Energy-dependent Efflux of Methotrexate in L1210 Leukemia Cells

EVIDENCE FOR THE ROLE OF AN ATPase OBTAINED WITH INSIDE-OUT PLASMA MEMBRANE VESICLES*

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Earlier studies from our laboratory (Dembo, M., Sirotnak F. M., and Moccio, D. M. (1984) J. Membr. Biol. 78, 9–17) suggested that methotrexate (MTX) efflux from L1210 cells was mediated predominantly by an ATP-dependent, outwardly directed, mechanism. To examine this process further, we utilized predominantly (74%) inside-out plasma membrane vesicle preparations derived from an L1210 cell variant (L1210/R24) with 15-fold reduced V_{max} for [3H]MTX influx. Efflux of [3H]MTX, under nonionic buffer conditions, in these inside-out membrane vesicles was temperature and ATP dependent (apparent K_m = 0.40 ± 0.06 mM), osmotically sensitive, and unaffected by protonophores. The presence of K^+, Na^+, Cl^-, and HCO_3^- at their physiological concentrations had no effect on [3H]MTX efflux. Other triphosphonucleotides (GTP and CTP), but not a nonhydrolyzable analogue, adenosine-5'-O-(3-thiotriphosphate) (ATP[S]), could also stimulate efflux, but to a lesser extent. Also, ATP[S] and orthovanadate were potent inhibitors of ATP-dependent efflux of [3H]MTX. Efflux experiments revealed a system with low saturability for [3H]MTX during efflux (apparent K_m = 46 ± 7 μM), but extremely high capacity (106 ± 15 pmol/min/mg protein), and a pH optimum in the range of 5.5–6. However, appreciable efflux was measured in the physiological range of pH 6.7–6.9. A number of inhibitors or coperoxidases for ATP-dependent [3H]MTX efflux in intact L1210 cells were inhibitors of ATP-dependent efflux in inside-out plasma membrane vesicles, including, cholate, bromosulphalein, verapamil, quinidine, and reserpine. These findings and other results showing that bromosulphalein will completely inhibit efflux are consistent with a role for an ATPase in [3H]MTX efflux, and suggest that the process under study is the bromosulphalein-sensitive, ATP-dependent route responsible for the majority of [3H]MTX efflux in intact L1210 cells.

Previous studies from this laboratory (1–3) have demonstrated that mediated transport of folate analogues, such as methotrexate, in L1210 leukemia cells involves separate systems for influx and efflux. In these cells, it was found that efflux of MTX was mediated primarily by an energy-dependent and outwardly directed mechanism. The activity of this efflux route is strictly dependent upon the level of intracellular ATP and is inopercable in ATP-depleted cells (2). The concept of an energy-dependent mechanism, which mediates efflux of MTX in tumor cells, was originally proposed by Hakala (4) using a Sarcoma 180 cell line. This worker observed that MTX accumulation at steady-state was undetectable but was dramatically increased when cells were exposed to 2,4-dinitrophenol, an inhibitor of oxidative phosphorylation. This result indicated that this effect may be due to inhibition of an active efflux process that requires metabolic energy. Subsequently, Goldman and co-workers (5, 6) carried out more detailed studies with L1210 cells using sodium azide to inhibit metabolic energy production. They demonstrated that the addition of 10 mM sodium azide increased the intracellular steady-state concentration of osmotically active MTX and that this effect was abolished by the addition of d-glucose (5, 6). They also showed that sodium azide had opposing effects on influx and efflux of MTX but also suggested that their data did not distinguish between the following possibilities, namely different effects of this agent on inward and outward flux mediated by the same system, or that two separate systems with different energy requirements were actually involved.

Our own studies, utilizing kinetic and energetic analysis, suggested (1–3) that the principal efflux route for MTX in ATP-replete L1210 cells was most likely distinct from the system mediating its influx. Additional evidence to support this notion was derived in experiments showing that each flux exhibited different specificities for various structural analogues (7), differing sensitivities to various biochemical and pharmacologic agents (2), and different growth-phase dependencies (8). In addition, in other studies from our laboratory, genetic variants of the L1210 cell were isolated (9, 10) with either increased or decreased influx of MTX and amount of the putative transporter (11) with no effect on efflux of this analogue. Finally, in related experiments, influx and efflux of [3H]MTX were functionally dissociated (12) in HL-60 cells following induction of terminal maturation.

