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# $Na^+$-dependent, active and $Na^+$-independent, facilitated transport of formycin B in mouse spleen lymphocytes

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**$Na^+$-dependent, active and $Na^+$-independent facilitated nucleoside transport were characterized in mouse spleen cells using rapid kinetic techniques and formycin B, a metabolically inert analog of inosine, as substrate. The Michaelis-Menten constants for formycin B transport by the two transporters were about 30 and 400 $\mu$M, respectively. The firstorder rate constant for $Na^+$-dependent transport was about 4-times higher than that for facilitated formycin B transport. The $Na^+$-dependent carrier is specific for uridine and purine nucleosides and accumulates formycin B concentratively in an unmodified form. Concentrative accumulation was inhibited by ATP depletion and gramicidin and ouabain treatment of the cells. Our data indicate a single $Na^+$-binding site on the $Na^+$-dependent nucleoside carrier and a Michaelis-Menten constant for $Na^+$ of about 10 mM. This transporter was not significantly inhibited by dipyridamole and nitrobenzylthioinosine, inhibitors of the facilitated transporter. The $Na^+$-independent, facilitated nucleoside transporter of spleen cells exhibits properties comparable to those of the carriers present in mammalian cells in general. The B lymphocytes remaining after depletion of spleen cell populations of T lymphocytes by incubation with a combination of T-cell specific monoclonal antibodies plus complement exhibited about the same activities of active and facilitated nucleoside transport as the original suspension.**

## Introduction

Transport across the plasma membrane is an essential and first step in the salvage of nucleosides by mammalian cells [1–3]. In salvage, transport is followed by simple, irreversible phosphorylation, which effectively activates the nucleosides and traps them intracellularly in phosphorylated form [2]. Two types of nucleoside transport systems have been identified in mammalian cells: (1) symmetrical, non-concentrative, facilitated transport with broad substrate specificity observed in all mammalian cells with a few exceptions [1–3] and (2) $Na^+$-dependent, concentrative transport associated with choroid plexus, epithelial cells of the kidney and intestine and mouse spleen cells [3–15]. The facilitated nucleoside transporter exists in two forms, which differ in sensitivity to inhibition by nitrobenzylthioinosine (NBTI) [3,16,17]. One form is strongly inhibited by nanomolar concentrations of NBTI (designated NBTI-sensitive), resulting from the binding of NBTI to high-affinity binding sites on the plasma membrane ($K_d \leq 1$ nM). The other form is not associated with such binding sites and is inhibited only by micromolar concentrations of NBTI (designated NBTI-resistant). Though some cells express only one or the other form, most cells express both forms in various proportions [3,18–20]. In many types of cells both forms exhibit similar sensitivity to inhibition by other inhibitors, such as dipyridamole, dilazep and lidoflazine, which are structurally unrelated to the nucleoside substrate of the transporter and to each other [17,18,21–23]. However, the facilitated nucleoside transporters of different species and cell types differ up to 1000-fold in their sensitivity to inhibition by the latter inhibitors [3,23]. $Na^+$-dependent nucleoside transport, in contrast, has been found to be highly resistant to inhibition by these inhibitors of facilitated nucleoside transport [4–15].

In a previous study [15] we have confirmed $Na^+$-dependent uridine uptake in mouse spleen cells [12], but found that it functions in conjunction with $Na^+$-independent, facilitated nucleoside transport. About 70% of the latter was in the NBTI-sensitive form, which corre-

Abbreviation: NBTI, nitrobenzylthioinosine.

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lated with the presence of about $10^4$ high-affinity, NBTI-binding sites/cell. Kinetic characterization of $Na^+$-dependent transport of uridine in spleen cells was complicated by its rapid phosphorylation in these cells. We found that the apparent concentrative accumulation of uridine in these cells reported by Darnowski et al. [12] reflected the intracellular accumulation of uracil nucleotides. In the present study we have further characterized the nucleoside transport systems of mouse spleen cells using as substrate a metabolically inert inosine analog, formycin B, which has been shown to be an efficient substrate for the facilitated nucleoside transporter of mammalian cells [24] as well as for the $Na^+$-dependent nucleoside transporters of rat and mouse intestinal epithelial cells [8,11].

