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# Altered expression of unidirectional extrusion routes for methotrexate and cholate in an efflux variant of L1210 cells

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The specificity and function of two unidirectional anion-efflux pumps in mouse L1210 cells were evaluated using a variant cell line selected for growth in the presence of cholate and bromosulfophthalein. Transport analysis revealed that cholate efflux in the variant L1210/C7 cell line had declined 8-fold, due to the loss of a bromosulfophthalein-sensitive efflux system, the major extrusion route for cholate in parental cells. Efflux measurements showed further that a bromosulfophthalein-sensitive efflux system for methotrexate was also absent in L1210/C7 cells. Total unidirectional efflux of methotrexate, however, was similar in the variant and parental cells, since the loss in the bromosulfophthalein-sensitive system was compensated by a rise in a second probenecid-sensitive route. The latter was identified from inhibitor studies to be the same system which acts as a minor efflux route for methotrexate in parental cells. These results support the hypothesis that L1210 cells contain a bromosulfophthalein-sensitive efflux system which mediates the unidirectional extrusion of either methotrexate or cholate, and a second probenecid-sensitive route which differs from the bromosulfophthalein-sensitive system in inhibitor specificity and also in its ability to transport methotrexate but not cholate.

## Introduction

Various studies indicate that L1210 mouse leukemia cells contain three routes for the efflux of methotrexate [1–7]. Two of these routes are unidirectional efflux pumps which have been separated by their response to changes in pH [1] and to various inhibitors such as BSP [1,4–7], probenecid [2,3,6,7] and vincristine [5–7]. The principal unidirectional system accounts for about 60% of total efflux and is inhibited by BSP and vincristine [5–7], whereas the second unidirectional component comprises about 25% of the total and is relatively insensitive to BSP and vincristine but can be inhibited by probenecid [3,5–7]. The remaining 15% of total methotrexate efflux in L1210 cells [5,7] occurs via the bidirectional reduced-folate carrier which can be separated from the two unidirectional efflux systems by exposure of the cells to NHS-methotrexate [8]. L1210 cells also contain an energy-dependent and BSP-sensitive efflux pump for cholate which functions unidirectionally outward and accounts for 85–90% of total cholate efflux [6]. Inhibitor studies have suggested that the efflux of cholate proceeds via the same BSP-sensitive system which mediates the unidirectional efflux of methotrexate [6,9].

The present study reports on the isolation and characterization of an efflux variant of L1210 cells which had been selected for resistance to growth inhibition by a combination of BSP and cholate. Efflux measurements showed that the variant L1210/C7 cell line had undergone two distinct changes during the selection procedure. The BSP-sensitive efflux routes for methotrexate and cholate that predominate in parental cells were absent in L1210/C7 cells, whereas a secondary probenecid-sensitive efflux activity for methotrexate had increased by 3-fold. The isolation of a variant cell line with an altered expression of efflux activities, combined with differences in ability to transport cholate and methotrexate, has allowed the unambiguous identification and separation of two anion-efflux routes in L1210 cells. Since the loss of one anion-efflux route in L1210/C7 cells is compensated by a rise in a second route, it also appears likely that these two

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Abbreviations: BSP, bromosulfophthalein; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate; NHS-methotrexate, *N*-succinimide ester of methotrexate; HBS, Hepes-buffered saline; HBBS, Hepes/bicarbonate-buffered saline.

efflux systems are structurally or functionally related.

## Materials and Methods

*Chemicals*

[2,4-$^3$H]Cholic acid (25 Ci/mmol) (New England Nuclear) and [3′,5′,9-$^3$H]methotrexate (20 Ci/mmol) (Moravek Biochemicals) were purified and stored as described previously [7], and NHS-methotrexate was synthesized by an established procedure [8]. Methotrexate, BSP (sulfobromophthalein), prostaglandin $A_1$, quinidine, reserpine, verapamil, probenecid, CCCP and antimycin A were obtained from Sigma.

*Cells*

Parental L1210 mouse leukemia cells and subline L1210/C7 were grown in maintenance cultures as described previously [8] in RPMI 1640 medium containing 3% fetal bovine serum and antibiotics. Transport measurements were performed with cells grown in the same medium in 500-ml portions in a shaking incubator. Cells were harvested at a density of $(0.9–1.3) \cdot 10^6$/ml, chilled to 4°C, collected by centrifugation at 4°C ($500 \times g$), and washed with HBS prior to suspension (at 4°C) in HBBS. Buffer compositions were: HBS: 20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.35 with NaOH. HBBS: 20 mM Hepes, 5 mM Na-bicarbonate, 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.35 with NaOH.