Although our earlier studies suggested (1, 2) that only a single energy-dependent route mediated efflux of MTX in L1210 cells, other workers (13, 14) presented evidence for an additional, albeit minor, unidirectional efflux route in these cells. In a reexamination of this question in our own laboratory (3), using the same methodological approaches, no evidence was obtained for a second energy-dependent route mediated efflux of MTX in L1210 cells.
ence for a second mediated efflux route could be generated. The basis for this discrepancy is unclear, but it could reflect differences in carrier involvement or activity among cell lines. In the present study, a significant efflux of MTX was found to be mediated by a \( \text{MgCl}_2 \)-dependent process. This efflux was sensitive to the presence of detergents, suggesting a role for membrane lipids in the efflux mechanism.

**EXPERIMENTAL PROCEDURES**

Cell Growth and Isolation—L1210/R24 cells for vesicle preparation were obtained as ascites suspensions from C57Bl X DBA/2Fl hybrid (C57Bl X DBA/2Fl) mice. These cells were maintained by intraperitoneal injection into mice without MTX treatment prior to harvesting. Cells were pelleted by centrifugation at 2500 rpm, washed twice with buffer, and resuspended to a concentration of 1 mg/ml in medium 2 (25 mM Tris-HCl, pH 7.2, 250 mM sucrose, and 0.5 mM dithiothreitol) in a Potter-Elvehjem homogenizer with a Teflon pestle at a concentration of 1 mg of buffer/g wet weight of cells for 20 strokes at 2500 rpm. The homogenate was centrifuged at 1000 x g for 10 min, and the supernatant was saved. The pellet was washed with buffer, centrifuged at 48,000 x g for 10 min. The supernatant was centrifuged at 45,000 x g for 20 min. The pellet was resuspended to a concentration of 4-6 mg/ml in medium 2 (25 mM Tris-HCl, pH 7.2, 100 mM mannitol, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 1 mM MnCl\(_2\)), as per protein determinations using \( \text{A}_{280/200} \) in H\(_2\)O. The suspension was incubated for 30 min on ice, and the vesicles were collected by centrifugation at 1400 x g for 12 min. The vesicles were resuspended in the same volume of medium 2 that was used to form them, shaken vigorously, and again collected at 1400 x g for 12 min. The vesicle pellet was diluted in resuspension buffer (25 mM Tris-HCl, pH 7.2, and 100 mM mannitol) to a final protein concentration of 2-4 mg/ml. A 4-5% aliphatic lipid vesicle suspension was purified using a discontinuous sucrose gradient, which consisted of 5 ml of 45% (w/w) sucrose, 10 ml of 893 mM sucrose, and 10 ml of 250 mM sucrose. All the sucrose solutions were prepared in 20 mM Tris-HCl, pH 7.2, and the gradient was centrifuged at 48,000 x g for 10 min. The vesicle layer at the 893 mM, 45% sucrose interface was collected and diluted 1:3 with medium 3 (20 mM Tris-HCl, pH 7.2, and 250 mM sucrose), and the vesicles were pelleted by centrifugation at 48,000 x g for 25 min. The pelleted vesicles were diluted in medium 3 to a final protein concentration of 5-7 mg/ml and put into dialysis tubing with a molecular mass cutoff of 12-14 kDa. The vesicles were dialyzed against 100 volumes of medium 3 for 1 h and again with fresh medium 3 overnight. The vesicles were collected using a 1400 x g spin for 12 min and stored on ice until transport experiments and marker enzyme assays could be performed. Approximately 1 mg of a preparation of inside-out plasma membrane vesicles was derived from 2.2 x 10\(^6\) cells. Intravesicular volume of the inside-out vesicle compartment alone, as determined by standard procedures (18), was 1.5 \mu l/mg protein (n = 3). Right-side-out plasma membrane vesicles were prepared by procedures already described in detail (19, 20) in our earlier publications.