**Experimental procedures**

*Spleen cells.* In most experiments spleens were provided as a byproduct of the production and titration of lactate dehydrogenase-elevating virus (LDV) in 4–6-week old Swiss mice [25]. However, in control experiments no significant differences in uridine transport and salvage [15] and in formycin B transport were observed between spleen cells from uninfected and LDV-infected mice. In a typical transport experiment, spleens were harvested from 30 to 60 uninfected or 1- or 5-day LDV-infected mice. Single cell suspensions free of erythrocytes were prepared in phosphate-buffered saline (pH 7.4), containing 5 mM glucose (PBS-glucose) or in RPMI containing 2.5% (w/v) bovine serum albumin (BSA) as described previously [15]. Cell viability was assessed by staining with trypan blue and the cells were enumerated with a Coulter electronic counter. In some experiments, where we measured both $Na^+$-dependent and -independent nucleoside transport, the cells were washed once in 0.145 M choline chloride containing 5 mM Tris-HCl (pH 7.4; Tris-choline chloride) and suspended as indicated in 0.145 M NaCl, LiCl, KCl or choline chloride containing 5 mM Tris-HCl (pH 7.4).

Where indicated, the spleen cell populations were depleted of T-lymphocytes by incubation with cytotoxic T-cell specific monoclonal antibodies (mAb) as described by Mueller et al. [26]. Suspensions of about $4 \cdot 10^7$ spleen cells/ml of RPMI plus BSA were mixed with 0.3 volume of culture fluid of each of hybridomas RL-172.4, 83.12.5 and JIJ-10 producing anti-CD4, anti-CD8 and anti-Thy1.2 mAbs, respectively, plus 0.2 volume of 'LOW TOX' rabbit complement (Accurate Scientific and Chemical Corp., Westbury, NY). After one hour of incubation at 37 °C, the residual cells were collected by centrifugation and resuspended in fresh medium. For measuring their responses to mitogenic stimuli spleen cells were suspended to $1 \cdot 10^6$ and $4 \cdot 10^6$ cells/ml in RPMI supplemented with 50 μM 2-mercaptoethanol plus 10% (v/v) fetal bovine serum. Samples of 100 μl of the suspensions were dispensed into 96-well tissue culture plates and mixed with 100 μl of solutions containing 0, 2, 6 and 20 μg concanavalin A or 20 and 50 μg lipopolysaccharide per ml. The plates were incubated at 37 °C for 1 and 2 days and then supplemented with 1 μCi [$^3$H]thymidine per well. After 6 h of incubation at 37 °C the cells were harvested with a PHD Cell Harvester (Cambridge Technologies, Inc., Cambridge, MA) and analyzed for radioactivity.

*Formycin B transport measurements.* Samples of cell suspension were supplemented with inhibitors and other substances as indicated in appropriate experiments. Then the time course of uptake of radiolabeled formycin B were measured under zero-*trans* conditions [1,3] at 25 °C using a dual syringe apparatus (12 time points/time course) or manual sampling for longer time points as described previously [1,3,17,27]. 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 in total cell material). Radioactivity/cell pellet was corrected for that attributable to extracellular space as estimated with [$^{14}$C]inulin [27]. Intracellular $H_2O$ space was measured with $^3H_2O$ [27].

For fractionating the acid-soluble pool of cells, the cells from replicate samples were centrifuged through an oil layer directly into a solution composed of sucrose and 0.5 M trichloroacetic acid [28]. The acid layer and the culture fluid were further processed and analyzed by ascending paper chromatography with several solvent systems as described previously [15,29].

$Na^+$-dependent formycin B transport was specifically measured in spleen cells in which facilitated transport was inhibited by treatment with dipyridamole or total uptake in $Na^+$-containing medium was corrected for $Na^+$-independent uptake (see later). $Na^+$-independent, facilitated transport was specifically measured in choline chloride-washed cells suspended in $Na^+$-free medium or in ATP-depleted cells. The spleen cells were depleted of ATP by incubation in PBS or Tris-choline chloride containing 5 mM KCN and 5 mM iodoacetate at 37 °C [30]. An appropriate integrated rate equation based on a simple carrier model was fitted to time courses of $Na^+$-independent equilibration of formycin B across the membrane assuming directional symmetry [1,15]. For estimation of the kinetic parameters of transport, eight formycin B concentrations were employed and the Michaelis-Menten parameters extracted by least-squares regression [1].