*Variant isolation*

The L1210/C7 subline was isolated by adapting parental L1210 cells in 10-ml cultures to RPMI 1640 medium containing 5% fetal bovine serum, antibiotics, 100 $\mu$M cholate and 100 $\mu$M BSP. Transfers were performed twice weekly for 20 weeks before cell morphology and growth rate approximated parental cells. Cultures were then screened for single clones by dilution into 100-mm soft agar Petri plates containing 30 ml of complete RPMI 1640 medium (5% fetal bovine serum and antibiotics) supplemented with 0.25% SeaKem LE agarose, 100 $\mu$M BSP and 300 $\mu$M cholate. Visible clones were removed from the plate after 10–12 days. L1210/C7 cells, which were indistinguishable from the parental cells either by growth rate or morphology, were grown in standard medium for at least three generations prior to use in transport measurements. Exposure of stock cultures for three days to standard medium containing 100 $\mu$M BSP and 300 $\mu$M cholate was performed monthly to prevent the reappearance of the parental phenotype.

*Measurements of growth inhibition*

The growth response of L1210 and L1210/C7 cells to varying concentrations of cholate, BSP and methotrexate was determined in 96-well plates by the procedure of Mosmann [10]. Growth was determined from the extent of reduction of a tetrazolium dye and was quantitated using a commercial plate reader with a 570 nm filter. The serum concentration in the growth medium was 5%, the time of exposure to inhibitors was 72–96 h, and the inoculum was obtained from cells grown in a shaking incubator. Data points represent the mean of at least three separate experiments performed in duplicate.

*Uptake and efflux measurements*

Cholate uptake was measured as described previously [6] in samples containing $2 \cdot 10^7$ cells, 100 nM [$^3$H]cholate, the desired additions and HBBS buffer in a final volume of 0.25 ml. Cholate efflux was measured [6] in cells that had been suspended to $(6–10) \cdot 10^7$/ml in HBBS, incubated for 15 min at 37°C with 100 nM [$^3$H]cholate, diluted and washed with HBBS, and resuspended to $2 \cdot 10^7$ cells/ml in HBBS. Loads for efflux measurements were typically $0.40 \pm 0.10$ and $0.65 \pm 0.10$ pmol [$^3$H]cholate/mg protein for L1210 and L1210/C7 cells, respectively.

Methotrexate uptake was measured in samples containing $2 \cdot 10^7$ ml cells, 10 $\mu$M [$^3$H]methotrexate, the desired additions and HBBS in a final volume of 1.0 ml. After incubation for the desired time at 37°C, the cells were chilled to 4°C, diluted with 8 ml of ice-cold HBS, collected by centrifugation, washed once with 8 ml of HBS, and analyzed for radioactivity as described previously [7]. Influx was determined by a similar procedure in cells incubated with varying concentrations of [$^3$H]methotrexate for 2 min at 37°C. Identical cell mixtures incubated at 4°C served as the controls for influx. Methotrexate efflux was measured in cells loaded at 10 $\mu$M [$^3$H]methotrexate and then treated with 5 $\mu$M NHS-methotrexate prior to efflux measurements [5,7]. The typical load of [$^3$H]methotrexate after 15 min at 37°C in L1210 and L1210/C7 cells was $110 \pm 20$ and $180 \pm 30$ pmol/mg protein, respectively. The latter values exceeded intracellular concentrations of dihydrofolate reductase by more than 10-fold. Determinations of $IC_{50}$ values for half-maximal inhibition of cholate and methotrexate efflux were performed as described previously [6,7]. Reported values represent the mean of at least three separate determinations. Protein concentrations were determined by the Biuret method [11] using bovine serum albumin as the standard.

*Cholate metabolism*

The metabolism of [$^3$H]cholate was measured in $2 \cdot 10^8$ L1210 cells that had been suspended in 10 ml HBBS or complete medium containing either 0.2 $\mu$M [$^3$H]cholate (10 000 000 cpm/nmol) or 10 $\mu$M [$^3$H]cholate (200 000 cpm/nmol) and incubated with

shaking in a sealed 25-ml Erlenmeyer flask containing a 5% $CO_2$ atmosphere. After 30 min or 24 h at 37°C, the cells and medium were separated by centrifugation at 4°C (5 min, $1000 \times g$). Cell pellets were washed twice with 5 ml HBS and the cellular contents were extracted by suspending in 1.0 ml of methanol/acetic acid/water (70:2:28). After vigorous mixing and brief probe sonication, the sample was clarified by centrifugation at 4°C (20 min, $24000 \times g$) and the supernatant (cytosolic fraction) was retained for analysis. The cell-free culture medium was diluted 1:1 with acetone, placed at −20°C for 16 h, and clarified (as above) by centrifugation at 4°C. Samples (200 μl) were analyzed by HPLC using an Altex $C_{18}$ Ultrasphere-ODS column (0.46 × 25 cm) and an isocratic solvent system [12] consisting of methanol and water (70:30) adjusted to pH 3 with phosphoric acid. Fractions (1.0 ml) were collected, placed at 37°C until dry, suspended in 200 μl water, transferred to scintillation vials with the aid of two 4-ml portions of ScintiVers BD (Fisher), and analyzed for radioactivity.

