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Na^+-dependent, active nucleoside transport in mouse spleen lymphocytes, leukemia cells, fibroblasts and macrophages, but not in equivalent human or pig cells; dipyridamole enhances nucleoside salvage by cells with both active and facilitated transport

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Formycin B influx studies have shown that P388 and L1210 mouse leukemia cells, mouse L929 cells, mouse RAW 309 Cr.1 cells, LK35.2 mouse B-cell hybridoma cells and cultured mouse peritoneal macrophages express both Na^+-dependent, active and nonconcentrative, facilitated nucleoside transport systems. In the mouse cell lines, active transport represented only a minor nucleoside transport component and was detected only by measuring formycin B uptake in the presence of dipyridamole or nitrobenzylthioinosine, strong inhibitors of facilitated, but not of active, nucleoside transport. Inhibition of facilitated transport resulted in the concentrative accumulation of formycin B in cells expressing active nucleoside transport. Concentrative formycin B accumulation was abolished by treatment of the cells with gramicidin or absence of Na^+ in the extracellular medium and strongly inhibited by ATP depletion or ouabain treatment. Mouse macrophages accumulated formycin B to 70-times the extracellular concentration in the absence of dipyridamole during 90 min of incubation at 37°C. Thus active transport represents a major nucleoside transport system of these cells, similarly as previously reported for mouse spleen lymphocytes. In contrast to the various types of mouse cells, active formycin B transport was not detected in human HeLa cells, human H9, Jurkat and CEM T lymphoidal cells and pig spleen lymphocytes. These cells expressed only facilitated nucleoside transport with kinetic properties similar to those of the facilitated transporters of other mammalian cells.

Introduction

Two principle types of nucleoside transport have been recognized in mammalian cells [1–3]. (1) Facilitated, non-concentrative transport with broad substrate specificity, which has been found, with a few exceptions, in all types of cells investigated. It exists in two forms; one form is strongly inhibited by nanamolar concentrations of nitrobenzylthioinosine (NBTI; designated NBTI-sensitive), resulting from the binding of NBTI to a high affinity, nucleoside transporter-associated binding site on the plasma membrane ($K_d \leq 1$ nM). The other form is not associated with this binding site and inhibited only by micromolar concentrations of NBTI (designated NBTI-resistant). Both forms are about equally sensitive to certain other nucleoside transport inhibitors, such as dipyridamole, but the sensitivity of facilitated transporters of different species to the latter inhibitors may differ up to 1000-fold [4].

(2) Na^+-dependent, active nucleoside transport, which has been detected in epithelial cells or brush border membranes of rat, mouse and rabbit kidney and intestine [5–10], as well as in rabbit choroid plexus [11,12] and mouse spleen cells [13–15]. At least two types of active nucleoside transporters have been distinguishes on the basis of substrate specificity [8,9], but both systems are resistant to inhibition by NBTI and dipyridamole [5–15]. The presence of active nucleoside transporters in epithelial cells of the kidney and intestine probably serves an important function in nutrient adsorption as suggested by their localization in brush border membranes. These cells also uniquely possess a Na^+-dependent, active sugar transporter [16]. In contrast, the potential physiological function of active nucleoside transport in mouse lymphocytes is unclear.

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Recently, it has been found that L1210 mouse leukemia cells also exhibit some Na^+-dependent, active nucleoside transport in addition to normal facilitated nucleoside transport (Belt and Paterson, personal communication). L1210 leukemia cells lack T- and B-cell surface antigens and have been used as a laboratory model for acute lymphocytic leukemia of childhood. We have confirmed the presence of active nucleoside transport in L1210 cells and have extended this finding to a variety of other types of mouse cells. In contrast, we found that various types of human and pig cells express only facilitated nucleoside transport. The operation of active nucleoside transport seems to explain the increased salvage of 2′-deoxyadenosine (dAdo) and other nucleosides in presence of dipyridamole by L1210 cells and perhaps other cells [17].

We have used formycin B as substrate in the present studies since it has been shown to be an efficient substrate for both the Na^+-dependent, active [7,8,15] and facilitated [18] nucleoside transporters of mammalian cells, but to be only very poorly phosphorylated by these cells and resistant to enzymatic phosphorolysis [7,18]. Thus it is an ideal substrate for measuring active and facilitated nucleoside transport unimpeded by metabolic reactions.

Experimental procedures

Cells. Single cell suspensions of mouse spleen cells free of erythrocytes were prepared in phosphate-buffered saline (pH 7.4), containing 5 mM glucose (PBS-glucose) as described previously [14]. Single cell suspensions of pig spleen cells were prepared in the same manner with the following modification. A fresh pig spleen (provided by the Department of Surgery, University of Minnesota Medical School) was cut into about 3-cm^2 pieces, which were briefly washed in distilled water and subsequently in PBS-glucose. Spleen cells were then dissociated by squeezing the tissue pieces in a garlic press.

Mouse peritoneal macrophages were isolated from about six-week old Swiss mice (Biolabs Inc., St. Paul, MN) and cultured in 24-well tissue culture plates (2 cm^2/well) with RPMI plus 10% (v/v) fetal bovine serum and 10% (v/v) L-cell-conditioned medium as described elsewhere [19]. The cultures were used for transport assays after 2 to 3 days of incubation with one medium change. Mouse L929 and RAW 309 Cr.1 cells (an Abelson leukemia virus-transformed mouse macrophage line obtained from the American Type Culture Collection) were routinely propagated in tissue culture T-flasks with RPMI plus 5% (v/v) fetal bovine serum and 5% (v/v) newborn calf serum [20]. For transport studies the cells were seeded into 24-well culture plates and the cultures used when confluent.

L1210 and P388 mouse leukemia cells and human HeLa cells were propagated in suspension culture on a gyrotory shaker as described previously [21]. Human T lymphocyte lines H9, Jurkat (kindly provided by M. Smith, University of North Carolina), CCRF-CEM (kindly supplied by B. Chesebro, Rocky Mountain Laboratories) and LK35.2 mouse B-cell hybridoma cells (obtained from the American Type Culture Collection) were maintained in culture in T-flasks in the same medium used for L929 and RAW 309 Cr.1 cells. For the propagation of LK35.2 cells the medium was supplemented with non-essential amino acids and 50 μM 2-mercaptoethanol. For transport assays the cells were propagated in 2-liter roller bottles in the appropriate medium. All cell lines were routinely examined for mycoplasma contamination by assaying cells and culture fluid for adenosine phosphorylase activity [22]. All cells used in the present study were free of mycoplasma.