*Determination of $Na^+$ content of cells.* Samples of $1.5 \cdot 10^8$ untreated and gramicidin-treated spleen cells were collected by centrifugation, washed once in Tris-

choline chloride and then analyzed for $Na^+$ concentration by flame photometry (conducted by R.H. Opaugh, 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 nucleosides and nucleobases, ouabain, gramicidin (from *Bacillus brevis*), concanavalin A and lipopolysaccharide 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). Anti-CD4, anti-CD8 and anti-Thy1.2 mAbs were kindly supplied by M. Jenkins.

## Results and Discussion

The data in Fig. 1A illustrate that the initial rate of uptake of 5 $\mu$M formycin B by a mouse spleen cell population was about 5 times higher in isotonic NaCl than in isotonic KCl, LiCl and choline chloride media. In contrast, the rate of uptake of 500 $\mu$M formycin B was about the same in all four media (Fig. 1B). These results are similar to those with uridine as a substrate [15] and consistent with the view that formycin B uptake by the spleen cells is mediated by two transport systems, a $Na^+$-dependent, high-affinity transporter as well as the broadly specific, $Na^+$-independent, low-affinity facilitated transporter operating, with a few exceptions, in all mammalian cells that have been investigated [3]. One difference between our earlier results with uridine as substrate and the present results with formycin B is that the ratio of $Na^+$-dependent transport (total minus $Na^+$-independent transport) to $Na^+$-independent transport at 5 $\mu$M in mouse spleen cells was greater for formycin B (approx. 4-fold) than for uridine (about equal). This difference has been confirmed in two additional experiments of the type shown in Fig. 1A and probably reflects differences in the affinities of uridine and formycin B for the two transport systems (see later).

Chromatographic analyses of acid extracts and the culture fluid of spleen cells that had been incubated for 10 min with 5 $\mu$M [$^3$H]formycin B in different types of media showed that the formycin B was metabolically inert, as previously reported from studies with a rat epithelial cell line [8] and P388 mouse leukemia cells [15]. In the mouse spleen cells, < 5% of the intracellular radioactivity was recovered in phosphorylated products and no other metabolic products were detected (data not shown).

The following experiments were designed to further characterize the $Na^+$-dependent and independent transport systems of the mouse spleen cells. Fig. 2A illustrates the $Na^+$-dependency of the uptake of 5 $\mu$M formycin B in greater detail. The $Na^+$ concentration yielding a 50% of maximum $Na^+$-dependent transport velocity ($K_{Na}$) was $9.8 \pm 2.5$ mM (Fig. 2B), a value similar to those observed for active transport of formycin B in a rat epithelial cell line [8] and for $Na^+$-dependent uridine uptake in our mouse spleen cells [15] and rabbit intestinal and rat renal brush-border membrane vesicles [13,14]. The data are consistent with a minimum $Na^+$:formycin B stoichiometry of 1 : 1. We have also analyzed the data in the Eadie-Scatchard plot of the Hill equation [31]:

