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# Separation and inhibitor specificity of a second unidirectional efflux route for methotrexate in L1210 cells *

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L1210 cells mediate the unidirectional and energy-dependent efflux of methotrexate. Efflux occurs primarily via a system which has a high sensitivity to prostaglandin $A_1$, vincristine, reserpine, verapamil, and bromosulfophthalein, but evidence has also been obtained for a second efflux component with a lower response to these inhibitors. Pretreatment of L1210 cells with low concentrations of vincristine reduces methotrexate efflux by three fold and uncovers a second efflux component with an inhibitor specificity which is distinctly different from the primary efflux route. Vincristine treatment increased by 8–20-fold the concentration required for half-maximal efflux inhibition by prostaglandin $A_1$, reserpine, bromosulfophthalein, and verapamil but had no effect on inhibition by probenecid, quinidine, or carbonylcyanide *m*-chlorophenylhydrazone. A selective block in the primary efflux system and retension of the second component was also achieved in cells exposed to low concentrations of prostaglandin $A_1$ or bromosulfophthalein. These results support prior conclusions that L1210 cells contain both a primary and secondary unidirectional efflux route for methotrexate. The second system has been difficult to detect and quantitate since it comprises only 25% of total unidirectional efflux and shows a relatively low response to various efflux inhibitors.

## Introduction

L1210 mouse leukemia cells contain multiple routes for the efflux of methotrexate [1–6], an antifolate employed in cancer chemotherapy. Efflux proceeds in part via the bidirectional reduced-folate influx system, which contributes up to 20% of total efflux in energy-replete cells [5]. Efflux via the reduced folate carrier system can be blocked specifically by brief treatment with an activated derivative of methotrexate (NHS-methotrexate) [4–6,7] and hence can be readily separated from other efflux components [4–6]. The remaining portion of methotrexate efflux is energy-dependent and it is functionally unidirectional since analyses of influx kinetics have not revealed a component with a corresponding inhibitor sensitivity [4,8,9]. The unidirectional efflux of methotrexate has been characterized primarily by its response to various inhibitors. The most effective compounds include $PGA_1$, VCR, reserpine, verapamil, BSP, and probenecid [5,6]. Since some of these latter compounds had been shown to produce biphasic inhibition kinetics [1,3–6], the possibility was raised that L1210 cells may contain a primary unidirectional efflux route for methotrexate with a high sensitivity to various inhibitors and a second system with a relatively low inhibitor sensitivity. Results from another laboratory, however, suggested that only a single route mediated the unidirectional efflux of methotrexate in L1210 cells [10].

The present study was initiated to pursue the question of multiple unidirectional efflux routes for methotrexate in L1210 cells. Our approach was to identify inhibitor conditions which could essentially block the primary efflux system for methotrexate without substantially affecting the activity of other possible efflux routes. L1210 cells exposed to low concentrations of VCR provided these desired inhibitor conditions, and a second efflux route for methotrexate with a distinct inhibitor specificity was identified. A comparison of assay procedures also suggested that in prior studies [10] the inability to observe this secondary route

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Abbreviations: Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonate: NHS-methotrexate, *N*-succinimide ester of methotrexate; HBS, Hepes-buffered saline; HBBS, Hepes/bicarbonate-buffered saline; HBBS/glucose, HBBS containing 5 mM glucose; TBBS, Tris/bicarbonate-buffered saline; BSP, bromosulfophthalein; VCR, vincristine; $PGA_1$, prostaglandin $A_1$.

could have resulted from differences in the experimental conditions.

## Materials and Method

*Chemicals.* [3′,5′,9-$^3$H]Methotrexate (20 Ci/mmol) (Moravek Biochemicals) was diluted with unlabeled methotrexate to a specific activity of 100000 cpm/nmol and purified by thin-layer chromatography [4]. Methotrexate, BSP (sulfobromophthalein), $PGA_1$, reserpine, verapamil, VCR, and probenecid were obtained from Sigma Chemical Co. NHS-methotrexate was prepared as described previously [7].

