrofolate reductase binding capacity [10]. The inset of Fig. 1 confirms that efflux of the major portion of exchangeable methotrexate can be characterized by a single exponential. Exposure of the cells to K^+ buffer markedly inhibited efflux of exchangeable methotrexate (Fig. 1, inset). In eight experiments, the efflux rate constant was 47.3 ± 8.3% ($P < 0.001$) of that observed in Na^+ buffer. Addition of 10 mM glucose to the K^+ buffer further inhibited efflux of exchangeable methotrexate to 25.7 ± 10.8% of that observed in Na^+ buffer while glucose alone had no measuable effect on efflux in Na^+ buffer. The time course of efflux in Fig. 1 suggests that the non-exchangeable fraction was increased. This was further substantiated by experiments that monitored efflux over longer intervals. The addition of 10 mM glucose had no effect on the non-exchangeable fraction in either buffer.

Effects of K^+ buffer with or without glucose on net transport of methotrexate

An analysis of the net transport of a solute requires that the cell water as well as the free and bound components be determined under each experimental

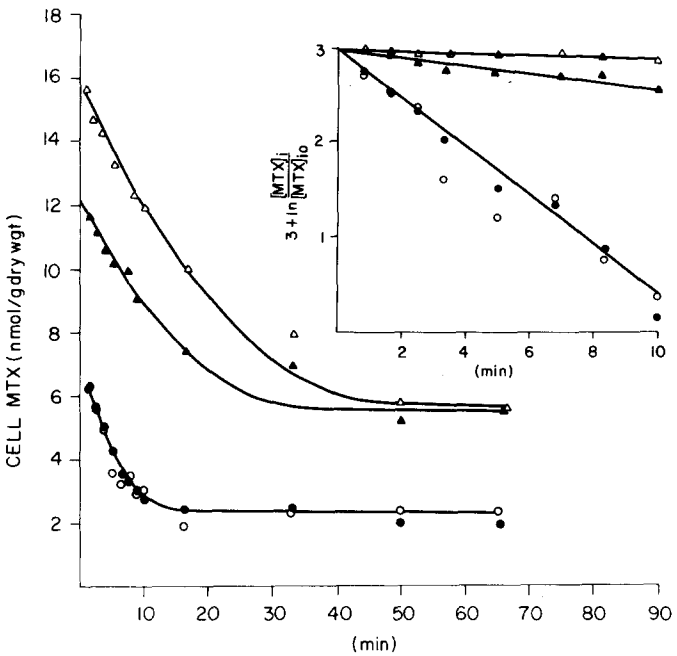

Fig. 1. Effect of K^+ buffer on methotrexate (MTX) efflux. Cells were incubated with 2 μM [^{3}H]methotrexate in either Na^+ or K^+ buffer with or without 10 mM glucose for 50 min. The cells were then washed twice in 0°C buffer and resuspended into each respective methotrexate-free buffer at 37°C; (●) Na^+ buffer; (○) Na^+ buffer + 10 mM glucose; (▲) K^+ buffer; (△) K^+ buffer + 10 mM glucose. The inset is a semilogarithmic plot of the fraction of exchangeable methotrexate remaining as a function of time after the cells were resuspended in methotrexate-free buffer. $[MTX]_{io}$ is the concentration of exchangeable intracellular methotrexate prior to resuspension and $[MTX]_i$ refers to the exchangeable intracellular methotrexate concentration at times following resuspension. Exchangeable methotrexate was determined from the total methotrexate level in the cell less the non-exchangeable fraction. A factor of 3 was added to each logarithmic function to eliminate negative values.

condition. Evaluation of the transmembrane electrochemical difference for methotrexate is further complicated by the fact that this agent is a bivalent anion at physiological pH [11], and therefore the membrane potential must be monitored and considered. Table III shows that exposure of these cells to K^+ buffer caused a marked increase in intracellular water and a decrease in the membrane potential as estimated by the rise in the Cl^- distribution ratio consistent with previous reports on the effects of a high [K^+] buffer on the Ehrlich ascites tumor [12,13]. Furthermore, intracellular [Na^+] was decreased and intracellular [K^+] was increased. The changes occurred rapidly and were constant within 10 min after the cells were exposed to K^+ buffer. Cell water and the membrane potential so determined were used in the following experiments to calculate intracellular concentrations and theoretical equilibrium values for intracellular methotrexate.