$$v_{NA}/[Na^+]^n = v_{Na}/K_{Na} + V_{max}/K_{Na}$$

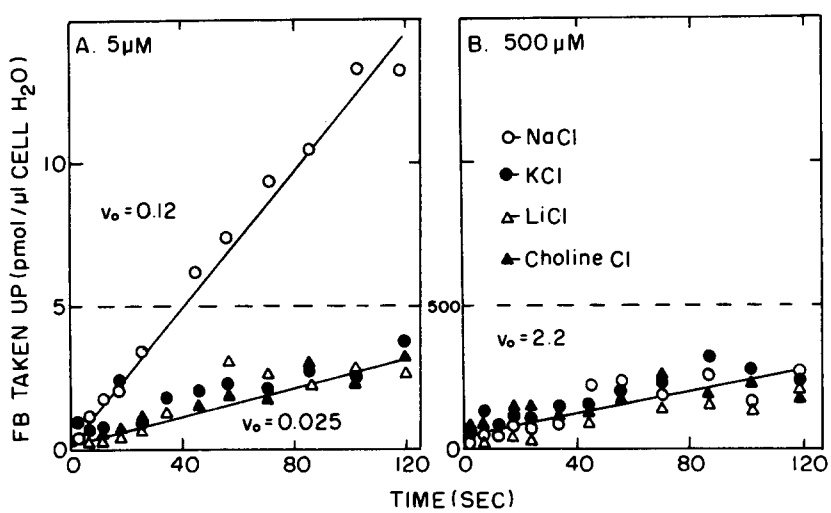


Fig. 1. $Na^+$-dependent and -independent formycin B transport by mouse spleen cells. Spleen cells were washed in Tris-choline chloride and suspended to $3.3 \cdot 10^7$ cells/ml in isotonic solutions of NaCl, KCl, LiCl or choline chloride. Then the uptake of 5 $\mu$M and 500 $\mu$M [$^3$H]formycin B (220 cpm/$\mu$l; irrespective of concentration) was measured at 25 °C by rapid kinetic techniques. Initial velocities of uptake ($v_0$) were estimated graphically and are expressed in pmol/$\mu$l cell water per s. The broken lines indicate the intracellular concentration of [$^3$H]formycin B equal to that in the medium.

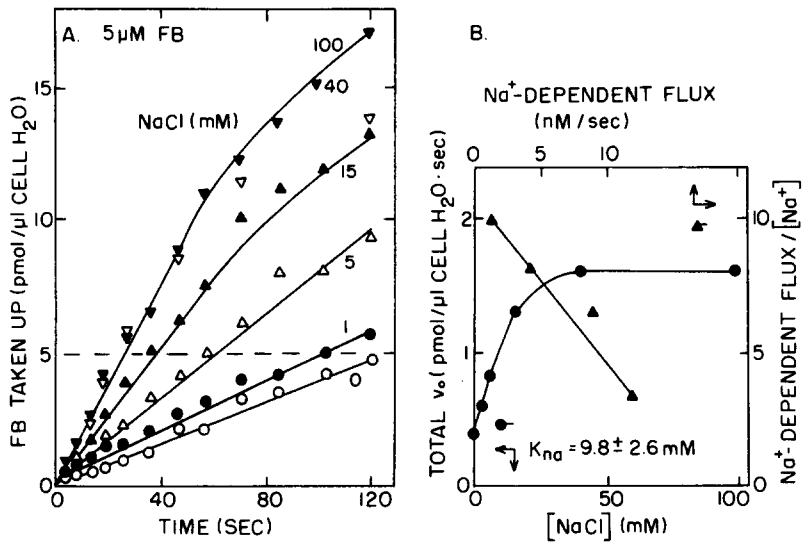


Fig. 2. Effect of $Na^+$ concentration on formycin B transport in mouse spleen cells. Samples of Tris-choline chloride washed cells were suspended in mixtures of isotonic Tris-choline chloride and Tris-NaCl to the indicated concentrations of $Na^+$ and a final cell density of $4.8 \cdot 10^7$/ml. Then the influx of 5 μM [$^3$H]formycin B (60 cpm/pmol) was measured at 25° C by rapid kinetic techniques (A). Initial velocities of total uptake ($v_0$) were estimated graphically from the initial linear portions of the uptake curves and are plotted as a function of $Na^+$ concentration in B (●———●). $Na^+$-dependent influx ($v_{Na}$) was calculated by subtracting the rate of uptake in the absence of NaCl from the rate of uptake in the presence of various concentrations of $Na^+$ and plotted as a function of $v_{Na}/[Na^+]^n$ (▲———▲, see text). $K_{Na}$ was calculated by fitting the Michaelis-Menten equation to the $v_{Na}$ values.