*Cells.* Parental L1210 mouse leukemia cells were grown as described previously [7] in RPMI 1640 medium containing 3% fetal bovine serum and antibiotics. Transport measurements were performed with cells (500 ml) grown in 1-liter capped flasks in a shaking incubator. Cells at a density of 0.9 to $1.3 \cdot 10^6$/ml were chilled to 4°C, collected by centrifugation at 4°C ($500 \times g$), washed with 100 volumes of HBS (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.35 with NaOH), and suspended (at 4°C) in the desired buffer: HBBS (Hepes/ bicarbonate-buffered saline), 20 mM Hepes, 5 mM Na-bicarbonate, 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.35 with NaOH; HBBS/glucose, HBBS containing 5 mM glucose; TBBS (Tris/bicarbonate-buffered saline), 10 mM Tris, 107 mM NaCl, 26.2 mM Na-bicarbonate, 5.3 mM KCl, 1.9 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.35 with HCl; or TBBS/glucose, TBBS plus 7 mM glucose.

*Efflux measurements.* Methotrexate efflux was measured in washed cells that had been suspended to $(6–10) \cdot 10^7$/ml in HBBS, incubated for 15 min at 37°C with 10 $\mu$M [$^3$H]methotrexate, diluted 5 fold with saline (5 mM phosphate, 160 mM Na-phosphate, pH 7.35), centrifuged at $1000 \times g$ (5 min, 4°C), and treated (5 min, 23°C) with 10 $\mu$M NHS-methotrexate to block efflux via the reduced-folate influx carrier [4,6]. After centrifugation, the cells were suspended to $3 \cdot 10^7$ per ml in the desired assay buffer. Assay mixtures (prepared in duplicate) consisted of substrate-loaded cells and additions in a final volume of 1.0 ml. After incubation for varying times at 37°C, the cells were diluted with 7 ml of ice-cold saline, collected by centrifugation at $1000 \times g$ (5 min, 4°C), suspended in 0.5 ml of saline, and analyzed for radioactivity in 10 ml of ScintiVerse (Fisher). Samples held at 0°C served as the control. Results were reported as retained substrate (in pmol/mg of cell protein) or relative rate determined from the first-order rate constant and were the mean of at least two separate determinations. Inhibitor specificity analysis involved measurements of efflux at varying concentrations of inhibitor at a time that approximated 50% release of the substrate by the control (without inhibitor), conversion of individual data points to a first-order rate constant (from the $t_{1/2}$ for efflux), and determination of percent inhibition relative to the control. $IC_{50}$ values for half-maximal efflux inhibition were determined from plots of percent inhibition versus inhibitor concentration. Protein concentrations were measured by the Biuret reaction [11] using bovine serum albumin as the standard. Theoretical competitive inhibition plots were derived from the Dixon equation.

*ATP analysis.* ATP levels were determined as described previously [4] in L1210 cells that had been washed and suspended in HBBS or TBBS to a density of $2 \cdot 10^7$/ml and combined with the desired additions in a final volume of 1.0 ml. After incubation of duplicate samples for the desired time at 37°C, cells were collected by centrifugation at 4°C (5 min, $1000 \times g$), suspended with vigorous mixing in 0.5 ml of 0.3 M sulfuric acid, neutralized with 0.5 ml 0.6 M NaOH, diluted with 4.0 ml 20 mM K-phosphate (pH 7) and assayed twice for ATP by the luciferin/luciferase assay of Cheer et al. [12]. The standard deviation was usually less than 10% for the mean of the four data points assayed in each experiment, whereas absolute values for ATP levels varied up to 20% in experiments performed on different days.

## Results

### *General features of methotrexate efflux in L1210 cells*

Previous studies have shown that methotrexate efflux in L1210 cells occurs rapidly and is comprised of multiple components [1–6]. Efflux follows approximately first-order kinetics for the release up to 70% of the accumulated [$^3$H]methotrexate under our standard conditions in which the substrate load ($110 \pm 20$ pmol/mg protein) greatly exceeds the level of dihydrofolate reductase ($9 \pm 2$ pmol/mg protein). This load is achieved in HBBS using 10 $\mu$M [$^3$H]methotrexate and an incubation interval of 15 min at 37°C. The short loading time (15 min) is desirable since it limits the possibility of adverse buffer effects or interference from the formation of intracellular [$^3$H]methotrexate polyglutamates. The $t_{1/2}$ for efflux is $3.0 \pm 0.4$ min ($n = 12$) in HBBS, and an increase to $2.5 \pm 0.3$ min ($n = 12$) occurs when 5 mM glucose is added during the efflux measurement. Glucose present during both loading and efflux does not produce any further increase in the $t_{1/2}$ for efflux, although a significant decrease (30–40%) occurs in the substrate load. In HBBS, efflux via the influx carrier comprises approximately 20% of total efflux [5], but this percentage decreases to $14 \pm 5$ ($n = 5$) when efflux is performed in the presence of 5 mM glucose. The remaining 86% of efflux is energy-dependent and functionally unidirectional. When the concentration of [$^3$H]methotrexate is reduced to 2 $\mu$M and glucose is present, separation of