Cells were incubated for 50 min with 1 μM [^{3}H]methotrexate in either Na^+ buffer or K^+ buffer following which the exchangeable intracellular methotrexate concentration was monitored for an additional 90 min. Fig. 2 shows only a small increase in the steady-state methotrexate level in K^+ buffer. The dashed lines in Fig. 2 represent the intracellular methotrexate concentration

TABLE III

EFFECT OF K^+ BUFFER ON INTRACELLULAR WATER, CHLORIDE DISTRIBUTION RATIO, MEMBRANE POTENTIAL AND INTRACELLULAR K^+ AND Na^+

Cells were incubated at 37°C for 50 min in Na^+ or K^+ buffer with or without 10 mM glucose. Portions were removed and determinations made as described in Materials and Methods. Data are expressed as the mean ± S.E. of the average of five determinations in the number of experiments indicated in parentheses. $[HOH]_i$/DW is the ratio of the intracellular water ($[HOH]_i$) to dry weight (DW) of a cell pellet. $[Cl^-]_i/[Cl^-]_e$ is the ratio of the intracellular ($[Cl^-]_i$) to extracellular ($[Cl^-]_e$) chloride concentration. $\Delta\Psi$ is the membrane potential determined as discussed in Materials and Methods.

	Na^+ buffer	Na^+ buffer + glucose	K^+ buffer	K^+ buffer + glucose
$[HOH]_i$/DW (μl/mg)	3.33 ± 0.10 (8)	3.20 ± 0.06 (5)	6.52 ± 0.22 (8)	6.51 ± 0.32 (6)
$[Cl^-]_i/[Cl^-]_e$	0.578 ± 0.027 (6)	0.497 ± 0.025 (4)	0.895 ± 0.021 (6)	0.851 ± 0.034 (5)
$\Delta\Psi$ (mV)	−15.68 ± 1.10 (6)	−18.79 ± 1.34 (4)	−2.98 ± 0.63 (6)	−4.40 ± 1.14 (5)
$[Na^+]$ (mM)	54.0 ± 7.4 (4)	49.5 ± 9.4 (4)	9.18 ± 3.57 (4)	9.53 ± 2.76 (4)
$[K^+]$ (mM)	125.5 ± 10.5 (4)	127.5 ± 11.1 (4)	151.9 ± 10.3 (4)	151.7 ± 12.2 (4)

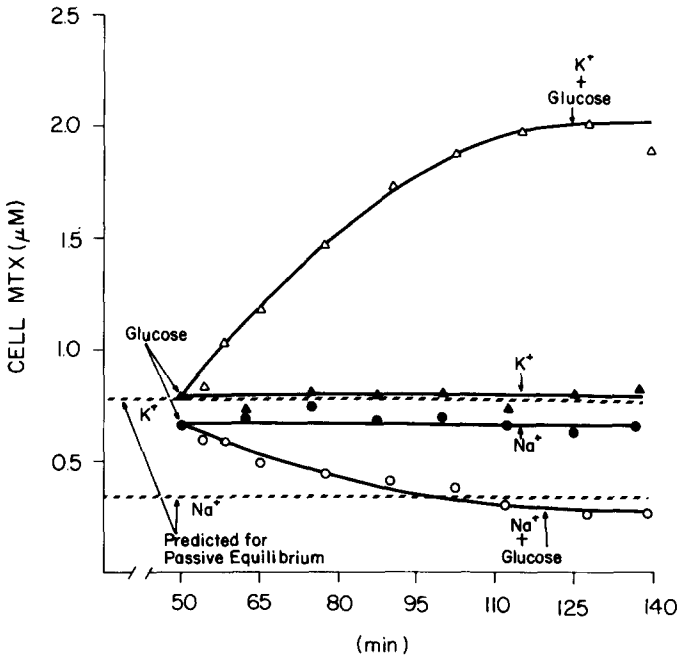

Fig. 2. Effect of K^+ buffer on the intracellular concentration of methotrexate (MTX) at the steady state with or without glucose. Cells in Na^+ buffer (●) or K^+ buffer (▲) were incubated in the presence of 1 μM methotrexate at 37°C. At 50 min, glucose was added to portions from the two groups (arrow) to attain a final concentration of 10 mM. (○) Na^+ buffer + glucose, (△) K^+ buffer + glucose. The upper (K^+ buffer) and lower (Na^+ buffer) dashed lines indicate the intracellular methotrexate concentration predicted for thermodynamic equilibrium calculated as discussed in Materials and Methods. Each data point was corrected for bound methotrexate and represents exchangeable drug alone.