## Results

*Isolation, growth characteristics and inhibitor sensitivity of L1210/C7 cells*

Various growth conditions were tested in an effort to isolate sublines of L1210 cells with altered efflux of methotrexate and/or cholate, two anions which appear from kinetic studies to share a common BSP-sensitive efflux pump in L1210 cells [6]. Growth conditions included the exposure of L1210 cells to increasing amounts of cholate and also to combinations of cholate and BSP. The latter conditions were used to isolate an L1210/C7 subline which differed from parental cells in the ability to tolerate high concentrations of cholate in the presence of BSP.

BSP produces a substantial increase in cholate toxicity in parental L1210 cells, but this property is lost in L1210/C7 cells. Whereas BSP alone (at 100 μM) has no effect on the growth of L1210 cells (compared to control cells without BSP), BSP combined with cholate (Table I) increases cholate toxicity by 6-fold. $IC_{50}$ values for cholate declined from 0.48 mM in L1210 cells without BSP to 0.08 mM in cells containing 100 μM BSP. Contrasting results were obtained with L1210/C7 cells, which showed only a small BSP-dependent increase in cholate toxicity (Table I). $IC_{50}$ values for cholate in L1210/C7 cells declined only 1.2-fold from 0.28 mM to 0.23 mM upon addition of 100 μM BSP. The inherent sensitivity to cholate was also higher in L1210/C7 cells than in parental cells (by 1.7-fold). Methotrexate was slightly less effective in inhibiting growth of L1210/C7 cells ($IC_{50} = 15 \pm 4$ nM) than parental cells ($IC_{50} = 10 \pm 3$ nM), and BSP (100 μM) produced a slight decrease in sensitivity to methotrexate ($IC_{50} = 20 \pm 5$ nM) in both cell lines. Growth of L1210/C7 cells under the selection conditions can thus be explained by a loss in the ability of BSP to increase cholate toxicity.

TABLE I

*Effect of BSP on the sensitivity of L1210 and L1210/C7 cells to growth inhibition by cholate*

The effect of 100 μM BSP on half-maximal growth inhibition by cholate ($IC_{50}$) was determined in 96-well plates as described in Materials and Methods.

| Cell line | Half-maximal growth inhibition by cholate (mM) | | Enhancement (-fold) |
|---|---|---|---|
| | − BSP | + BSP | |
| L1210 | 0.48 ± 0.07 | 0.08 ± 0.03 | 6.0 |
| L1210/C7 | 0.28 ± 0.04 | 0.23 ± 0.05 | 1.2 |

*Cholate uptake and efflux by L1210/C7 cells*

Prior studies with L1210 cells have established the presence of a unidirectional and energy-dependent efflux system for cholate which can be blocked by various inhibitors [6,7]. Efflux via this system is rapid ($t_{\frac{1}{2}} = 1.5 \pm 0.2$ min) and first-order in cells loaded either at 50 nM [$^3$H]cholate [6] or at concentrations of [$^3$H]cholate up to 10 μM. Above 10 μM, first-order kinetics are retained, whereas the $t_{\frac{1}{2}}$ for efflux begins to increase, suggesting that saturation of the efflux system can be achieved, but only at relatively high levels of this substrate. The influx of cholate is insensitive to inhibitors and increases linearly at substrate concentrations between 50 nM and 100 μM [6], and hence exhibits the properties of passive diffusion.

The decreased sensitivity of L1210/C7 cells to the combination of cholate and BSP suggested that these cells have acquired an altered ability to accumulate or extrude cholate. Transport measurements in HBBS (Fig. 1) revealed that L1210/C7 cells exhibit a significant change in cholate efflux, but not influx. Initial uptake proceeded at approximately the same rate in both L1210 and L1210/C7 cells lines, but total accumulation of cholate was higher in L1210/C7 cells. The difference in uptake was 1.6-fold after 15 min (Fig. 1), and by 30 min (Table II), cholate uptake in the variant cells was greater than twice the level of L1210 cells. When efflux inhibitors (BSP, probenecid or prostaglandin $A_1$) were added to the incubation mixture (Table II), cholate uptake after 30 min was unchanged or only slightly increased in L1210/C7 cells, whereas the same additions to parental cells increased uptake by 2.5-fold. Efflux inhibitors increased cholate uptake in parental cells to approximately the same level that was achieved in variant cells in the absence of inhibitors.