Formycin B transport measurements. As indicated in appropriate experiments, cells were suspended in basal medium 42B (BM42B; Ref. 23) or PBS-glucose or overlayed with RPMI when assayed in 24-well plates. Or the cells were first washed with isotonic choline chloride containing 5 mM Tris-HCl, pH 7.4 (Tris-choline chloride) and then suspended in or overlayed with different media. Where indicated, cells were pretreated with various inhibitors or depleted of ATP by incubation in glucose-free medium containing 5 mM KCN and 5 mM iodoacetate at 37 °C for 10–20 min [24]. Then time courses of uptake of [^{3}H]formycin B were measured under zero-*trans* conditions at 25 °C or 37 °C. Formycin B uptake by suspensions of cells was measured using a dual syringe apparatus (12 time points/time course) or by manual sampling for longer time points as described previously [1,2,17,25,26]. The concentration of [^{3}H]formycin B was kept constant in all samples of an experiment, while the specific radioactivity was altered by addition of unlabeled formycin B. The procedures involve separating the cells from the medium by rapid centrifugation through an oil layer and analyzing the cell pellet for radioactivity. Radioactivity/cell pellet was corrected for that attributable to extracellular space as estimated with [^{14}C]inulin [24]. Intracellular H_2O space was measured with ^3H_2O [25]. For estimating initial facilitated transport velocities (v_{12}^{zt}) an appropriate integrated rate equation based on a simple carrier model was fitted to short-term time courses of equilibration of formycin B across the membrane assuming directional symmetry and equal mobility of substrate-loaded and empty carrier [1,17,25]. For estimation of the kinetic parameters of facilitated transport, six or seven formycin B concentrations were employed and the Michaelis-Menten parameters extracted by least-squares regression [1].

Formycin B uptake by adhering cells propagated in 24-well tissue culture plates was measured by adding sequentially, in timed intervals, [^{3}H]formycin to the desired concentration to 11 wells in a plate. At the

completion of the incubation, the medium was dumped out and the wells were rinsed rapidly three times (within 15 s) with ice-cold balanced salt solution (BSS) containing 10 μM dipyridamole and 1 mM phloridzin to inhibit both facilitated and active nucleoside transport (see later). Then the cells in each well were lyzed in 1 ml water with the help of scraping with a rubber policeman and the lysates analyzed for radioactivity. To estimate the efficiency of the rinsing in removing unincorporated substrate, the cells in one well of a plate were overlayed with 0.5 ml of distilled water, rather than isotonic medium, plus [^{3}H]formycin B. The radioactivity retained in these wells was insignificant in relation to that taken up by the cells ($\leq$ 100 cpm/well). The cells from another well/plate (except in the case of macrophages) were removed by trypsinization and enumerated in a Coulter counter. Macrophages were enumerated by counting the cells in five fields in an inverted microscope and converting this number to cells/well on the basis of the areas of the microscopic field and the well [20].

Analysis of metabolism of formycin B. Suspended cells, after incubation with [^{3}H]formycin B for varying lengths of time, were collected by centrifugation through an oil layer into a solution composed of sucrose and 0.5 M trichloroacetic acid [25]. Cells propagated in 24-well plates were washed with BSS after incubation with [^{3}H]formycin B as described already and then extracted with 0.5 M trichloroacetic acid. The acid extracts were further processed and analyzed by ascending paper chromatography as described previously [23] using two solvents: 28 (composed of 30 ml 1 M ammonium acetate (pH 5) and 70 ml 95% ethanol) and 40 (composed of 39 ml butanol, 22 ml ethylacetate, 22 ml ammonium hydroxide and 17 methanol) [18].

Determination of Na^+ content of cells. Samples of $4 \cdot 10^7$ untreated or gramicidin-treated L1210 cells were collected by centrifugation, washed once in Tris-choline chloride and then analyzed for Na^+ concentration by flame photometry (conducted by R.H. Ophaug, School of Dentistry, University of Minnesota).

Materials. [^{3}H]Formycin B was purchased from Moravek Biochemicals (Brea, CA) and diluted to the desired specific radioactivity with unlabeled formycin B. Unlabeled formycin B, ouabain, and gramicidin (from *Bacillus brevis*), were obtained from Sigma (St. Louis, MO) and unlabeled NBTI from Calbiochem (San Diego, CA). Dipyridamole (Persantin) was a gift from Geigy Pharmaceuticals (Yonkers, NY).

Results

Active formycin B transport in L1210 and P388 mouse leukema cells, and LK35.2 mouse B-cell hybridoma cells

Rapid kinetic studies initially failed to detect Na^+-dependent active transport of formycin B in L1210 and

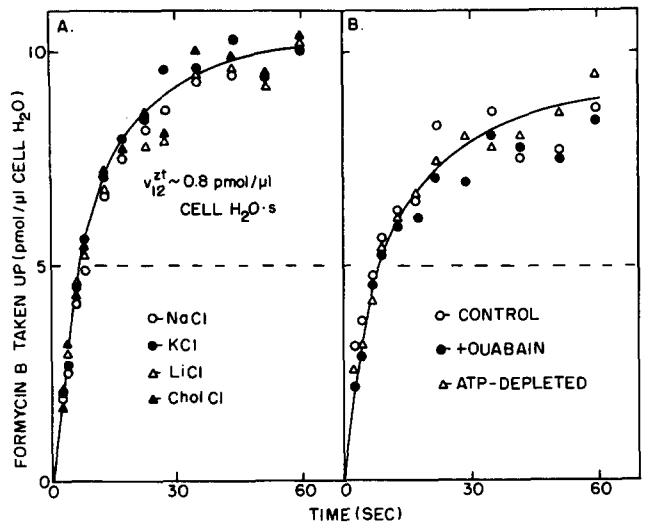

Fig. 1. Initial zero-*trans* uptake of formycin B by L1210 cells suspended in different media (A) and effects of ouabain and ATP depletion (B). (A) L1210 cells were washed once in Tris-choline chloride and suspended to $3.8 \cdot 10^7$ cells/ml as indicated in isotonic, Tris-buffered NaCl, KCl, LiCl or choline chloride. Then the uptake of 5 μM [^{3}H]formycin B (53 cpm/pmol) was measured at 25°C by rapid kinetic techniques. The initial velocity of entry (v_{12}^{z1}; in pmol/μl cell per s) was estimated graphically from the initial linear portion of the uptake curve. (B) Samples of a suspension of $3.1 \cdot 10^7$ cells/ml of Tris-saline were supplemented where indicated with 3 mM ouabain or 5 mM KCN plus 5 mM iodoacetate. After 20 min of incubation at 37°C, the suspensions were equilibrated at 25°C and the uptake of 5 μM [^{3}H]formycin B was measured as in (A). The broken lines indicate the intracellular concentration of formycin B equal to that in the extracellular fluid.