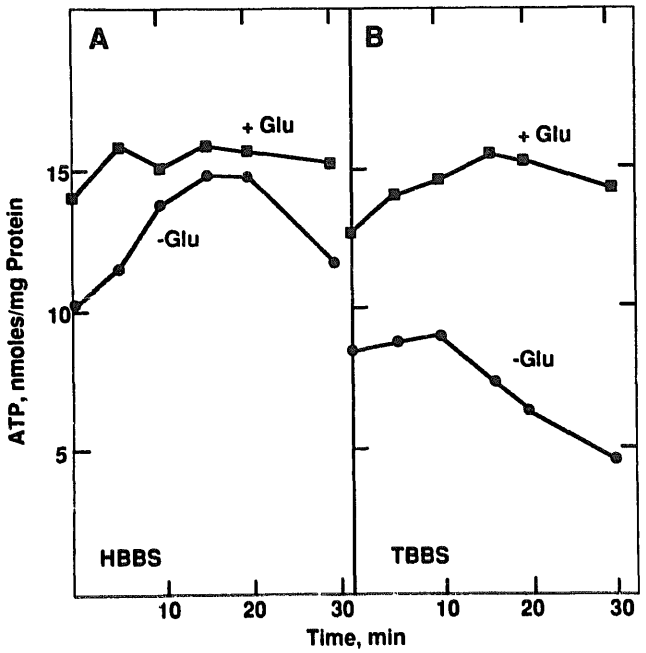


Fig. 1. Effect of time and buffer composition on ATP levels in L1210 cells in HBBS (A) or TBBS (B). L1210 cells in 1.0 ml HBBS or TBBS were incubated for the indicated times at 37°C in the absence and presence of glucose, collected by centrifugation and analyzed for ATP as described in Materials and Methods. Data points represent the mean of at least two determinations of duplicate samples (assayed twice) with standard deviations usually less than 10%. Concentrations of glucose (Glu): HBBS, 5 mM; TBBS, 7 mM. Initial buffer pH, 7.35.

efflux routes would be difficult since the substrate load is only 1.5-fold [2] to 2.5-fold [10] above the levels of dihydrofolate reductase.

ATP levels in L1210 cells vary with the time of incubation at 37°C (Fig. 1A). The level of ATP in freshly-harvested cells suspended in HBBS is lower initially and then gradually rises (about 35%) to a maximum after 15–20 min at 37°C. Upon addition of 5 mM glucose, ATP levels rise immediately and are maintained at a maximum for 30 min, but this maximum is only slightly higher than the ATP level of cells incubated for 15 min in HBBS without glucose (Fig. 1A). The same rapid rise and plateau level of ATP was also observed with 2 mM glucose (not shown). Since efflux is initiated after a 15-min load period, the modest stimulation of methotrexate efflux by glucose, in spite of energy-dependent efflux components, can be explained by the small effect of glucose on ATP levels under the buffer conditions and timing intervals employed.

The amount of cellular ATP is also consistent with physiologically competent cells. The value of 15 nmol ATP/mg protein (Fig. 1) corresponds to 24 nmol/$10^7$ cells and hence approximates prior estimates (28–30 nmol/mg protein) for fully-energized L1210 cells [10]. From the intracellular volume of L1210 cells [6] and 15 nmol/mg protein, the cytoplasmic concentration of ATP is about 4 mM.

The initial rise in ATP with cells suspended in HBBS without glucose indicates that L1210 cells under these conditions are highly active metabolically and can restore ATP levels to nearly optimal levels without an external energy source. However, it is unclear why these cells exhibit an initially low level of ATP, but temperature stress during the harvesting of cells is one factor. It has been noted repeatedly that cells which are harvested at room temperature by centrifugation and then washed and suspended in cold buffer have a 20–30% lower initial level of ATP (and methotrexate efflux) than cells chilled first in growth medium, collected by centrifugation at 4°C, and then suspended in cold buffer (the standard procedure). ATP and efflux, however, are each restored to optimal levels in cells handled by either procedure upon addition of 5 mM glucose.