**Transport Experiments**—This procedure is similar to that employed by Horio et al. (21) and uses the same concentration ratios for the ATP-regenerating system as Kamoto and Toyoshima (22). Millipore HAWP 0.45 mm filters were rinsed in double-distilled H\(_2\)O three times and stored in medium 3, pH 7.4, for at least 1 h. A 20-\mu l aliquot of vesicles (50-70 \mu g of membrane protein) was preincubated with 30 \mu l of non-ionic transport buffer with an ATP-regenerating system (25 mM MES, pH 6.1, with Mg\(_2\), 225 mM sucrose, 45 mM Tris-phosphocreatine, and 90 units of creatine phosphokinase) in a siliconized glass test tube for 30 s at 37 °C. The reaction was started by adding 50 \mu l of MMS (25 mM MES, pH 6.1 with Mg\(_2\), and 250 mM sucrose) containing 10 mM Tris-ATP and two desired final concentration of [\(^{3}H\)]MX. After an appropriate amount of time at 37 °C, the reaction was stopped by dilution with 9 ml of ice-cold medium 3, pH 7.4. The vesicles were collected by rapid filtration using a Millipore filter apparatus with 11 mm Hg of suction applied. The filters were rinsed three times with 9-m1 volumes of buffer, allowed to dry, and dissolved in scintillation mixture. [\(^{3}H\)]MX accumulation into the vesicles was determined by liquid scintillation counting, and the binding to the surface after a 5-s exposure to [\(^{3}H\)]MX at 37 °C was subtracted from the values obtained for each time course. Alternatively, surface binding was determined by back extrapolating the 37 °C time course to the origin. Each time point was performed in duplicate for each vesicle preparation, and graphed points represent the average of at least three experiments done on three different days. All inhibitors used were present at the specified concentrations in the non-ionic transport buffer with and without [\(^{3}H\)]MX.

**Vesicle Sidedness and Contamination Marker Assays**—The orientation of the vesicles was determined by using an alkaline phosphatase (EC 3.1.3.1) assay (23) for the ecto- or outside plasma membrane marker, and a glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) assay (24) for the endo or inside plasma membrane marker. Both the enzyme activities in the reaction mixtures contain 100 \mu g of membrane protein, and 250 mM sucrose was added to the reaction buffers to prevent vesicle lysis during the enzyme activity measurements. The sidedness orientation was determined by performing duplicate assays of each enzyme with and without 0.2% (v/v) Triton X-100. The detergent was added to the vesicles before the assays were done. The percent of vesicle sidedness was calculated by dividing the enzymatic activity without detergent by the activity in the presence of detergent for each marker enzyme. In control experiments, we first showed that 0.2% Triton X-100 has no direct effect on the activity of the marker enzymes employed. Membrane experiments utilized invasculated membrane fragments or inside-out vesicle preparations that were sonicated to disrupt the intact vesicles. The alkaline phosphatase activity was used to determine the population of vesicles that was right-side-out, and the inside-out population was determined by subtraction. The same was done to determine the inside-out vesicle population using the glyceraldehyde-3-phosphate dehydrogenase assay, and the right-side-out population was determined by subtraction. The overall sidedness was the value obtained by averaging the results. In order to determine the purity of the vesicles as compared to other organelles, assays for succinic dehydrogenase (EC 1.3.9.1), NADPH-cytochrome c reductase (EC 1.6.2.3), and acid phosphatase (EC 3.1.3.2) were done (25-27). Using the method of Newkirk and Waite (28), the extent of contamination by mitochondrial membranes, endoplasmic reticulum, and lysosomes in the vesicle preparations was determined. All of these assays were based on the protein content of the vesicle preparations, determined by the method of Lowry (29) using a bovine serum albumin standard curve, and showed that the content of the preparation was 94 ± 6% (n = 12) plasma membrane.

Chemicals—[\(^{3}H\)]MX was obtained from Moravek Biochemicals, City of Industry, CA. This was purified to >98% by high performance liquid chromatography (30) prior to use for transport experiments. Tri-ATP, phosphocreatine, and creatine phosphokinase were obtained from Sigma. All other chemicals were analytical reagent. The results are presented below.
Evidence that protons present in the nonanionic buffers employed had no involvement in [3H]MTX accumulation in inside-out vesicles was verified by showing (data not given) that the addition of protonophores (2,4-dinitrophenol, carbonyl cyanide p-(trifluoromethoxy) phenyldrazone, and oligomycin) had no effect on the accumulation observed.