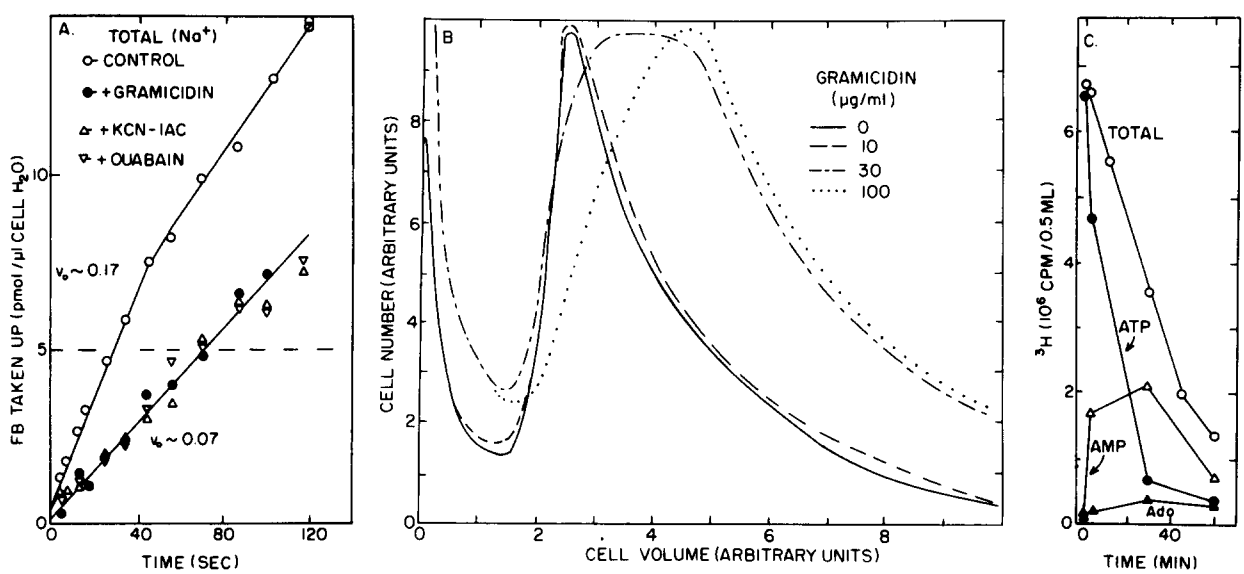


Fig. 3. Effects of ATP depletion and treatment with gramicidin and ouabain on total formycin B transport in mouse spleen cells (A), effect of gramicidin treatment on cell volume (B), and ATP depletion of mouse spleen cells by incubation in glucose-free medium containing KCN and iodoacetate (C). (A) Samples of the suspension of $2.5 \cdot 10^7$ spleen cells/ml of Tris-NaCl were supplemented as indicated with 100 μg gramicidin/ml, 2.5 mM ouabain, or 5 mM KCN plus 5 mM iodoacetate and incubated at 37° C for 30, 20 or 10 min, respectively. Then the uptake of 5 μM [$^3$H]formycin B (40 cpm/pmol) was measured at 25° C by rapid kinetic techniques. The broken line indicates the intracellular concentration of [$^3$H]formycin B equal to that in the medium. Initial uptake velocities ($v_0$) were estimated graphically and are expressed in pmol/μl cell water per s. (B) Samples of a suspension of $7.5 \cdot 10^7$ spleen cells/ml of PBS-glucose were incubated with the indicated concentrations of gramicidin/ml at 37° C for 20 min and then analyzed for cell volume distribution by means of a Coulter counter attached to a multichannel analyzer. (C) A suspension of $6 \cdot 10^7$ spleen cells/ml of PBS-glucose was supplemented with 25 μM deoxycoformycin to inhibit adenosine deaminase and then incubated with [$^3$H]adenosine (2 μCi/ml; approx. 0.05 μM) at 37° C for 10 min. The cells were collected by centrifugation and suspended to the same density in glucose-free basal medium containing 5 mM KCN and 5 mM iodoacetate. At various times of further incubation at 37° C, the cells from duplicate 0.5 ml of suspension were collected by centrifugation through oil and analyzed for radioactivity. The acid soluble pools were extracted from replicate samples of cells and analyzed chromatographically [30]. The amounts of radioactivity in ATP (ADP), AMP and adenosine were estimated on the basis of the chromatographic analyses and the total cell-associated radioactivity.