P388 cells. As shown in Fig. 1, the initial equilibration of 5 μM formycin B across the plasma membrane in L1210 cells was the same whether the cells were suspended in isotonic, Tris-buffered NaCl, KCl, LiCl or choline chloride and was not affected by pretreatment of the cells with ouabain or ATP-depletion of the cells. Pretreatment of the cells with gramicidin (200 μg/ml) also had no effect, but caused an about doubling in cell volume upon longer time periods of incubation (> 30 min; data not shown). Formycin B accumulated intracellularly to a higher concentration than present extracellularly (Fig. 1) but this was not unexpected, since this low level of concentrative accumulation of formycin B and other purine nucleosides has been observed in all mammalian cells investigated [17,27], and probably reflects their binding to some intracellular component(s). Thus the data in Fig. 1 indicated that facilitated transport is the primary route of entry of formycin B into L1210 cells. Results essentially identical to those shown in Fig. 1 were obtained for P388 cells (data not shown).

A Na^+-dependent, active formycin B transport component, however, became apparent upon longer incubation of the cells in the presence of dipyridamole (Fig. 2A). It has been observed in rat epithelia cells [7] and mouse spleen cells [14,15], that dipyridamole, by inhibit-

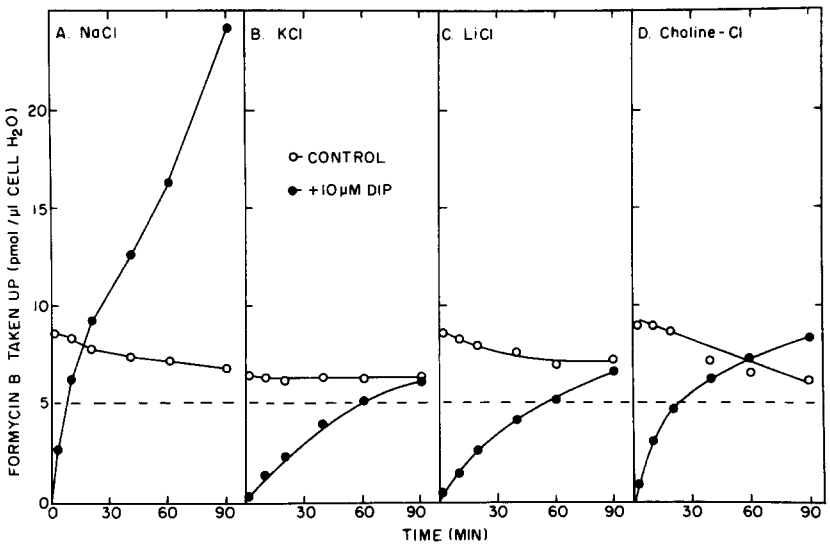

Fig. 2. Long-term uptake of formycin B by L1210 cells suspended in different media in the presence and absence of dipyridamole. L1210 cells were washed in Tris-choline chloride and suspended to $3 \cdot 10^7$ cells/ml in isotonic. Tris-buffered NaCl (A), KCl (B), LiCl (C), or choline chloride (D). One half of each suspension was supplemented with 10 μM dipyridamole (DIP; ●) and then all suspensions with 5 μM [^{3}H]formycin B (37 cpm/pmol). At various times of incubation at 25 °C the cells from 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity Radioactivity per cell pellet was corrected for that trapped in extrcellular space and converted to pmol/μl cell water on the basis of an experimentally determined cell water space. All values are averages of duplicate samples. The broken lines indicate the intracellular concentration of formycin B equal to that in the extracellular fluid.

ing backflow of formycin B via facilitated transport [18], enhances the concentrative accumulation of formycin B via the Na^+-dependent active transporter. Similarly, as shown in Fig. 2A, dipyridamole strongly inhibited the initial influx of formycin B via facilitated transport in L1210 cells [25], but then caused a slow

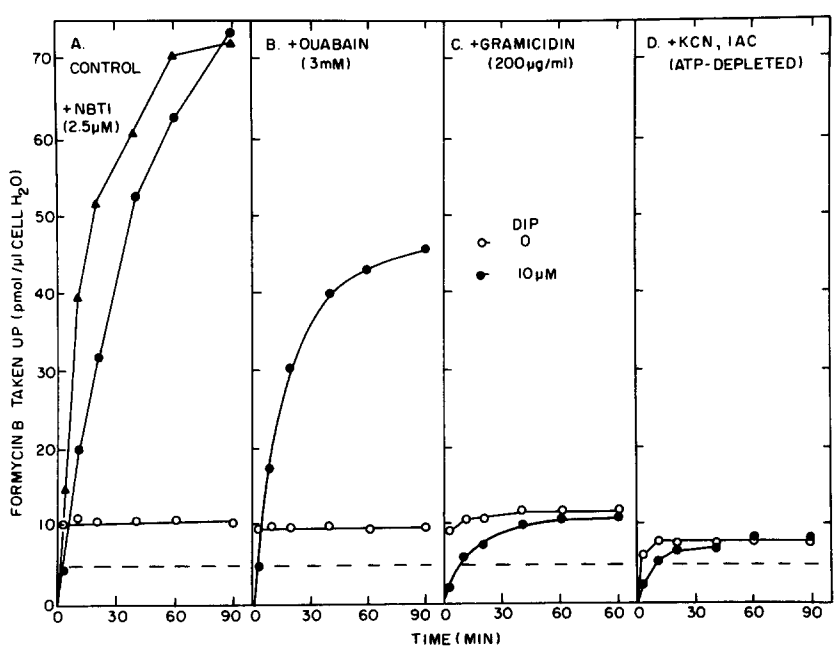

Fig. 3. Effects of ouabain, gramicidin and ATP-depletion on the dipyridamole-mediated concentrative accumulation of formycin B by L1210 cells. Samples of a suspension of $2 \cdot 10^7$ L1210 cells/ml of isotonic Tris-NaCl were supplemented as indicated with 3 mM ouabain (B), 200 μg gramicidin/ml (C) or 5 mM KCN plus 5 mM iodoacetate (D). After 20 min of incubation at 37 °C the suspensions were equilibrated at 25 °C and a portion of each suspension was supplemented with 10 μM dipyridamole (DIP; ●) and another portion in A with 2.5 μM NBTI (▲). Then the uptake of 5 μM [^{3}H]formycin B was measured as described in the legend to Fig. 2. The broken lines indicate the intracellular concentration of formycin B equal to that in the extracellular fluid.