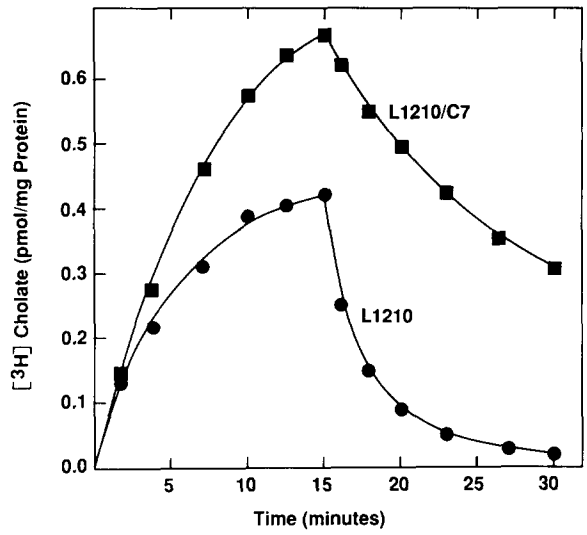


Fig. 1. Comparison of cholate uptake and efflux by L1210 and L1210/C7 cells. Uptake was measured in cells suspended in HBBS containing 100 nM [$^3$H]cholate, incubated for the indicated times at 37°C and analyzed for accumulated radioactivity (see Materials and Methods). Efflux was measured in cells incubated with 100 nM [$^3$H]cholate for 15 min, recovered by centrifugation, washed and analyzed for the time-dependent release of the labeled substrate.

The efflux of cholate by parental and variant cells was substantially different (Fig. 1). L1210 cells exposed to [$^3$H]cholate for 15 min and then washed to remove extracellular substrate exhibited a $t_{\frac{1}{2}}$ of 1.5 min for 50% extrusion of the accumulated substrate, whereas L1210/C7 cells treated similarly showed a $t_{\frac{1}{2}}$ of 12 min, an 8-fold decrease in rate.

*Methotrexate uptake and efflux in L1210/C7 cells*

Prior evidence for a shared efflux pump for cholate and methotrexate in L1210 cells [6] led to a comparison of methotrexate transport (in HBBS) in the parental and variant cell lines. Methotrexate influx and total uptake after 15 min (Fig. 2) and after 30 min (not shown) were each 2-fold higher in L1210/C7 cells, whereas methotrexate efflux was approximately the same in both cell lines (Fig. 2). The $t_{\frac{1}{2}}$ for efflux was 3.5 min in the mutant cells, compared with 3.0 min in the parent. Analysis of initial influx versus methotrexate concentration showed that the $K_t$ for methotrexate influx in HBBS (5.4 $\mu$M) was the same in both cell lines, whereas the $V_{max}$ for methotrexate had increased about 2-fold in L1210/C7 cells (35.0 pmol/min per mg protein) relative to the parent (17.8 pmol/min per mg protein).

TABLE II

*Effect of efflux inhibitors on the uptake of cholate in L1210 and L1210/C7 cells*

Uptake was measured at 0.1 $\mu$M [$^3$H]cholate in cells suspended in HBBS containing the indicated concentration of inhibitor and incubated for 30 min at 37°C.

| Cell line | Addition | Concentration ($\mu$M) | Cholate uptake (pmol/mg protein) | Stimulation (-fold) |
|---|---|---|---|---|
| L1210 | None | – | 0.48 | 1.0 |
| | BSP | 200 | 1.23 | 2.6 |
| | Probenecid | 1000 | 1.20 | 2.5 |
| | Prostaglandin $A_1$ | 2 | 1.10 | 2.3 |
| L1210/C7 | None | – | 1.00 | 1.0 |
| | BSP | 200 | 1.13 | 1.1 |
| | Probenecid | 1000 | 1.09 | 1.1 |
| | Prostaglandin $A_1$ | 2 | 0.98 | 1.0 |

*Inhibitor specificity of the unidirectional efflux components for cholate and methotrexate efflux in L1210/C7 cells*

The efflux components for cholate (Fig. 1) and methotrexate (Fig. 2) in L1210/C7 cells were characterized further using efflux inhibitors. Cholate efflux in L1210/C7 cells was only partially responsive to inhibitors (Fig. 3B). The slow efflux in these cells ($t_{\frac{1}{2}} = 12$ min) with no addition (line 1, Fig. 3B) could be reduced by an additional 2-fold ($t_{\frac{1}{2}} = 20$–25 min) by 10 $\mu$M antimycin A (line 4, Fig. 3B) or by 2 mM probenecid (line 3, Fig. 3B), whereas a lower inhibition was observed with 200 $\mu$M BSP (line 2, Fig. 3B). Glucose (5 mM) increased efflux only slightly (10%), indicating that the slow efflux in these cells was not due to limitations in cellular energy. This low dependence for glucose is consistent with prior studies which