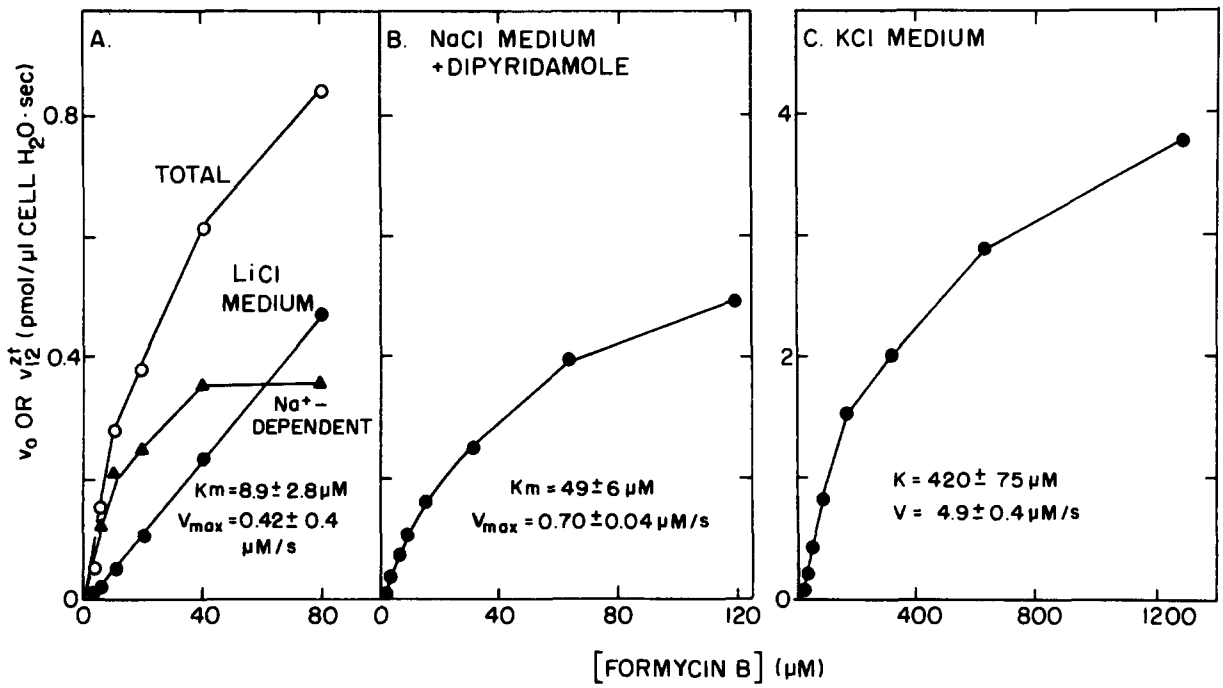


Fig. 4. Estimation of kinetic parameters of $Na^+$-dependent and independent formycin B transport in mouse spleen cells. In A, Tris-choline chloride washed spleen cells were suspended in isotonic Tris-NaCl or Tris-LiCl to $3 \cdot 10^7$ cells/ml and then time courses of uptake of 1, 4, 10, 20, 40, and 80 μM [$^3$H]formycin B (207 cpm/μl, irrespective of concentration) were measured at 25° C by rapid kinetic techniques. In B, a suspension of $7.5 \cdot 10^7$ spleen cells/ml PBS-glucose was incubated with 20 μM dipyridamole for 2 min and then time courses of uptake of 1, 2, 4, 8, 16, 32, 64, and 128 μM [$^3$H]formycin B (200 cpm/μl, irrespective of concentration) were assayed at 25° C in samples of suspension. Initial transport velocities ($v_0$) were estimated graphically from the uptake time courses. In A, $v_0$ for $Na^+$-dependent formycin B transport was calculated by subtracting $v_0$ for uptake in LiCl medium from $v_0$ for total uptake at each formycin B concentration. The Michaelis-Menten equation was fitted to the $v_0$ values. In C, choline-chloride washed spleen cells were suspended in Tris-KCl to $7.5 \cdot 10^7$ cells/ml. Then the zero-*trans* uptake of 10, 20, 40, 80, 160, 320, 640 and 1280 μM [$^3$H]formycin B (250 cpm/μl, irrespective of concentration) was measured by rapid kinetic techniques. An appropriate integrated rate equation based on the simple carrier model was fitted to the pooled data assuming directional symmetry [15,24], and the kinetic parameters extracted by least-squares regression analysis [1,3]. All kinetic parameters are presented ± S.E. of the estimate.

where $v_{NA}$ is the velocity of $Na^+$-dependent formycin B transport and $n$ is the Hill coefficient. The linearity of the plot $v_{Na}/[Na^+]$ vs. $v_{Na}$ (Fig. 2B) indicates $n = 1$, that is a single $Na^+$-binding site on the $Na^+$-dependent formycin B carrier.