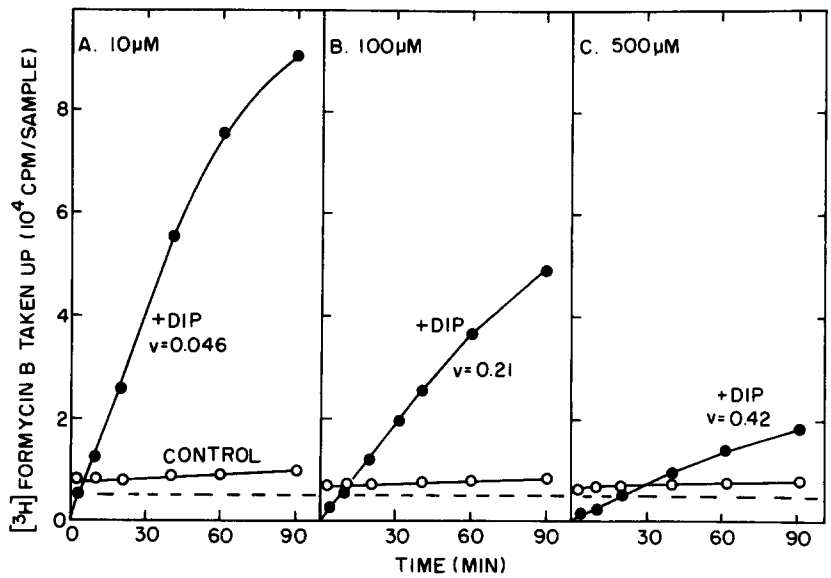

Fig. 4. Dipyridamole-mediated concentrative accumulation of formycin B by L1210 cells as a function of the formycin B concentration. Samples of a suspension of $2.5 \cdot 10^7$ cells/ml of BM42B were supplemented, where indicated, with 20 μM dipyridamole (DIP). Then the uptake of 10, 100 and 500 μM [^{3}H]formycin B (433 cpm/μl, irrespective of concentration) was measured as described in the legend to Fig. 2. The broken lines indicate the intracellular concentration of ^{3}H equal to that in the extracellular fluid. Velocities of concentrative accumulation of formycin B (v; in pmol/μl cell water per s) were estimated from the initial linear portions of the uptake curves.

accumulation of formycin B to 4-times the extracellular concentration over the 90-min incubation period at 25° C. NBTI was at least as effective as dipyridamole in stimulating the long-term concentrative accumulative of formycin B in L1210 cells (Fig. 3A). This concentrative accumulation of formycin B in the presence of dipyridamole was not observed when the cells were suspended in isotonic media containing KCl, LiCl, or choline chloride (Figs. 2B–D). It was also completely abolished by preincubation of the cells with 200 μg gramicidin/ml or depleting the cells of ATP (Figs. 3C and D). Preincubation of the cells with 3 mM ouabain

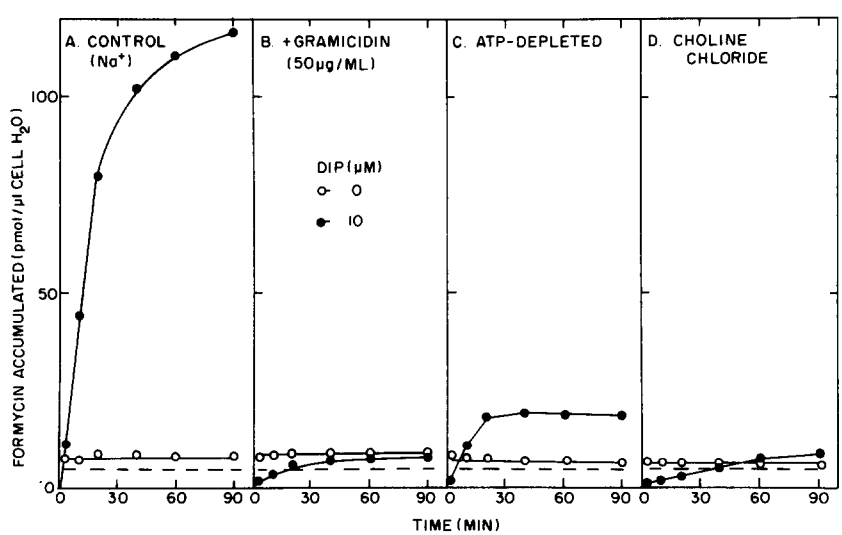

Fig. 5. Na^+-dependent concentrative uptake of formycin B by P388 mouse leukemia cells. One sample of a suspension of $1.4 \cdot 10^7$ P388 cells/ml of BM42B was supplemented with 50 μg gramicidin/ml (B) and one sample remained untreated (A). The cells from another sample of the suspension were collected by centrifugation and suspended to the same density in glucose-free BM42B containing 5 mM KCN and 5 mM iodoacetate (C). These suspensions were incubated at 37° C for 20 min. The cells from a fourth sample of the original suspension were washed once in Tris-choline chloride and suspended to the original density in the same (D). One half of each suspension (A–D) was supplemented with 10 μM dipyridamole (DIP). Then all suspensions were equilibrated at 25° C and the uptake of 5 μM [^{3}H]formycin B (67 cpm/pmol) was measured as described in the legend to Fig. 2. All points represent averages of duplicate samples. The broken lines indicate the intracellular concentration of formycin B equal to that in the extracellular fluid.

reduced the dipyridamole effect but did not abolish it (Fig. 3B). Preincubation of the cells with 1.5 or 6 mM ouabain had about the same effect as preincubation with 3 mM ouabain, while preincubation with at least 30 μg gramicidin/ml was required to completely abolish the concentrative accumulation of formycin B in the presence of dipyridamole (data not shown). The gramicidin treatment increased the Na^+ content of the cells about 7-fold (from about 8 to 55 $\mu g/10^7$ cells) indicating the effectiveness of the treatment in abolishing the transmembrane Na^+-gradient.