ATP Dependence for Intravesicular Accumulation of [3H]MTX—The results in Fig. 1 show a typical time course experiment measuring intravesicular [3H]MTX accumulation at 37 °C in the presence and absence of 5 mM ATP. A concentration of 20 μM [3H]MTX was employed in these experiments. These data show a very rapid initial uptake of [3H]MTX in the presence of ATP with a deviation from a constant time course by 30 s of incubation and an approach to apparent steady-state by 160 s of incubation. The intravesicular concentration of [3H]MTX at this time was 2–2.5-fold greater than the extravesicular concentration. This was confirmed by high performance liquid chromatography (30) analysis of intravesicular [3H]MTX, which also showed that the accumulation of radioactivity was only in the form of authentic MTX. In contrast, minimal uptake was observed in the absence of ATP or at 0 °C in the presence of 5 mM ATP. This latter component of uptake most likely represents passive diffusion of this folate analogue since it was insensitive (data not shown) to all of the agents shown to be inhibitors of ATP-dependent accumulation (see a later section of the text and Table II). The ATP-independent uptake was consistent with our prior studies (19, 20), which showed that a much larger fraction of total uptake of [3H]MTX was attributable to passive diffusion in membrane vesicle preparations in contrast to intact cells. This can be explained by the far greater surface to volume ratio that is characteristic of vesicles, which are only 1/10,000 the size of intact cells.

Other data given in Fig. 2 demonstrate that [3H]MTX associating with inside-out plasma membrane vesicles in the presence of ATP was osmotically sensitive. These data show that the association of [3H]MTX with the vesicles is proportional to intravesicular volume and, thus, the time course obtained in each case is actually a measure of transport into the vesicles, rather than binding to the vesicle surface. A similar result was obtained (data not shown) for uptake of [3H]MTX in the absence of ATP.

In experiments, like those shown in Figs. 1 and 2, measurements of ATP-dependent [3H]MTX uptake in inside-out plasma membrane vesicles were made in the presence of Na+, K+, Cl−, and HCO3− at concentrations found in the internal compartment of intact cells. In these experiments, the trans-

### TABLE 1

Summary of data pertaining to the determination of membrane vesicle sidedness

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Marker enzyme</th>
<th>Enzyme activity</th>
<th>Sidedness</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No detergent</td>
<td>Plus detergent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>× 10−4 (units/mg protein)</td>
<td>%</td>
</tr>
<tr>
<td>Inside-out</td>
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<td></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (endo)</td>
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<td>96.0</td>
</tr>
<tr>
<td>Right-side-out</td>
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<td>32.0</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (endo)</td>
<td>6.9</td>
<td>98.7</td>
</tr>
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* Average.
by ATP was appreciably ablated when either inhibitor was

stimulators of ATP-dependent uptake. Stimulation of initial uptake

the ATPase inhibitor, orthovanadate, were both potent inhib-

than that observed in the absence of ATP. In the second

ment (Fig. 3A) ATP, but not the nonhydrolyzable analogue

hydrolysis of this triphosphonucleotide. In the first experi-

of [3H]MTX was obtained under the nonionic buffer conditions

port buffer was supplemented with 100 mM KCl, 10 mM NaCl,

and 4 mM NaHCO₃ and osmolality adjusted with sucrose. In

a comparison between unsupplemented and supplemented buffer done at both pH 6.1 and 6.8, we found (data not shown) no effect of these ions, together or alone, on the rate of ATP-dependent accumulation of [3H]MTX.