ATP levels in L1210 cells vary in different patterns depending on the buffer employed. In a Tris/bicarbonate-buffered saline (TBBS) containing 7 mM glucose, a buffer system employed in other efflux studies with methotrexate [2,10,13], results comparable to HBBS/glucose were obtained (Fig. 1B): an initial time-dependent rise in ATP was observed; an approximately constant level of ATP was maintained for 30 min; and the absolute level of ATP was comparable to that for L1210 cells in HBBS/glucose. A different pattern, however, was apparent in TBBS without glucose (Fig. 1B). ATP levels were lower initially but a recovery phase was not observed during short time intervals, and a decline began after 10 min which continued until ATP levels were less than 50% of optimal levels by 30 min. These results show that the activity of energy-dependent efflux routes for methotrexate would be suppressed when measured in TBBS without glucose, even after relatively short time intervals of exposure to this buffer, and that more adverse effects would be likely at the longer exposure times.

Significant differences were also observed between HBBS and TBBS in their ability to maintain the extracellular pH at 37°C. TBBS contains a lower level of buffer (10 mM Tris) and a much higher level of bicarbonate (26.2 mM) than HBBS (20 mM Hepes and 5 mM bicarbonate, respectively) and hence would be expected to have a lesser capacity for preventing a pH rise due to the release of $CO_2$. To quantitate the buffering capacities of these solutions, samples containing $3 \cdot 10^7$ cells in 1.0 ml of buffer (pH 7.35) were incubated for varying times at 37°C (in $16 \cdot 100$ mm tubes open to the atmosphere) and monitored for changes in pH. The TBBS sample increased in pH by nearly one-half of a unit (to pH 7.83) when incubated for 30 min at 37°C, whereas the pH of the HBBS

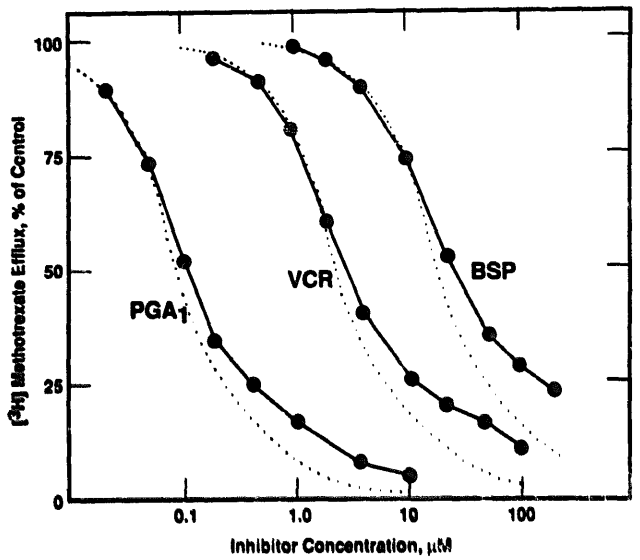


Fig. 2. Concentration dependence for the inhibition of methotrexate efflux by $PGA_1$, VCR and BSP in L1210 cells. Cells were loaded by incubation (in HBBS) with 10 $\mu$M [$^3$H]methotrexate (15 min, 37°C), treated with 10 $\mu$M NHS-methotrexate (5 min, 23°C) and then analyzed for [$^3$H]methotrexate efflux in HBBS/glucose for 2 and 5 min at 37°C in the presence of the indicated concentrations of $PGA_1$ or BSP. Data for VCR were obtained similarly except that the indicated concentrations of VCR were added only during the substrate-loading step, which was extended to 20 min at 37°C. Plotted values (percent of control) were calculated from the first-order rate constant for efflux at each inhibitor concentration divided by efflux in the control without inhibitor times 100. The dotted lines are theoretical plots for competitive inhibition of a single efflux component.

sample increased to a much lesser extent (to pH 7.42) under the same conditions. Glucose added to TBBS slowed but could not prevent the rise in pH with this buffer (pH 7.48 after 30 min), whereas a slight acidification was observed in HBBS containing glucose (pH 7.27 after 30 min).