The concentrative accumulation of formycin B in L1210 cells in the presence of dipyridamole was concentration dependent and saturable (Fig. 4). The Michaelis-Menten constant for the active transporter seems to be lower than that for the facilitated transporter (see later), but cannot be accurately assessed in L1210 cells because it represents only a minor transport component. Chromatographic analysis of acid extracts of the cells indicated that after 90 min of incubation with [^{3}H]formycin B at 25 °C either in the presence or absence of dipyridamole < 10% of the intracellular radioactivity was associated with phosphorylated products of formycin B (data not shown).

Like L1210 cells, P388 cells concentratively accumulated formycin B in the presence of dipyridamole (Fig. 5A) and the concentrative accumulation was abolished by treatment of the cells with gramicidin (Fig. 5B) or the absence of Na^+ (Fig. 5D), and was greatly reduced by ATP depletion of the cells (Fig. 5C) or ouabain treatment (data not shown). Similar results were obtained for suspensions of LK35.2 mouse B-cell hybridoma cells (data not shown).

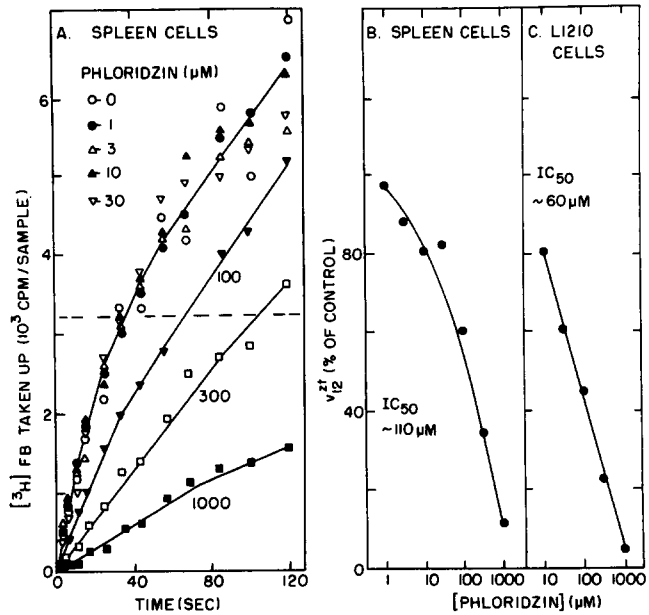

Fig. 6. Inhibition of formycin B transport in mouse spleen lymphocytes (A,B) and L1210 cells (C) by phloridzin. (A) Samples of a suspension of $1 \cdot 10^8$ spleen cells/ml of PBS-glucose were supplemented with the indicated concentrations of phloridzin and then the uptake of 5 μM [^{3}H]formycin B (54 cpm/pmol) was measured by rapid kinetic techniques. A first-order rate equation [15] was fitted to the uptake time courses to estimate initial entry velocities (v_{12}^{zt}) which are plotted as a function of the phloridzin concentration in frame (B). The inhibition of formycin B transport by L1210 cells was determined in the same manner in samples of a suspension of $3.4 \cdot 10^7$ cells/ml, except that the formycin B concentration was 500 μM (0.28 cpm/pmol) and that v_{12}^{zt} was estimated by integrated rate analysis [1,2] assuming a Michaelis-Menten constant of 300 μM (Table I).

Effect of phloridzin on formycin B transport in mouse spleen cells

For measuring Na^+-dependent, active and facilitated formycin B transport in cultures of adhering cells (see below) it is advantageous to be able to include inhibitors in the stop solution that inhibit both transport systems in order to effectively prevent the loss of substrate from the cells during rinsing of the cells to remove unincorporated substrate. Facilitated transport is readily inhibited by dipyridamole, but equivalent inhibitors for Na^+-dependent active nucleoside transport have not been described. However, recently Lee et al. [9] and Jarvis [10] reported that cold buffer containing 1 mM phloridzin is an effective stop solution for measuring Na^+-dependent, active transport in rat renal and rabbit intestinal brush border membranes. We have, therefore, examined the effect of phloridzin on total uptake of formycin B by mouse spleen lymphocytes, since Na^+-dependent, active transport represents a major nucleoside transport component of these cells [15]. Figs. 6A and B illustrate a concentration-dependent inhibition of uptake of 5 μM formycin B by these cells. The concentration of phloridzin causing 50% inhibition of the initial rate of uptake (IC_{50}) was about 110 μM and the maximum inhibition was about 90%. Since under the conditions of the assay about 60% of uptake reflected Na^+-dependent, active transport and about 40% facilitated transport [15], these data indicate that both transport systems were inhibited by phloridzin. Facilitated nucleoside transport by human erythrocytes has previously [28] been shown to be inhibited by phloretin, the non-glycosylated analog of phloridzin, with an IC_{50} of about 200 μM. Furthermore, the data in Fig. 6C show that the facilitated transport of formycin B (measured at 500 μM) by L1210 cells was inhibited by phloridzin with a similar IC_{50} (about 60 μM). This compares with an IC_{50} of 50–400 nM for the inhibition by dipyridamole of the facilitated transport of nucleosides, including formycin B [15], in various types of mouse cells [4]. Thus the use of a combination of both 1 mM phloridzin and 10 μM dipyridamole in a stop solution effectively blocks both Na^+-dependent, active and facilitated nucleoside transport.