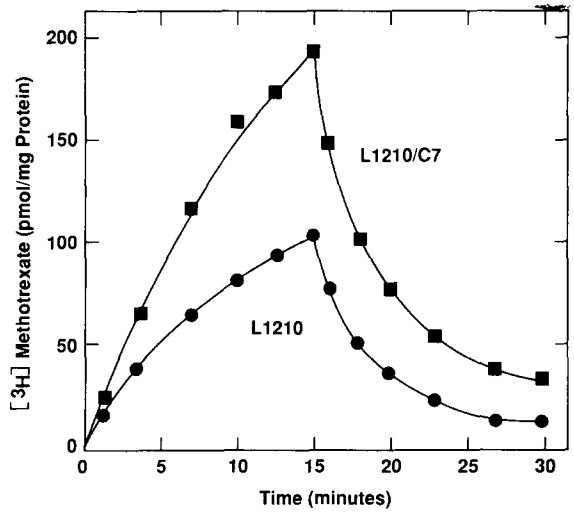


Fig. 2. Uptake and efflux of methotrexate by L1210 and L1210/C7 cells. Uptake was measured in cells suspended in HBBS containing 10 $\mu$M [$^3$H]methotrexate, incubated for the indicated times at 37°C and analyzed for accumulated radioactivity (see Materials and Methods). Efflux was measured in cells incubated with 10 $\mu$M [$^3$H]methotrexate for 15 min, recovered by centrifugation, treated with NHS-methotrexate and analyzed for the time-dependent release of the labeled substrate.

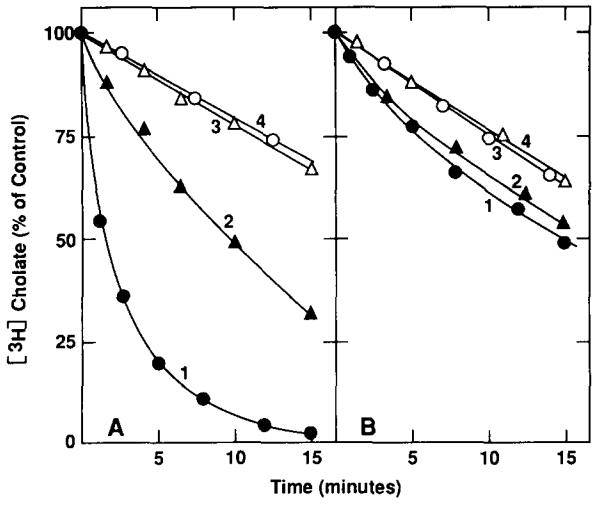


Fig. 3. Effect of various additions on cholate efflux by L1210 cells (A) and L1210/C7 cells (B). Additions: line 1, none; line 2, 200 $\mu$M BSP; line 3, 2 mM probenecid; line 4, 10 $\mu$M antimycin A.

had shown that L1210 cells in HBBS buffer retain high levels of ATP [7]. The substantially higher efflux of cholate in parental cells and its response to efflux inhibitors is shown in Fig. 3A.

The inhibitor specificity of methotrexate efflux in L1210 (Fig. 4A) and L1210/C7 (Fig. 4B) was also evaluated. A comparable and nearly complete inhibition of methotrexate efflux was observed in both cells lines with 2 mM probenecid and 10 $\mu$M antimycin A, but a different response was observed with BSP. The latter compound (at 200 $\mu$M) reduced methotrexate efflux (line 2 vs. line 1) in parental cells by 70%, whereas the same concentration of BSP decreased

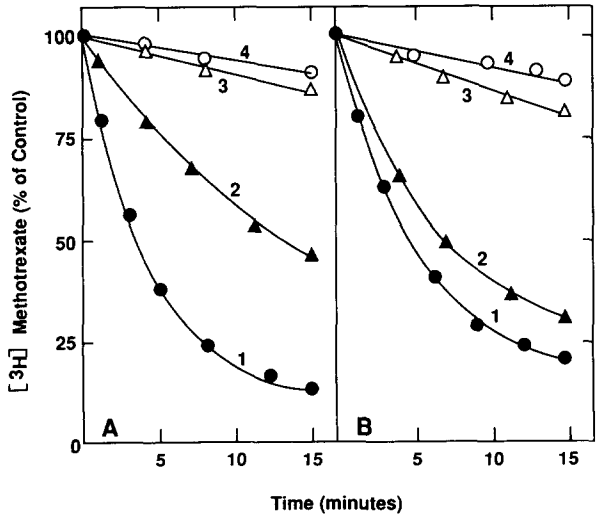


Fig. 4. Effect of various additions on methotrexate efflux in L1210 cells (A) and L1210/C7 cells (B) pretreated with NHS-methotrexate. Additions: line 1, none; line 2, 200 $\mu$M BSP; line 3, 2 mM probenecid; line 4, 10 $\mu$M antimycin A.