The uptake of 5 μM formycin B by spleen cells in Tris-NaCl was inhibited about 60% by preincubation with 50 μg gramicidin/ml (Fig. 3A). The ionophore gramicidin abolishes $Na^+$ and $K^+$ gradients across the membrane of human erythrocytes [32], and in the case of the mouse spleen cells, incubation with 30 or 100 μg gramicidin/ml at 37° C for 20 min increased the $Na^+$ content of the cells from about 4 to 14 μg/$10^7$ cells. It also caused a 50–100% increase in the size of the cells (Fig. 3B). A prolonged incubation (longer than 20 min) of the cells in isotonic KCl had a similar effect on cell size (data not shown). The results illustrate the dependence of active formycin B transport on the transmembrane $Na^+$-gradient. In agreement with this conclusion, pretreatment of the cells with 2.5 mM ouabain also decreased the uptake of 5 μM formycin B about 60% (Fig. 3A).

Fig. 3C shows that incubation of the spleen cells in glucose-free medium containing 5 mM KCN and 5 mM iodoacetate at 37° C resulted in a rapid loss of ATP, but that about 60 min of incubation were required to completely deplete the cells of adenine nucleotides. A 10-min incubation with KCN-iodoacetate, however, has previously been found sufficient to reduce uridine phosphorylation by the spleen cells > 90% [15]. Such a 10-min incubation of the spleen cells in the KCN-iodoacetate medium reduced the uptake of 5 μM formycin B in NaCl medium by about 60% (Fig. 3A), whereas it had little effect on formycin B uptake by the cells in choline chloride medium (data not shown). The results indicate that $Na^+$-dependent formycin B uptake was energy dependent.

Dipyridamole reduced the uptake of 5 μM formycin B in a concentration-dependent manner, but only to a maximum of about 50%, whereas the residual uptake of formycin B by ATP-depleted cells and spleen cells suspended in isotonic KCl was inhibited about 70% (data not shown). NBTI at a concentration of 2 μM had no significant effect on formycin B uptake by cells suspended in NaCl, but reduced the residual uptake by ATP-depleted cells about 40% (data not shown). The results are similar to those previously reported for uridine uptake by spleen cells [15] and are consistent

with the view that $Na^+$-dependent nucleoside transport is largely resistant to inhibition by dipyridamole and NBTI [4–15]. The synergistic inhibition of formycin B uptake by the KCN-iodoacetate and dipyridamole treatments is consistent with the view that the former inhibits $Na^+$-dependent, whereas the latter inhibits $Na^+$-independent, facilitated nucleoside transport. The failure of NBTI to inhibit formycin B uptake by ATP-depleted spleen cells more than 40% indicates that a considerable proportion of the $Na^+$-independent, facilitated transporter is present in the NBTI-resistant form. This conclusion was also indicated by studies of the facilitated transport of uridine in these cells, which suggested that 30–40% of the transporter is present in NBTI-resistant form [15].

The kinetic parameters of $Na^+$-dependent formycin B transport were assessed in two ways. In one experiment the rate of uptake of various concentrations of formycin B was measured both in NaCl and LiCl media (Fig. 4A), and the rate of $Na^+$-dependent transport was taken as the difference between the rates observed in the two types of media. A fit of the Michaelis-Menten equation to the transport rates yielded $K_m = 9$ $\mu$M and $V_{max} = 0.42$ pmol/$\mu$l cell water per s. In the second approach, $Na^+$-dependent formycin B transport was measured in spleen cell populations treated with 20 $\mu$M dipyridamole to inhibit its $Na^+$-independent, facilitated transport (Fig. 4B). The $K_m$ and $V_{max}$ values estimated by this method were $49 \pm 6$ $\mu$M and $0.70 \pm 0.04$ pmol/$\mu$l cell water per s, respectively, in one experiment (Fig. 4B) and $34 \pm 12$ $\mu$M and $2.0 \pm 0.2$ pmol/$\mu$l cell water per s, in another experiment (data not shown). The latter values were somewhat higher than those based on the rate of $Na^+$-dependent transport calculated by subtracting the rate of uptake in LiCl medium from the total uptake rate (Fig. 4A). The reasons for these discrepancies are not clear at present; both approaches are subject to a number of experimental uncertainties. The lower $K_m$ value (9 $\mu$M) is similar to that reported for $Na^+$-dependent uridine transport by choroid plexus [4] and rabbit intestinal brush-border membrane vesicles [14], whereas the higher $K_m$ value (approx. 40 $\mu$M) is comparable to that reported for $Na^+$-dependent formycin B transport in mouse intestinal epithelial cells [11] and uridine transport in mouse spleen cells [12,15].