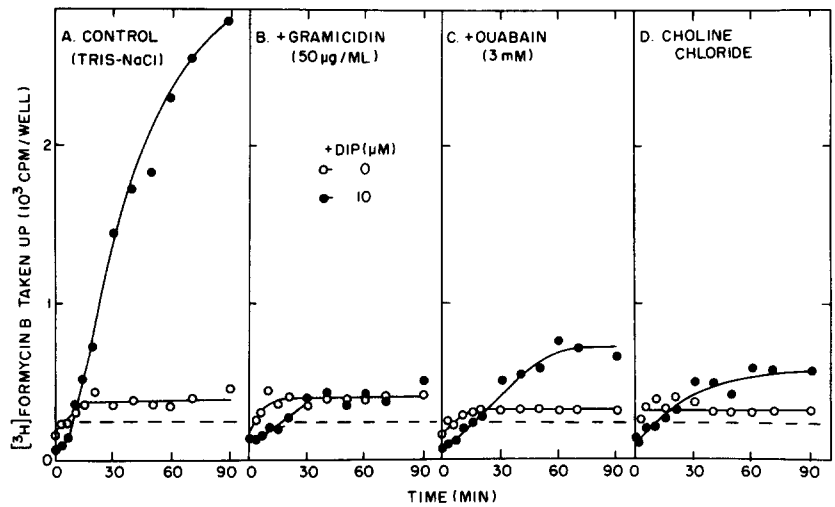

Fig. 7. Formycin B uptake by L929 cells in isotonic NaCl (A) and choline chloride (D) and effects of gramicidin (B) and ouabain (C) on formycin B uptake. L929 cells were propagated in 24-well tissue culture plates to confluency ($2.5 \cdot 10^5$ cells/well). (A–C) The wells were rinsed with RPMI and then received 0.5 ml of RPMI, which contained, where indicated, 50 μg gramicidin/ml (B) or 3 mM ouabain (C). After 10 min of incubation at 37° C, 11 wells in each plate were supplemented with dipyridamole (DIP) to 10 μM. Then at timed intervals during incubation at 37° C (1–89 min) [^{3}H]formycin B was added to 5 μM (120 cpm/pmol) to these wells as well as 11 equivalent wells not treated with dipyridamole. At 90 min the medium was dumped out and the wells were rapidly rinsed three times with ice-cold BSS containing 10 μM dipyridamole and 1 mM phloridzin. The cells were scraped into 1 ml of water with a rubber policeman and the lysates analyzed for radioactivity. The cells in one well from each plate were overlayed with 0.5 ml water (instead of RPMI) plus [^{3}H]formycin B to assess the efficiency of rinsing out unincorporated substrate. The cells from another well were removed by trypsinization and enumerated in a Coulter counter. (D) [^{3}H]Formycin uptake was measured in the same way as described for (A–C), except that the wells were initially rinsed with Tris-choline chloride and then received 0.5 ml Tris-choline chloride instead of RPMI. The broken lines indicate the intracellular concentrations of [^{3}H]formycin B equal to that in the extracellular fluid.

Na^+-dependent active formycin B transport in mouse L929 cells, RAW 309 Cr.1 cells and resident peritoneal macrophages

Fig. 7A illustrates that monolayer cultures of L929 cells rapidly accumulated 5 μM formycin B to a steady-state level that amounted to about 1.5-times the extracellular concentration but that, in the presence of 10 μM dipyridamole, they slowly accumulated 5 μM formycin B to about 10-times the extracellular concentration. The results are similar to those observed for mouse spleen cells [15] and L1210 and P388 mouse leukemia cells (cf. Figs. 2A, 5A and 7A). The concentrative accumulation of formycin B in the presence of dipyridamole was greatly reduced in a medium in which NaCl was replaced by choline chloride (Fig. 7D) or if the cells were pretreated with gramicidin or ouabain

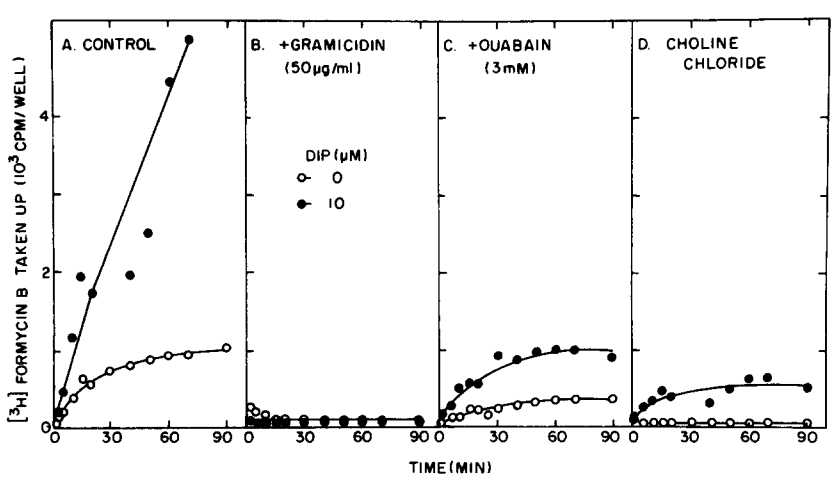

Fig. 8. Na^+-dependent concentrative uptake of formycin B by cultured mouse peritoneal macrophages. The experiment was conducted as described in the legend to Fig. 7, except with 2 day cultures of mouse macrophages ($2 \cdot 10^4$ cells/well). The intracellular concentration of [^{3}H]formycin B equal to that in the extracellular fluid was about 15 cpm/well.

(Figs. 7B and C). Similar results were obtained for primary cultures of resident mouse peritoneal macrophages (Fig. 8) and a line of Abelson virus-transformed mouse macrophages, RAW 309 Cr.1 (data not shown). However, in the case of mouse macrophages and RAW 309 Cr.1 cells. Na^+-dependent, concentrative transport seems to be the primary or major nucleoside transport system. Macrophages accumulated formycin B to about 70-times the extracellular concentration in the absence of dipyridamole over a 90-min incubation period (Fig. 8A). This concentrative uptake of formycin B was almost completely inhibited by gramidicin treatment (Fig. 8B) or the absence of Na^+ (Fig. 8D) and greatly reduced by ouabain treatment (Fig. 8C). In the presence of dipyridamole the macrophages accumulated formycin B to about 350-times the extracellular concentration (Fig. 8A). This increased concentrative accumulation of formycin B in the presence of dipyridamole indicates that the macrophages also express facilitated nucleoside transport. RAW 309 Cr.1 cells accumulated 5 μM formycin B to 7- and 60-times the extracellular concentration in the absence and presence of dipyridamole, respectively (data not shown).

Formycin B uptake by these types of cells was measured in monolayer cultures in 24-well tissue culture plates. Formycin B uptake was stopped by rapid rinsing of the wells with ice-cold BSS containing 10 μM dipyridamole and 1 mM phloridzin, but we found that rinsing with ice-cold BSS without inhibitors yielded comparable results.