that would be expected from the Nernst equation if methotrexate were passively distributed across the cell membrane based on the Cl^- distribution ratio as an estimation for the membrane potential. In Na^+ buffer, the steady-state level of methotrexate is greater than the level predicted for passive distribution (lower dashed line). However, in K^+ buffer, the observed intracellular methotrexate concentration was close to the calculated equilibrium

TABLE IV

EFFECT OF K^+ BUFFER ON THE METHOTREXATE DISTRIBUTION RATIO

Cells were incubated with 1 μM [^{3}H]methotrexate at 37°C and intracellular methotrexate concentrations determined after a steady state for intracellular drug was achieved. $[MTX]_i$ is the concentration of exchangeable intracellular methotrexate and $[MTX]_e$ is the extracellular methotrexate concentration. Expected $[MTX]_i/[MTX]_e$, the distribution ratio expected for an equilibrating system, was calculated from the Nernst equation, as described in Materials and Methods.

	Expected $[MTX]_i/[MTX]_e$	Measured $[MTX]_i/[MTX]_e$	Measured/ expected	(*n*)
Na^+ buffer	0.280	0.819 ± 0.061	2.93	11
Na^+ buffer + glucose	0.237	0.414 ± 0.061	1.75	4
K^+ buffer	0.781	0.735 ± 0.047	0.94	12
K^+ buffer + glucose	0.678	1.501 ± 0.095	2.21	8

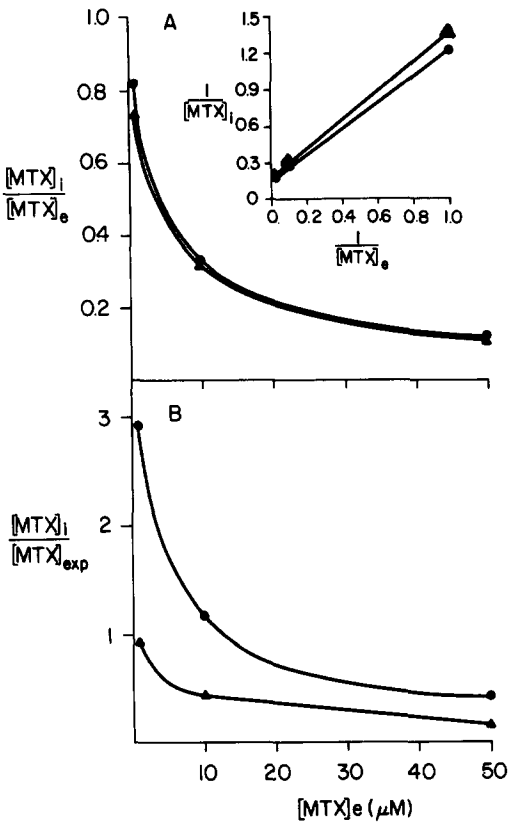

Fig. 3. The relationship between the extracellular methotrexate (MTX) concentration and (A) the distribution ratio or (B) the ratio of the measured intracellular methotrexate concentration to that expected for an equilibrating system in Na^+ (●) or K^+ buffer (▲). Cells were incubated at 37°C with 1, 10 or 50 μM [^{3}H]-methotrexate and intracellular methotrexate concentrations were determined after the cells had attained a steady state. $[MTX]_i$ is the exchangeable intracellular methotrexate concentration, $[MTX]_e$ is the extracellular methotrexate concentration and $[MTX]_{exp}$ is the intracellular methotrexate concentration expected for an equilibrating system.