Results presented in Fig. 3 suggest that stimulation by ATP of [3H]MTX uptake in inside-out membrane vesicles is not the result of an allosteric effect but occurs as a result of hydrolysis of this triphosphonucleotide. In the first experiment (Fig. 3A) ATP, but not the nonhydrolyzable analogue ATP-S, stimulated initial uptake of [3H]MTX. Uptake of [3H]MTX in the presence of this analogue was no different than that observed in the absence of ATP. In the second experiment (Fig. 3B), these data show that ATP-S as well as the ATPase inhibitor, orthovanadate, were both potent inhibitors of ATP-dependent uptake. Stimulation of initial uptake by ATP was appreciably ablated when either inhibitor was added to the vesicle suspension. This latter result further suggests that ATP-S not only will not replace ATP as a stimulator of [3H]MTX uptake, but will also effectively pre-
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Fig. 3. Requirement for ATP hydrolysis of [3H]MTX accumulation into inside-out plasma membrane vesicles. A, vesicles were prewarmed to 37°C in MMS buffer with regenerating system for 30 s with either no addition, or 5 mM ATP, or 5 mM ATPγS in the same buffer added along with 20 μM [3H]MTX to start intravesicular accumulation. B, the reaction conditions were the same as in A. No ATP or 5 mM ATP with or without 1 mM ATPγS or 50 μM orthovanadate were added together with 20 μM [3H]MTX. Average of three experiments ± standard error of the mean of <25%.

Fig. 4. pH dependence for [3H]MTX accumulation into inside-out membrane vesicles. See legends for Fig. 1 and Table II for methodologic details. [3H]MTX efflux was measured over a 12-s interval at 37°C and corrected for non-ATP-dependent accumulation. To avoid altering the Mg concentration during these experiments, either Trizma base or acetic acid was used to adjust the pH of HEPES-Mg-sucrose and MES-Mg-sucrose buffers. Average of three experiments ± standard error of the mean < 20%.

Inhibition of [3H]MTX efflux by bromosulfophthalein is shown in Fig. 5. The importance of these data relates to the fact that they document complete inhibition of ATP-dependent efflux in the range of 50 to 100 μM of this organic anion. Interestingly, another inhibitor of [3H]MTX efflux in intact L1210 cells, the organic anion probenecid (3, 13, 14, 33), did not inhibit [3H]MTX accumulation (data not shown) in these membrane vesicles. This result is consistent with our earlier observations (3) on the interaction of this organic anion with the efflux process for [3H]MTX in intact L1210 cells and will be discussed in a later section.

Concentration Response for ATP and the Effect of Other Triphosphonucleotides on Intravesicular Accumulation of [3H]MTX—The effect of different ATP concentrations on initial accumulation of [3H]MTX (12-s interval) in inside-out membrane vesicles is given in Fig. 6. The response curve shows saturation kinetics in the concentration range of 0–5 mM ATP and was corrected for the amount of accumulation of [3H]MTX occurring in the absence of ATP. From the double-reciprocal plot of these data given in Fig. 5, a single saturable component was derived with an apparent Kₗ of 0.40 ± 0.06 mM and a Vₗ of 34.4 ± 5 pmol/min/mg protein at 20 μM [3H]MTX. Other triphosphonucleotides are able to replace ATP as mediators of intravesicular accumulation of [3H]MTX but are less effective than ATP. Two of these, GTP and CTP, are approximately equal (data not shown) but were only 50% as effective as ATP when initial influx velocity was compared at concentrations of 5 mM. As a point of reference, we note that energetically replete L1210 cells obtained under optimum growth conditions contain 4.8 ± 0.5 mM ATP (see Ref. 2).
ular Accumulation in the Presence of ATP—The concentration response for the initial accumulation of [3H]MTX in inside-out membrane vesicles is given in Fig. 7. In these experiments, initial accumulation at each concentration of [3H]MTX was linear with time (data not shown) within the 12 s interval employed for the measurements. The response curve obtained shows saturation kinetics only in a relatively high concentration range of 20–60 μM [3H]MTX. From the double-reciprocal plot shown (Fig. 7), a single, poorly saturable component is delineated (apparent $K_m = 46 \pm 7 \mu M$), but with a very high capacity ($V_{max} = 104 \pm 15 \text{ pmol/min/mg protein}$). The lower value for $V_{max}$ (initial accumulation) obtained from the data on ATP concentration response shown in Fig. 6 reflects less than saturating [3H]MTX concentrations employed in those experiments.