*Biphasic inhibition of methotrexate efflux*

Prior studies in HBBS have provided evidence that the inhibition of unidirectional methotrexate efflux in L1210 cells by VCR [5], $PGA_1$ [6], and BSP [6] is biphasic. When these same experiments were repeated in HBBS/glucose, similar biphasic plots were obtained (Fig. 2). Each of these compounds inhibited total unidirectional efflux by 70–80% at relatively low concentrations, whereas a further reduction in efflux required concentrations of inhibitor beyond that expected of a single efflux component. The divergence form monophasicity can be seen by a comparison of the data (solid lines) and the calculated competitive inhibition curves (dotted lines) in Fig. 2. Concentrations for 50% inhibition of total efflux by $PGA_1$, VCR, and BSP were 0.11, 4.0, and 24 $\mu$M, respectively, whereas calculations of half-maximal inhibition of the highly-sensitive portion of efflux (75% of the total) gave slightly lower respective values of 0.09, 3.0, and 18 $\mu$M. Half-maximal inhibition of the less-sensitive second phase occurred at about 1.5 $\mu$M $PGA_1$, 70 $\mu$M VCR, and 300 $\mu$M BSP. Values reported previously [5,6] for half-maximal inhibition of unidirectional methotrexate efflux by $PGA_1$, VCR, BSP, and several other compounds in HBBS without glucose are essentially the same as reported here with HBBS/glucose (see Table I), indicating that glucose does not alter the sensitivity of methotrexate efflux to these inhibitors. Similar biphasicity for inhibitors in the presence (Fig. 2) and absence [5,6] of glucose indicates further that glucose has no appreciable effect on the proportion of efflux via the two efflux components. Measurements [5,6] of efflux inhibition with time (2, 5, and 10 min) had established previously that each of the inhibitors in Table I (except VCR) exerts its inhibitory effect on efflux immediately after or within the first minute of incubation at 37°C (without glucose). Efflux analyses at different times (2 and 5 min) with $PGA_1$ and BSP in the presence of glucose (see Fig. 2) also failed to reveal any appreciable change in inhibitor effectiveness during efflux measurements with these compounds.

*Separation of efflux components by pretreatment with VCR*

The possibility was explored that a second unidirectional efflux component for methotrexate could be isolated by blocking the primary efflux route with low concentrations of VCR. An assay procedure was developed in which parental cells were loaded with [$^3$H]methotrexate in the presence of a concentration of VCR (15 $\mu$M) which produced a substantial inhibition of the BSP-sensitive route ($IC_{50} = 3$ $\mu$M) but mostly spared the secondary route. Since the inhibitory effects of VCR accumulate with time and are not immediately reversible [5], VCR was added during the loading procedure and then (for convenience) was omitted during treatment with NHS-methotrexate and the assay for efflux (in HBBS/glucose). Efflux in cells treated with NHS-methotrexate and VCR ($t_{1/2} = 7.2$ min) was reduced nearly 3-fold relative to parental cells treated with NHS-methotrexate alone ($t_{1/2} = 2.8$ min), but further inhibition could be achieved. The remaining component was inhibited by $PGA_1$, reserpine, verapamil, and BSP, but concentrations required for half-maximal inhibition were 8–20-fold higher than in the untreated control (Table I). The extent of inhibition exceeded 80% by each compound except BSP, whose concentration was not raised above 300 $\mu$M to avoid possible damage to the cell membrane. In contrast, half-maximal inhibition by CCCP, quinidine, and probenecid remained relatively unchanged after VCR treatment (Table I), and high concentrations of CCCP (20 $\mu$M) and probenecid (2 mM) each produced nearly complete inhibition (90–95%) of the second component.

TABLE I

*Effect of VCR pretreatment on the inhibitor sensitivity of the unidirectional efflux of methotrexate in L1210 cells*

L1210 cells were loaded with [$^3$H]methotrexate in the absence or presence of 15 $\mu$M VCR, treated with NHS-methotrexate and evaluated for efflux in HBBS/glucose after 3 min (control cells) or 7 min VCR-treated cells) in the presence of varying concentrations of inhibitor. Concentrations for 50% inhibition of methotrexate efflux ($IC_{50}$) were determined from plots of percent efflux activity remaining versus log inhibitor concentration. Values represent the mean of three or more determinations with standard deviations that were usually less than 20%.

| Inhibitor | Concentration ($\mu$M) for 50% inhibition of methotrexate efflux ($IC_{50}$) | | |
|---|---|---|---|
| | control | −glucose [a] | +VCR |
| $PGA_1$ | 0.11 | 0.10 | 1.2 |
| Reserpine | 1.0 | 1.0 | 8.0 |
| BSP | 24 | 21 | 300 |
| Verapamil | 12 | 15 | 200 |
| CCCP | 0.3 | 0.3 | 0.3 |
| Probenecid | 75 | 64 | 110 |
| Quinidine | 105 | 95 | 100 |

[a] From Ref. 6.