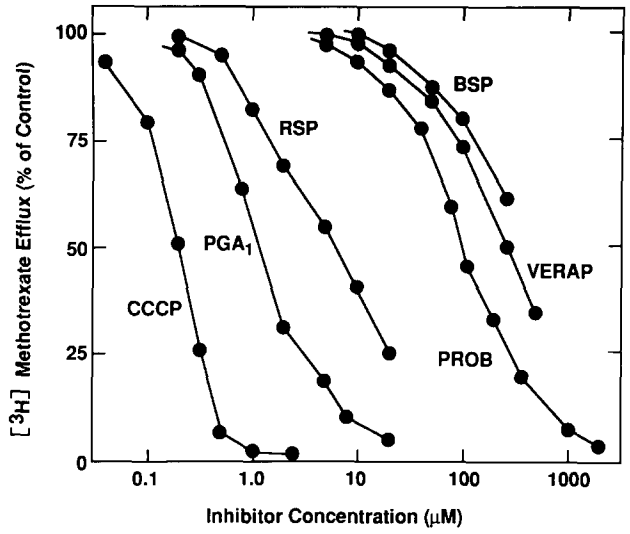


Fig. 5. Concentration dependence for the inhibition of methotrexate efflux by CCCP, prostaglandin $A_1$ ($PGA_1$), reserpine (RSP), probenecid (PROB), verapamil (VERAP) and BSP in L1210/C7 cells. Cells were loaded by incubation (in HBBS) with 10 $\mu$M [$^3$H]methotrexate (15 min, 37°C), treated with 5 $\mu$M NHS-methotrexate (2 min, 23°C) and then analyzed for [$^3$H]methotrexate efflux (4 min, 37°C) in the presence of the indicated concentration of inhibitor. Plotted values were calculated from the first-order rate constant for efflux at each inhibitor concentration divided by efflux in the control without inhibitor $\times 100$.

efflux only 25% in the mutant cells. Glucose had only a slight stimulatory effect (2–10%) on methotrexate efflux in either cell line. It thus appeared that the prominent BSP-sensitive efflux component for methotrexate in parental cells was less active or absent in L1210/C7 cells.

Methotrexate efflux in L1210/C7 cells responded poorly to increasing concentrations of BSP and inhibition failed to reach 50% at the highest concentration of BSP employed (200 $\mu$M) (Fig. 5). This result differs substantially from parental cells which show a major BSP-sensitive component ($IC_{50} = 21$ $\mu$M) that constitutes 70–75% to total unidirectional efflux [6,7]. The response of methotrexate efflux in L1210/C7 cells to several other inhibitors is also shown in Fig. 5. Their order of effectiveness (CCCP, prostaglandin $A_1$, reserpine, probenecid, verapamil, BSP) also differs substantially from that obtained when these same compounds are employed as inhibitors of methotrexate efflux in parental cells [6,7]. An analysis of the data in Fig. 5 for multiple efflux routes as described previously [7] did not provide evidence for more than a single efflux component for methotrexate. Each efflux inhibitor in this study was also examined for the rate of appearance of maximal inhibition. This was achieved by measuring the release of methotrexate at one-minute intervals for 5 min using an inhibitor concentration which produced roughly 50% inhibition. With the exception of vin-

TABLE III

*Inhibitor sensitivity of unidirectional efflux routes for methotrexate in L1210/C7 and L1210 cells*

Half-maximal inhibition of methotrexate efflux ($IC_{50}$) was determined from plots of efflux activity vs. inhibitor concentration (see Fig. 5). Inhibitors were added just prior to the initiation of efflux, except vincristine, which was added during the loading interval (15 min at 37°C) with [$^3$H]methotrexate [5]. Efflux in L1210/C7 cells was measured after 4 min at 37°C.

| Inhibitor | Half-maximal inhibition of efflux ($\mu$M) | | |
|---|---|---|---|
| | L1210/C7 cells | L1210 cells Probenecid-sensitive route [a] | L1210 cells BSP-sensitive route [b] |
| CCCP | 0.21 | 0.3 | 0.29 |
| Prostaglandin $A_1$ | 1.1 | 1.2 | 0.10 |
| Reserpine | 6.2 | 8.0 | 1.0 |
| Probenecid | 98 | 110 | 64 |
| BSP | > 200 | 300 | 21 |
| Verapamil | 250 | 200 | 15 |
| Quinidine | 105 | 100 | 95 |
| Vincristine | 65 | 43 [c] | 3 [c] |

[a] From Ref. 7.
[b] From Ref. 6.
[c] From Ref. 5.

cristine (see Table III), the extent of inhibition by each of the inhibitors did not change significantly over time (i.e., inhibition was approx. 50% during both the first and last minute of the time-course).