**DISCUSSION**

These studies sought evidence as to the nature of an ATP-dependent mechanism mediating MTX efflux in L1210 cells. The results appear to document the direct involvement of ATP in efflux of this folate analogue with inside-out plasma membrane vesicles. A role for electrogenic ions and electro-chemical potential differences mediated by ion motive ATPases appears highly unlikely. The stimulation of [3H]MTX efflux by ATP, but not by ATPγS, which was observed in some experiments suggests that the ATP dependence reflects a requirement for hydrolysis of this triphosphonucleotide. Thus, a role for ATP as an allosteric effector, analogous to ATP gating of ion channels, seems to be ruled out. These results and the findings that orthovanadate, a transition state analogue for phosphate release from ATPases (34), and NEM, an inhibitor of membrane ATPases (35), are potent inhibitors of [3H]MTX efflux lead us to conclude that the mechanism involved is an outwardly directed membrane ATPase. The observation that the requirement for ATP could be fulfilled, albeit to a lesser extent, by other triphosphonucleotides like GTP and CTP (36), further supports the role of an ATPase in efflux of [3H]MTX.

The different effects of vanadate and NEM have been utilized (35) to distinguish between various membrane ATPases. Therefore, it was of interest to find that [3H]MTX efflux in inside-out membrane vesicles was sensitive to the presence of both agents. On the other hand, the low pH optimum demonstrated for ATP-dependent efflux of [3H]MTX is characteristic (37) of some, but not all, ATPases that have been examined in this manner. The significance of a relatively low pH optimum observed for ATP-dependent efflux of [3H]MTX is unclear since it is widely known that intracellular pH in tumor cells during growth is in the range of 6.7–6.9. However, there are many examples in the literature for cytosolic enzymes that exhibit pH optima outside of the...
prevailing intracellular pH range for mammalian cells. As in those cases, efflux of [3H]MTX in intact L1210 cells by this high capacity process would operate under physiological conditions at a significant, but lower, level than that level sustained at the pH optimum for this process. Under such conditions, this efflux process would be expected to modulate (1–3, 13, 14, 30) the extent of accumulation of intracellular exchangeable [3H]MTX. Moreover, these observations are consistent with earlier findings by Henderson et al. (38), who showed that the predominate (bromosulfophthalein sensitive) efflux route for [3H]MTX in intact L1210 cells was markedly stimulated by lowering internal pH below neutrality. Additional work will be required to address this issue and to establish the identity and physiological permeant for the system under study here.

Other evidence from these studies bears upon the question of the relationship between the ATPase, seemingly elucidated in inside-out plasma membrane vesicles, and the predominant route for [3H]MTX efflux (29) in intact L1210 cells. In addition to the similar ATP and pH dependencies in each case, inhibition of [3H]MTX efflux by a highly diverse group of agents occurs at similar concentration ranges in either system. However, the inhibitory potency of quinidine on [3H]MTX efflux was 10-fold greater in thevesicle system than with intact L1210 cells. This may be accounted for by the fact that accessibility of this agent to the permeant binding site in these studies supports the notion expressed earlier that the process under study here is the bromosulfophthalein-transport system.

L1210 cells where there is a limitation (1, 2, 5–7, 13, 15, 16, 32), who showed that the predominate (bromosulfophthalein sensitive) efflux route for [3H]MTX in intact L1210 cells was markedly stimulated by lowering internal pH below neutrality. Additional work will be required to address this issue and to establish the identity and physiological permeant for the system under study here.

In both cases, evidence was obtained for their function in outwardly directed flux of cytotoxic agents and their identity as ATPases. Also, both systems were inhibited by orthovanadate and modulators of multidrug resistance, including, verapamil, quinidine, and reserpine. However, despite these similarities, it is clear that both systems under discussion are not one and the same. Tumor cells that express the multidrug-resistant phenotype and elaborate P-glycoprotein do not exhibit (40) cross-resistance to methotrexate. Also, we have recently shown that MTX efflux is unaltered in multidrug-resistant cells. It now seems likely that the ATPase we have apparently elucidated as a mediator of MTX efflux in L1210 cells may be part of the large ATP-binding cassette of membrane proteins (41, 42) that serve many functions in tumor cells.

REFERENCES


* F. M. Sirotnak, unpublished results.