When glucose was removed from the efflux assay, initial efflux declined by 10–20%, but no significant change was noted in $IC_{50}$ values for the efflux inhibitors either in control cells (cf. Table I and Ref. 6) or in cells treated with VCR (data not shown). When the effectiveness of each inhibitor (in VCR-treated cells) was measured at a storter time interval (3.5 min) relative to standard conditions (7 min), no change was noted in any of the $IC_{50}$ values.

Separation of a primary and secondary unidirectional efflux route for methotrexate could also be accomplished by adding low concentrations of $PGA_1$ (0.4 $\mu$M) or BSP (100 $\mu$M) directly to the efflux assay. Methotrexate efflux was reduced by 3–4-fold under these conditions, and the remaining efflux exhibited a 20-fold lower sensitivity to the further addition of $PGA_1$ or BSP (Table II). Probenecid, conversely, showed approximately the same capacity for inhibiting methotrexate efflux regardless of the presence of low levels of $PGA_1$ or BSP (Table II).

*Effect of efflux inhibitors on ATP levels in L1210 cells*

The structural diversity of methotrexate efflux inhibitors and their differential effects on separate efflux routes suggests that inhibition may occur by several mechanisms. Since an established means for efflux inhibition is energy deprivation [4,6,13,14]; the various efflux inhibitors employed in this study were screened for an effect on ATP levels. Measurements were performed in parental L1210 cells at inhibitor concentrations which were comparable to or greatly exceeded

TABLE II

*Effect of low concentrations of $PGA_1$ and BSP on the inhibitor sensitivity of methotrexate efflux in L1210 cells*

L1210 cells were loaded with [$^3$H]methotrexate, treated with NHS-methotrexate and evaluated for efflux after 3 min (control cells) or 7 min ($PGA_1$- or BSP-treated cells) at varying concentrations of the indicated inhibitors in HBBS/glucose: with no addition (control); plus 0.4 $\mu$M $PGA_1$ (+$PGA_1$); or plus 100 $\mu$M BSP (+BSP). Concentrations for 50% inhibition of methotrexate efflux ($IC_{50}$) were determined from plots of percent efflux activity remaining versus log inhibitor concentration. Values represent the mean of two or more determinations with standard deviations that were less than 20%.

| Inhibitor | Concentration ($\mu$M) for 50% inhibition of methotrexate efflux ($IC_{50}$) | | |
|---|---|---|---|
| | control | +$PGA_1$ | +BSP |
| $PGA_1$ | 0.11 | 1.5 | 1.1 |
| BSP | 24 | 260 | 300 |
| Probenecid | 75 | 105 | 95 |

the concentration needed to achieve half-maximal inhibition of methotrexate efflux. The results (Table III) show that only the inhibition of methotrexate efflux by CCCP could be attributed to an effect on energy metabolism. ATP levels were reduced by 80–90% at a concentration of CCCP (0.5 $\mu$M) which exceeded by about 2-fold the concentration of CCCP (0.3 $\mu$M) required to inhibit efflux by 50%. ATP levels were also reduced by probenecid, but this occurred only at a concentration (2 mM) which was substantially higher than required for half-maximal inhibition of efflux (100

TABLE III

*Effect of efflux inhibitors on ATP levels in L1210 cells*

ATP levels were measured as described in Materials and Methods in L1210 cells that had been preincubated in HBBS for 15 min at 37°C and then exposed for 5 min (at 37°C) to the indicated compounds. Values represent the mean of two separate experiments with duplicate samples assayed twice. Standard deviations were usually less than 10%.

| Addition | Concentration ($\mu$M) | ATP (pmol/mg protein) |
|---|---|---|
| None | – | 14.4 |
| CCCP | 0.5 | 1.5 |
| | 5.0 | 0.4 |
| $PGA_1$ | 20 | 13.9 |
| Reserpine | 20 | 15.7 |
| Probenecid | 500 | 10.8 |
| | 2000 | 4.3 |
| BSP | 50 | 12.9 |
| | 200 | 12.9 |
| Verapamil | 100 | 13.7 |
| | 400 | 11.4 |
| Quinidine | 100 | 14.7 |
| | 500 | 11.8 |
| VCR | 50 | 13.9 |
| Glucose | 5000 | 17.2 |

μM). The remaining compounds were relatively ineffective in reducing intracellular ATP even at levels 20-fold higher than the concentration required for half-maximal inhibition of efflux. In control experiments, ATP levels were not affected by incubating cells (in HBBS) with 10 μM methotrexate for 15 min at 37°C either with or without treatment with 10 μM NHS-methotrexate (for 5 min at 23°C).