level (upper dashed line). When cells at the steady state with methotrexate were exposed to 10 mM glucose, markedly different results were obtained in the two buffers (Fig. 2). In Na^+ buffer, glucose decreased intracellular methotrexate to a level approaching a passive distribution. In K^+ buffer, addition of glucose caused a rapid net uptake of methotrexate to a level well above equilibrium. Table IV summarizes the data from a series of these experiments. The measured ratio of the exchangeable methotrexate concentration in the intracellular water to the extracellular methotrexate concentration (distribution ratio) in Na^+ buffer exceeds the equilibrium ratio by 2.9-fold. Addition of 10 mM glucose reduced the ratio to 1.7 ($P < 0.01$). In contrast, the distribution ratio in K^+ buffer was not significantly different from equilibrium at this extracellular methotrexate level. However, addition of 10 mM glucose in K^+ buffer increased the chemical gradient for methotrexate 2.2-fold greater than its expected equilibrium ratio ($P < 0.001$). If the extracellular methotrexate concentration was increased, the methotrexate distribution ratio at the steady-state decreased

in both buffers (Fig. 3a), and a reciprocal plot of the intracellular ($[MTX]_i$) vs. extracellular concentration of methotrexate ($[MTX]_e$) (adsorption isotherm) was linear (Fig. 3a, inset) as reported previously for the L1210 leukemia [1]. Thus, as $[MTX]_e$ becomes very high, $1/[MTX]_i$ approaches a limit, the reciprocal of which is the maximum $[MTX]_i$ which can be achieved at the steady state due to the saturable influx process. This $[MTX]_i$ value was calculated to be 5.86 μM in Na^+ buffer and 5.57 μM in K^+ buffer. This is consistent with an asymmetrical transport system with a saturable influx process and a low affinity or non-saturable efflux process which apparently also persists in the presence of K^+ buffer. Fig. 3a indicates further that the distribution ratios for methotrexate at the steady state are the same in both buffer systems at all extracellular methotrexate concentrations. However, as shown in Fig. 3b, when the methotrexate gradient is interpreted in the context of the change in membrane potential as estimated by the Cl^- distribution ratio, the ratio of the observed steady-state methotrexate distribution ratio to the expected distribution ratio for equilibrium is much lower in K^+ than Na^+ buffer. Thus, while intracellular concentrations of methotrexate at the steady state are similar in both buffers, the energetics of the system in terms of electrochemical-potential differences are markedly altered in K^+ buffer.

Similar to influx, addition of iodoacetate to cells in Na^+ buffer reversed the effect of glucose on net transport from inhibition to stimulation. As seen in Fig. 4, iodoacetate raised the net intracellular methotrexate level after a long lag period, while addition of glucose reduced this parameter. However, addition of both glucose and iodoacetate produced an immediate increase in the net level of intracellular methotrexate. Thus, the stimulatory effects of glucose seen in K^+ buffer could be produced in Na^+ buffer when glycolysis was inhibited by iodoacetate.

Other aspects of net transport in K^+ buffer were similar to those observed in Na^+ buffer. NaN_3 increased the intracellular concentration of methotrexate at the steady state in both buffers. Also under both conditions, net efflux of methotrexate was observed when cells at the steady state were exposed to (±)-5-formyltetrahydrofolate (countertransport). Finally, 1 mM ouabain did not alter the steady-state methotrexate level in either Na^+ or K^+ buffer.

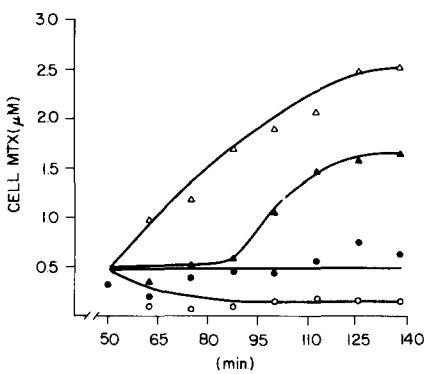

Fig. 4. Effect of glucose and/or iodoacetate on net uptake of exchangeable methotrexate (MTX) in Na^+ buffer. Cells were incubated in Na^+ buffer with 1 μM [^{3}H]methotrexate at 37°C. At 50 min, iodoacetate or both were added to portions of the suspension. (●) Control, (○) 10 mM glucose, (▲) 1 mM iodoacetate, (△) 1 mM iodoaceate + 10 mM glucose.