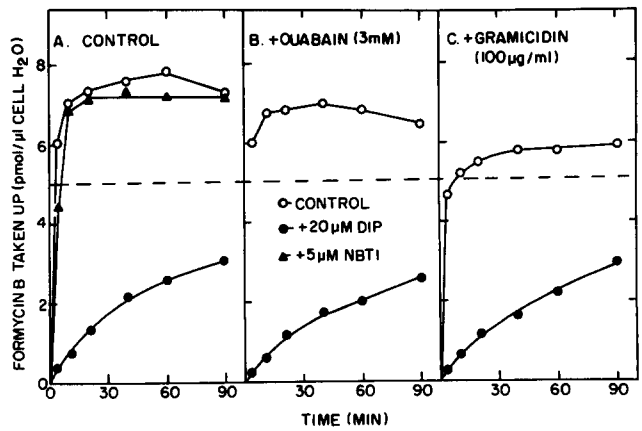

Fig. 9. Long-term uptake of formycin B by HeLa cells in the presence and absence of dipyridamole and lack of effect of ouabain and gramicidin. Samples of a suspension of $6 \cdot 10^6$ HeLa cells/ml of BM42B were supplemented where indicated with 3 mM ouabain or 100 μg gramicidin/ml and incubated at 37 °C for 20 min. Then the suspensions were equilibrated at 25 °C, a portion of each suspension was supplemented with 20 μM dipyridamole (DIP) or 5 μM NBTI (in A) and the uptake of 5 μM [^{3}H]formycin B (70 cpm/pmol) was measured as described in the legend to Fig. 2. The broken lines indicate the intracellular concentration of formycin B equal to that in the extracellular fluid.

Human HeLa, H9, Jurkat and CEM cells and pig spleen lymphocytes do not express Na^+-dependent, active nucleoside transport

In contrast to all the mouse cells investigated, in HeLa cells (Fig. 9A), H9, Jurkat and CEM human T-lymphoidal cells (Figs. 10A and C, and data not

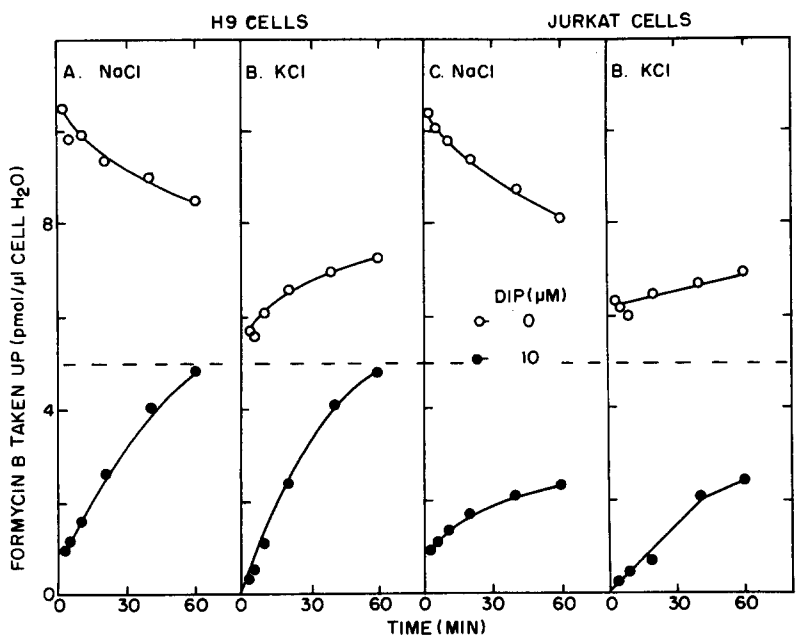

Fig. 10. Long-term uptake of formycin B by H9 (A,B) and Jurkat (C,D) human lymphoblastoid cells in isotonic NaCl or KCl in the absence and presence of dipyridamole. The cells were washed in Tris-choline chloride and then suspended to about $2 \cdot 10^7$ cells/ml in Tris-NaCl or Tris-KCl containing 5 mM D-glucose and 1 mM D-glutamine and where indicated 10 μM dipyridamole (DIP). Then the uptake of 5 μM [^{3}H]formycin B (65 cpm/pmol) was measured at 25 °C as described in the legend to Fig. 2. The broken lines indicate the intracellular concentration of formycin B equal to that in the medium.

shown) and pig spleen lymphocytes (Fig. 11A) dipyridamole did not effect a long-term concentrative accumulation of 5 μM formycin B. The presence of 2.5 μM NBTI was equally ineffective (Figs. 9A and 11A). NBTI strongly inhibited formycin B uptake in pig spleen lymphocytes (Fig. 11A) as previously reported for pig erythrocytes [29], but had relatively little effect on long-term formycin B uptake by HeLa cells since only 50% of facilitated nucleoside transport in these cells is NBTI-sensitive [2]. Furthermore, formycin B uptake in the absence and presence of dipyridamole was about the same whether HeLa cells were suspended in isotonic choline chloride or NaCl (data not shown) or H9 and Jurkat cells in isotonic KCl or NaCl (Fig. 10) or whether the various types of cells were treated with ouabain (Figs. 9B and 11B), or gramicidin (Figs. 9C and 11C) or ATP-depleted (data not shown). Similar results were also obtained whether formycin B uptake was measured in suspensions of HeLa cells or monolayer cultures of these cells in 24-well plates (data not shown).

Kinetic parameters for facilitated transport of formycin B by various cell lines

The foregoing results indicate that all human cell lines investigated, as well as pig spleen lymphocytes express only facilitated nucleoside transport. We have determined the kinetic parameters for the facilitated transport of formycin B by L1210, LK35.2, HeLa, H9, Jurkat and CEM cells and pig spleen lymphocytes using rapid kinetic techniques combined with integrated rate analysis of time courses of equilibration of six formycin B concentrations across the membrane and compared the values to those previously obtained for P388 cells [17] and mouse spleen lymphocytes [15] under identical experimental conditions (Table I). The Michaelis-Menten constants were comparable for all cell types analyzed, whereas the maximum velocities varied considerably as previously observed with other nucleosides as substrate [1,2].

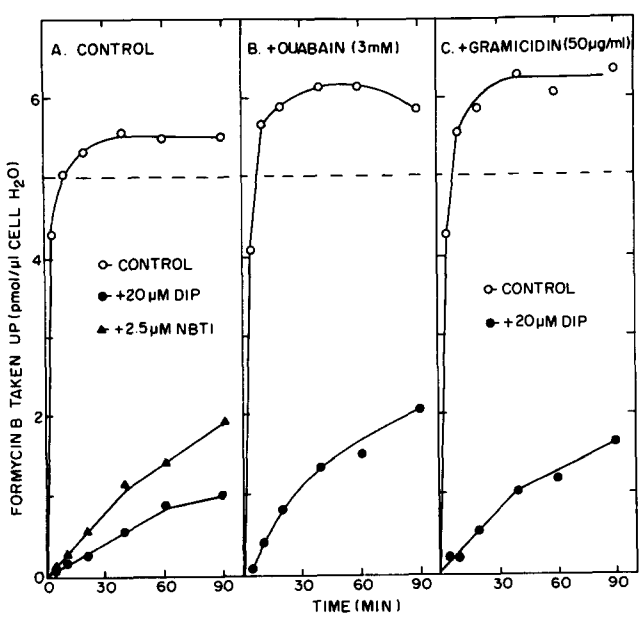

Fig. 11. Long-term uptake of formycin B by pig spleen lymphocytes in the absence and presence of dipyridamole and lack of effect of ouabain and gramicidin. Samples of a suspension of $1.1 \cdot 10^8$ pig spleen lymphocytes were supplemented with 3 mM ouabain or 50 μg gramicidin/ml and incubated at 37 °C for 20 min. Then a portion of each suspension was supplemented where indicated with 20 μM dipyridamole (DIP) or 2.5 μM NBTI (in A) and the uptake of 5 μM [^{3}H]formycin B (61 cpm/pmol) was measured as described in the legend to Fig. 2. The broken lines indicate the intracellular concentration of formycin B equal to that in the extracellular fluid.