## Discussion

The present study has addressed the hypothesis that the unidirectional efflux of methotrexate in L1210 cells consists of multiple components. Previous reports had shown that a majority of methotrexate efflux proceeds via an efflux component which exhibits energy-dependence and a high sensitivity to inhibition by BSP, VCR, $PGA_1$, reserpine, and verapamil, but biphasicity in the inhibition profiles for several compounds had also suggested that a second unidirectional route might also be present [3,5,6]. These prior results have now been extended by the development of an assay for the second route in which L1210 cells are exposed to concentrations of biphasic inhibitors which would substantially reduce the high-affinity system but show minimal effects on the low-affinity route. The biphasic inhibitors employed in this study were VCR, $PGA_1$, and BSP. A compilation of inhibitor responses by control cells and cells exposed to low levels of VCR, $PGA_1$, or BSP (Tables I and II) provides evidence for a primary and secondary efflux route for MTX with distinctly different inhibitor sensitivities. The major component is characterized by a relatively high sensitivity to $PGA_1$, VCR, reserpine, verapamil, and BSP, whereas comparable inhibition of the secondary route requires 8–20-fold higher concentrations of these compounds to achieve the same level of inhibition. Probenecid, CCCP, and quinidine comprise a second set of compounds which inhibit both efflux systems approximally equally. Since CCCP is a metabolic inhibitor, energy appears to be required in approximately equal amounts to drive each system. However, interdiction of an energy source can not explain the similarity in inhibition of both systems by probenecid or quinidine since these latter compounds affect efflux (Table I) at concentrations which have relatively little effect on ATP levels (Table III).

An additional feature of unidirectional MTX efflux is that inhibitors of the prominent route invariably inhibit to some extent the secondary route, suggesting that the two systems have common structural features. These similarities could reflect the presence of two separate systems with overlapping substrate specificities. A broad substrate specificity has already been implicated for the primary BSP-sensitive route for methotrexate since this same system also appears to mediate the efflux of cholate [6]. Alternatively, the two efflux routes may arise from a single transport protein which can exist in two different forms that do not readily interconvert. The notion of shared efflux substrates as diverse in structure as methotrexate and cholate has led to the proposal that the physiological function of these efflux systems is to export a diverse range of large and potentially toxic metabolic anions [6,15,16].

The proportion of total unidirectional efflux which proceeds via each route has been difficult to determine. Estimates from biphasic inhibition profiles suggest that the major component comprises about 75% of total unidirectional efflux and probably constitutes a single efflux system. The remaining 25% of unidirectional efflux may also represent a single route as judged by its apparent monophasic response to several efflux inhibitors, but its lower activity and relatively poor inhibitor responsiveness could have obscured additional routes with different inhibitor patterns. Studies performed elsewhere [2,10,13] support the notion of a unidirectional efflux of methotrexate, but only a single unidirectional route was observed [10]. The latter study [10] also reported that probenecid (added directly to loaded cells) was much less effective as an efflux inhibitor than had been reported in a previous study by the same group [2] or by us [3,6], and a different pattern of inhibition was also noted for verapamil [10]. The possibility was raised that differences between these prior studies and our results reported here and elsewhere [5,6] may have arisen from our use of HBBS without glucose, but the present results show that glucose has no effect on $IC_{50}$ values for various efflux inhibitors (Table I) and does not change the contribution to total efflux by the two unidirectional efflux components. In addition, cellular ATP levels (see Fig. 1) remain high in HBBS, compared with HBBS plus glucose, and are not significantly different from ATP levels in the TBBS/glucose buffer used in the prior study [10]. Our results suggest that the assay buffer in combination with longer incubation periods and other experimental differences masked the secondary efflux route for methotrexate in the prior study [10] and altered the response of efflux to probenecid and verapamil. HBBS has advantages over TBBS in maintaining cellular ATP levels in the absence of glucose (Fig. 1) and in maintaining the external pH during the incubation of cells at 37°C with or without added glucose.

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