Inhibitor sensitivities for methotrexate efflux are compared in Table III. Data are included from the present study for methotrexate efflux in L1210/C7 cells, and for two other activities identified previously in parental L1210 cells that are sensitive to probenecid and BSP [5,6] or only to probenecid [7]. The results show that the inhibitor specificity of methotrexate efflux in L1210/C7 cells is in close agreement with that of the probenecid-sensitive route in parental cells but is substantially different from the BSP-sensitive system.

*Cholate metabolism by cells*

The compilation of results from short-term efflux measurements with long-term growth studies led to an evaluation of the ability of L1210 and L1210/C7 cells to metabolize cholate. Intracellular and extracellular samples were prepared after incubation of cells with 10 $\mu$M [$^3$H]cholate for: 30 min at 37°C in HBBS buffer; 30 min at 37°C in complete growth medium; and 24 h at 37°C in complete medium; or with 0.2 $\mu$M [$^3$H]cholate for 30 min at 37°C in HBBS. Evaluation by HPLC (see Materials and Methods) indicated that no significant metabolism of the [$^3$H]cholate had occurred under any of these conditions. In each case a single peak was observed which eluted at 30 ± 1 ml and was coincident with the [$^3$H]cholate standard. Recovery of total radioactivity for each sample was 95 ± 5%.

## Discussion

The physiological function, specificity and common features of the unidirectional efflux systems for methotrexate and cholate in L1210 mouse cells have been investigated by comparing the growth features and efflux characteristics of parental L1210 cells and an efflux variant which had been selected for resistance to a combination of cholate and BSP. Resistant L1210 sublines were pursued to determine whether cells with altered cholate efflux also exhibit comparable changes in the efflux of methotrexate. Parallel changes in cholate and methotrexate efflux would be consistent with inhibitor studies which had suggested that these anions share a common BSP-sensitive efflux mechanism in L1210 cells [6,9]. A combination of BSP and cholate was included in the selection protocol, since BSP had been observed to increase cholate toxicity by 6-fold in parental cells (Table I), presumably by blocking cholate efflux [6]. The prominent growth feature of the isolated L1210/C7 clone was the loss in ability by BSP to enhance cholate toxicity (Table I). A modest 1.7-fold rise in the inherent sensitivity of the variant cells to cholate was also observed. Another change was noted which appeared unrelated to efflux pumps. The influx of methotrexate via the reduced-folate carrier system was found to increase 2-fold in L1210/C7 cells. The basis for this increase is unclear, but folate limitation may have developed from the presence of BSP during the selection period since BSP is a potent inhibitor of the influx system [13] that is required for the uptake of essential folate compounds. Hence, up-regulation of the influx carrier might have occurred to relieve a folate deficiency induced by BSP.

Transport measurements revealed that L1210/C7 cells do not exhibit appreciable levels of the BSP-sensitive cholate efflux route that is the predominant extrusion mechanism for cholate in parental cells. The time-course for cholate uptake (Fig. 1) showed that the initial influx of cholate was relatively unchanged in L1210/C7 cells but that total uptake was elevated due to an 8-fold reduction in cholate efflux. The extent of elevation in cholate uptake can be attributed solely to a loss of the BSP-sensitive efflux component, since the same elevated level of cholate uptake that occurs in L1210/C7 cells (after 30 min at 37°C) is also obtained in parental cells by the addition of an efflux inhibitor (BSP, probenecid or prostaglandin $A_1$, Table II). Moreover, the ability of efflux inhibitors to enhance cholate uptake (by 2.5-fold) in parental cells is not observed in L1210/C7 cells (Table II). Total uptake of cholate, however, increased by less than the 8-fold predicted by the extent of loss in efflux. The basis for

this discrepancy remains unclear, although a contributing factor might be the inability to reach a steady state after 30 min at 37°C. L1210/C7 cells retain a small inhibitor-sensitive component which mediates about 60% of the remaining portion of cholate efflux (Fig. 3B), but the specificity of this route is clearly different from the BSP-sensitive efflux system of parental cells [6] and is more closely aligned with the minor probenecid-sensitive efflux system for methotrexate [7]. The inhibitor-insensitive portion of cholate efflux in L1210/C7 cells mediates about 40% of the total and has the properties of passive diffusion. A similar absolute amount of cholate efflux had been assigned previously to passive diffusion in parental cells [6], but in the latter case it represented a much smaller portion (5%) of total efflux.