Discussion

Uphill transport of many amino acids and other solutes appears to be derived, at least in part, from the electrochemical potential difference for Na^+ and K^+ across the cell membrane [14—17]. Hence, high [K^+], low [Na^+] buffers have been utilized frequently to evaluate these Na^+-dependent transport mechanisms [18,20]. Unlike Na^+-dependent systems, however, methotrexate influx in the Ehrlich ascites tumor is not immediately affected by replacement of extracellular Na^+ with either K^+ or Li^+ [3]. This suggests that a ternary complex consisting of Na^+ or K^+, methotrexate and the carrier is not formed during the translocation of methotrexate into the cell. The present studies demonstrate, however, that longer exposure of cells to a high [K^+], low [Na^+] buffer profoundly alters transport of methotrexate.

First, incubation of cells with K^+ buffer for 30 min reduces influx by 27% and the efflux rate constant by 53%. These changes are consistent with the observed increase in the net exchangeable intracellular methotrexate level when expressed in units of cell dry weight. However, since the intracellular water is increased in cells exposed to K^+ buffer, the intracellular methotrexate concentration at the steady state is similar to that observed in Na^+ buffer. Although the chemical gradient for methotrexate across the cell membrane does not change, the electrochemical potential for methotrexate is markedly reduced due to a fall in the membrane potential. Uphill transport of methotrexate appears to persist, however, since the transmembrane chemical gradient decreases as the extracellular methotrexate is increased, indicating continued asymmetry in bidirectional fluxes compatible with a lower K_m value for influx than efflux. Furthermore, the steady-state intracellular methotrexate levels in both buffers are related to the extracellular concentration by an adsorption isotherm (Fig. 3a, inset), predicting that transmembrane electrochemical potential differences for methotrexate should be achieved at low extracellular levels. Hence, from the slope of Fig. 3a it is calculated than an extracellular methotrexate concentration of less than 0.53 μM in K^+ buffer will result in a methotrexate distribution ratio greater than that predicted for equilibrium. This, however, is difficult to confirm experimentally since at low extracellular methotrexate levels, the exchangeable intracellular component is very small compared to the larger bound fraction. In contrast, the extracellular concentration of methotrexate in Na^+ buffer need only be less than 14.8 μM for the methotrexate distribution ratio to be greater than the equilibrium value.

The conclusion that the electrochemical potential difference for intracellular methotrexate at the steady state is markedly reduced in K^+ buffer is based on the Cl^- distribution ratio as an estimation of the membrane potential and thus, may be subject to some inaccuracy. Certain errors are inherent in all methods for determining membrane potential [16] and, therefore, the true magnitude of this parameter is still uncertain. The Cl^- distribution ratio is a valid estimation of the membrane potential only if (i) all cell Cl^- is osmotically active, (ii) the ion is passively distributed across the cell membrane, (iii) measurements are taken only after thermodynamic equilibrium has been achieved, and (iv) Cl^- is equally distributed throughout the cell with no compartmentation. While assumptions i and ii appear to be valid [12,21], iii requires that enough time be

allowed for complete equilibration of Cl^- across the membrane. Although the half-time for Cl^- equilibration has been shown to be longer than previously thought, 60 min compared to 2 min [15], our measurements were taken 50 min after transfer to K^+ buffer at 37°C and the distribution did not change with further incubation, suggesting that thermodynamic equilibrium had been attained. The fourth assumption, however, is seriously questioned by reports of compartmentation of Cl^- in the cytoplasm [13] or sequestration within the nucleus [22]. Hence, there is probably some error in estimating the membrane potential by the Cl^- distribution ratio. Nevertheless, the values obtained agree well with those determined by microelectrode impalement [12,23], and while the actual magnitude of the membrane potential remains in question, it has been well established by several different methods that Ehrlich cells are depolarized when exposed to high K^+ concentrations [12,13]. Therefore, since the chemical gradient for methotrexate remains unchanged in K^+ buffer, the electrochemical potential must be reduced.