TABLE I

Kinetic parameter for the facilitated transport of formycin B in various types of cells

Formycin B equilibration across the membrane was measured by rapid kinetic techniques at six formycin B concentrations (ranging from 40 to 1280 μM). An appropriate integrated rate equation was fitted to the pooled data assuming directional symmetry of the carrier and equal mobility when loaded and empty [1,2,18]. All values are stated ± standard error of the estimate. K_m, Michaelis-Menten constant; V_{max}, maximum velocity. Cell densities were as follows: $1 \cdot 10^7$ HeLa cells/ml, $1.9 \cdot 10^7$ H9 cells/ml, $2.2 \cdot 10^7$ Jurkat cells/ml, $2.2 \cdot 10^7$ CEM cells/ml, $4.0 \cdot 10^7$ L1210 cells/ml, $1.4 \cdot 10^7$ LK35.2 cells/ml and $1.1 \cdot 10^8$ pig spleen lymphocytes/ml. The values for P388 cells and mouse spleen lymphocytes are from Refs. 18 and 15, respectively.

Cells	K_m (μM)	V_{max} (pmol/μl cell water·s)
Human HeLa cells	345 ± 49	12.1 ± 0.6
Human H9 T-lymphocytes	254 ± 25	40.4 ± 1.4
Human Jurkat T lymphocytes	303 ± 40	44.9 ± 2.0
Human CEM T lymphocytes	335 ± 72	39.0 ± 2.8
Pig spleen lymphocytes	561 ± 69	19.2 ± 1.0
Mouse L1210 leukemia cells	199 ± 15	37.9 ± 1.1
Mouse LK35.2 B-cell hybridoma	183 ± 21	10.1 ± 0.4
Mouse spleen lymphocytes	187 ± 13	5.3 ± 1.3
Mouse P388 leukemia cells	278 ± 9	44.5 ± 0.6

Discussion

There is little doubt that various types of mouse cells express some Na^+-dependent nucleoside transport in addition to facilitated nucleoside transport. The cells expressing active transport are from different sources including lymphocytes, leukemia cells, macrophages and fibroblast. However, except for spleen lymphocytes [14,15] and peritoneal macrophages, active transport represents only a minor component of the total nucleoside transport capacity of the cells. Indeed, analyses of initial time courses of transmembrane equilibration of 5 μM formycin B failed to detect active nucleoside transport in L1210 and P388 cells. It thus must represent < 10% of total nucleoside transport at this concentration. Active transport became only apparent upon long-term incubation when facilitated transport was inhibited by treatment of the cells with dipyridamole or

NBTI. On the basis of data in Fig. 4 and Table I we estimate that the V_{max} for active nucleoside transport in L1210 cells is only about 1% of that for facilitated nucleoside transport. In contrast, Na^+-dependent, active transport seems to be the primary nucleoside transport system of mouse peritoneal macrophages. In these cells, formycin B efflux by the facilitated transporter seems to be too slow to counteract active formycin B influx so that the cells concentratively accumulate formycin B to high concentrations even in the absence of dipyridamole.

The Michaelis-Menten constant for Na^+-dependent, active nucleoside transport is difficult to determine in cells in which it coexists with facilitated nucleoside transport. However, it clearly is considerably lower than that for facilitated transport. Previously, we estimated apparent K_m values of 10–40 μM for Na^+-dependent, active formycin B transport in mouse spleen lymphocytes [15]. A more accurate value is probably about 5 μM, which we determined in rat peritoneal macrophages, which exhibit only Na^+-dependent, active nucleoside transport (Plagemann, submitted for publication). The latter value is comparable to the apparent K_m values estimated for Na^+-dependent uridine transport in rabbit intestinal brush border membrane vesicles [10].

In contrast to the mouse cells, no significant Na^+-dependent, active nucleoside transport was detected in pig spleen lymphocytes and human HeLa, H9, Jurkat and CEM cells; the cells only express facilitated transport. This is the first report on the kinetic properties of the facilitated nucleoside transporters of human T-lymphoidal cell lines (Table I). Their kinetic properties, however, are comparable to those of the transporter of HeLa cells and other types of cells [1,2]. The number of cell types examined in this study is too small to decide whether the widespread presence of Na^+-dependent active nucleoside transport among mouse cells is unique for this species. However, the lack of active nucleoside transport systems in comparable pig and human cells raises doubts as to any physiological significance of Na^+-dependent, active transport systems in tissue cells other than epithelial cells of the kidney and intestine and perhaps choroid plexus and macrophages.

On the other hand, active nucleoside transport in L1210 cells may explain the increased sensitivity of L1210 cells to dAdo toxicity in the presence of dipyridamole, which correlates with an increased accumulation of dATP [17,30]. We have demonstrated that, although dipyridamole strongly inhibits the initial influx of dAdo in L1210 cells, upon long-term incubation it enhances the uptake of dAdo and its intracellular phosphorylation [17]. The results were similar to those reported in the present study for formycin B (Figs. 2A and 3A), except that in the case of dAdo the incoming nucleoside is phosphorylated. We postulate that the concentrative accumulation of dAdo in the presence of dipyridamole enhances its phosphorylation by mass action.

The results suggest that dipyridamole may be a potentially useful adjunct in chemotherapy with nucleoside analogs involving cells that express both active and facilitated nucleoside transport. Indeed, recently it has been shown that dipyridamole enhances the inhibition of human immunodeficiency virus replication by 2′,3′-dideoxycytidine in cultured human monocytes [31]. According to our hypothesis these human monocytes, like mouse macrophages should express active nucleoside transport.

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