Methotrexate efflux in L1210/C7 cells exhibits a different inhibitor specificity when compared with parental cells. The prominent BSP-sensitive route for methotrexate in L1210 cells is absent in the variant cell line, and it has been replaced by a BSP-insensitive activity. Moreover, the inhibitor specificity of this BSP-insensitive component corresponds closely to a minor probenecid-sensitive efflux route that has been reported previously for methotrexate in parental cells (Table III). These findings indicate that L1210/C7 cells lack the prominent BSP-sensitive efflux system of parental L1210 cells and have aquired elevated levels of the normally less-active probenecid-sensitive route. The probenecid-sensitive activity in L1210/C7 cells is expressed at 3-fold normal levels and hence approximates the composite of the two efflux activities for methotrexate in parental cells. The residual probenecid-sensitive efflux for cholate in L1210/C7 cells (Fig. 3B) did not exhibit a similar 3-fold increase in activity and hence appeared to be a different efflux system. The isolation of an efflux variant lacking the prominent BSP-sensitive activity of parental cells and expressing elevated levels of the minor probenecid-sensitive system supports the prior conclusion that L1210 cells express both a major and minor efflux route for methotrexate [6,7]. Other studies had suggested that the unidirectional efflux of methotrexate in L1210 cells proceeds only via the BSP-sensitive system [14]. The efflux variant also provides a convenient system for measuring this minor route.

A parallel loss of the BSP-sensitive efflux routes for methotrexate and cholate in L1210/C7 cells provides further evidence for a shared efflux component for these two anions. The simultaneous down-regulation of efflux activities in L1210/C7 cells for both methotrexate and cholate compliments prior kinetic evidence in parental L1210 cells which showed that the efflux of these anions exhibits a comparable sensitivity to various inhibitors [6]. Inhibitor studies have also provided evidence that cholate shares a common unidirectional efflux route with cAMP in C6 rat glioma cells [15]. The ability of unidirectional efflux systems to accommodate anions, such as methotrexate and cholate [6,9] or cholate and cyclic AMP [15], suggests that these efflux pumps have an unusually broad substrate specificity and hence may function to expel many structurally diverse intracellular anions [6,9,15]. Similar ATP-dependent and unidirectional efflux pumps have been described previously for other anions including oxidized glutathione and glutathione-*S*-conjugates [16–19], organic anion dyes [20–22], penicillin [23] and substituted leukotrienes [23,24], but the extent to which various anions share efflux routes, the number of routes available for each anion substrate, and the relationship to the cholate and methotrexate efflux pumps of L1210 mouse cells remains unclear. The identification of at least two anion efflux systems for methotrexate in L1210 cells, and the finding that one of these efflux systems also mediates the efficient efflux of cholate, supports the hypothesis that a family of broadly-specific anion efflux pumps may be responsible for mediating unidirectional anion efflux in L1210, as well as other mammalian cells.

BSP increases the toxicity of cholate in parental L1210 cells by 6-fold, presumably by increasing cholate accumulation via a block in cholate efflux. Methotrexate toxicity, however, does not undergo a similar increase in L1210 cells, even though cholate and methotrexate share a major BSP-sensitive efflux route. These differential effects by BSP can be explained by the presence of additional influx and efflux routes for methotrexate that are not shared with cholate. BSP has no effect on the influx of cholate [6], but it is a potent inhibitor of the influx of methotrexate [4,13]. Hence, a reduction in methotrexate uptake by BSP could neutralize or reverse any increase in methotrexate sensitivity that occurs by a block in methotrexate efflux by BSP. Methotrexate efflux also proceeds to a greater extent via BSP-insensitive routes which do not contribute to the efflux of cholate [6,7].

The importance to L1210/C7 cells in acquiring enhanced activity of the probenecid-sensitive efflux route is not clear, although it is possible that this second route increased to compensate for the loss of the BSP-sensitive system. This explanation would be feasible if the two efflux systems have a common function and overlap in substrate specificity. Hence, an increase in the probenecid-sensitive system might be necessary for the extrusion of common anion substrates which might otherwise become toxic to cells lacking the BSP-sensitive system. The coincident rise in one efflux activity to an extent comparable to the loss of the other activity also raises the possibility that the two efflux systems for methotrexate may represent two forms of the same transport system. The altered efflux characteristics of L1210/C7 cells might then be explained by

a single genetic change affecting a regulatory site on a component of the efflux system.

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