Methotrexate transport in the Ehrlich ascites tumor and other cells is unusual in that most metabolic poisons inhibit efflux and stimulate, rather than inhibit, net uptake of methotrexate, suggesting an energy-dependent exit mechanism [6]. Sustained, if not enhanced, uphill transport in the presence of metabolic inhibitors requires an energy source coupled to the methotrexate transport system other than the hydrolysis of ATP to account for this phenomenon. Based on the observation that the presence of organic phosphates in the extracellular compartment, in particular the adenine nucleotides, reduced influx and the electrochemical potential difference for methotrexate, it was suggested [3,5] that the high electrochemical potential difference for these solutes across the cell membrane may serve as an energy source that produces a countertransport of methotrexate into the cell. This process would not immediately be affected by depletion of ATP, since there would initially be little change in the total intracellular organic phosphate levels [3,5]. As shown in this study, addition of iodoacetate, which inhibits glycolysis at the glyceraldehyde-phosphate dehydrogenase step, has little effect on influx and raises the steady-state level of methotrexate only after a long lag period in Na^+ buffer. However, if glycolysis is inhibited with iodoacetate in the presence of glucose, methotrexate influx is stimulated and net levels of methotrexate are rapidly increased. Addition of glucose to cells in the presence of iodoacetate results in a marked build-up of fructose 1,6-diphosphate and a reduction in ATP, while iodoacetate alone has little effect on these pools [24]. The stimulation of methotrexate transport by glucose in cells inhibited with iodoacetate may be the result of (i) ATP depletion with inhibition of an exit pump and (ii) build-up of organic phosphates within the cell that retard efflux of methotrexate resulting in an increase in the net cell concentration. The stimulation of methotrexate influx and net transport by glucose in K^+ buffer may have a similar basis, since this cation inhibits glycolysis [25] and stimulates respiration [26,27]; effects produced also by iodoacetate [28,29]. However, the levels of organic phosphate intermediates have not as yet been determined in the presence of high $[K^+]$.

Of particular interest is the observation that K^+ buffer converts the methotrexate transport system from one incapable of homo*trans*-stimulation to one

in which homo*trans*-stimulation is prominent. Presumably, *trans*-stimulation occurs because during influx the rate at which the unloaded carriers reorient from the inner to the outer cell membrane interface is rate-limiting to the carrier cycle and the carrier flux is accelerated as it reorients to the outer interface in the loaded state. In previous studies and with Na^+ buffer reported here, *trans*-stimulation is observed in cells preloaded with tetrahydrofolate cofactors but not non-labeled methotrexate. The absence of homoexchange does not necessarily exclude a shared carrier for the bidirectional fluxes of methotrexate, but can be accounted for if the mobility of the methotrexate-carrier complex is the same as the mobility of the unloaded carrier alone. The firm demonstration of homoexchange in K^+ buffer does, however, provide strong evidence that under these conditions, bidirectional flows of methotrexate are mediated, at least in part, by a common carrier. The basis for emergence of homoexchange in K^+ buffer is unclear. It is unlikely that this is related simply to alterations in energy metabolism (i.e., inhibition of glycolysis or respiration), since homo*trans*-stimulation for methotrexate cannot be demonstrated in the presence of a variety of metabolic poisons (Fry, D.W. and Goldman, I.D., unpublished results). Nor can this effect be due to the accumulation of higher levels of intracellular non-labeled methotrexate, since steady-state methotrexate levels are identical in Na^+ or K^+ buffers. On the other hand, since methotrexate is a bivalent anion, depolarization of the cell may play a critical role in determining the mobility of the methotrexate-carrier complex. Alternatively, the structural fluidity of the membrane in the microenvironment of the methotrexate carrier may be sensitive to changes in neighboring monovalent cations.

It is apparent that the characteristics of methotrexate transport are influenced not only by the anionic [3—5], but also by the cationic composition of the extracellular compartment. This is an important consideration experimentally, since studies on transport of methotrexate and other folates have been conducted in high $[K^+]$ buffers [30—33]. Since cells swell and are depolarized in these media, these changes in physical properties of the cells must be considered in experimental design and analysis of the data, particularly in evaluation of electrochemical potential differences for methotrexate. In addition, increases in extracellular $[K^+]$ produce changes in bidirectional fluxes of methotrexate, exchange diffusion and qualitative and quantitative alterations in transport energetics. Hence, the ionic composition of the medium is an important determinant of the characteristics of the transport system for methotrexate and perturbations of its composition requires precise knowledge of how this will influence the physical characteristics of the cell and each transport parameter.

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