The kinetic parameters for $Na^+$-independent facilitated formycin B transport in mouse spleen cells were measured in cells suspended in KCl medium. Integrated rate analysis of time courses of transmembrane equilibration of 10, 20, 40, 80, 160, 320, 640, and 1280 $\mu$M formycin B assuming directional symmetry [3] yielded $K$ and $V$ values of $420 \pm 75$ $\mu$M and $4.9 \pm 0.4$ pmol/$\mu$l cell water per s, respectively (Fig. 4C). The $K$ value observed for facilitated formycin B transport in spleen cells are comparable to that observed for P388 mouse leukemia cells (about 280 $\mu$M; Ref. 24). Furthermore, the turnover numbers for NBTI-sensitive, facilitated formycin B transport by mouse spleen and P388 cells seem comparable. This conclusion is based on about $7 \cdot 10^{11}$ and $3.8 \cdot 10^{12}$ high-affinity NBTI binding sites per $mm^2$ surface area of spleen cells and P388 cells, respectively [15] and an about 9-fold higher $V_{max}$ for NBTI-sensitive, facilitated transport of formycin B in P388 cells (approx. 45 pmol/$\mu$l cell water per s; Ref. 24) than in spleen cells.

Other experiments have shown that the initial rate of zero-*trans* entry and exit of 100 $\mu$M formycin B by spleen cells suspended in KCl medium were the same ($v_{12}^{zt} = v_{21}^{zt}$; data not shown) indicating directional symmetry of the facilitated nucleoside transporter of these cells [1,3]. Similarly, the initial rates of zero-*trans* entry and equilibrium exchange of 1 mM formycin B were the same ($v_{12}^{zt} = v^{ee}$; data not shown), which indicates equal mobility of formycin B-loaded and empty carrier [1,3]. Furthermore, the zero-*trans* uptake of 100 $\mu$M [$^3$H]formycin B by spleen cells suspended in KCl medium was inhibited by all purine and pyrimidine nucleosides tested (added simultaneously with [$^3$H]formycin B in unlabeled form at a concentration of 1 mM); the degree of inhibition was about 80% for inosine and adenosine and decreased in the order uridine, thymidine, formycin B, and deoxycytidine (data not shown). These results are similar to the inhibition of uridine uptake by other nucleosides in mouse spleen cells [15] and of the facilitated transport of formycin B in P388 cells [24]. Thus, in kinetic properties, substrate specificity and NBTI and dipyridamole sensitivity facilitated nucleoside transport in mouse spleen cells is comparable to that in various mouse cell lines [1,3].

In contrast to facilitated formycin B transport, the $Na^+$-dependent transport of 5 $\mu$M [$^3$H]formycin B measured in dipyridamole-treated spleen cells suspended in PBS-glucose ($v_{12}^{zt} = 0.18$ pmol/$\mu$l cell water per s) was inhibited about 92% by 100 $\mu$M unlabeled adenosine and inosine, about 56% by 100 $\mu$M uridine and formycin B, but not significantly affected by 100 $\mu$M thymidine, cytidine or deoxycytidine (data not shown). The results are in agreement with those reported for the inhibition of $Na^+$-dependent uptake of uridine by other nucleosides in mouse spleen cells [15]. Combined, the results indicate that uridine and formycin B are substrates for the same $Na^+$-dependent transporter and that the mouse spleen cells express the uracyl-purine nucleoside-specific $Na^+$-dependent transporter that has been reported to be present in mouse intestinal epithelial cells [11]. The second $Na^+$-dependent nucleoside transporter detected in mouse epithelial cells that efficiently transports thymidine, but not formycin B, guanosine or inosine, seems to be lacking in mouse spleen cells [15].

The concentrative nature of the $Na^+$-dependent nucleoside transporter was assessed by measuring the

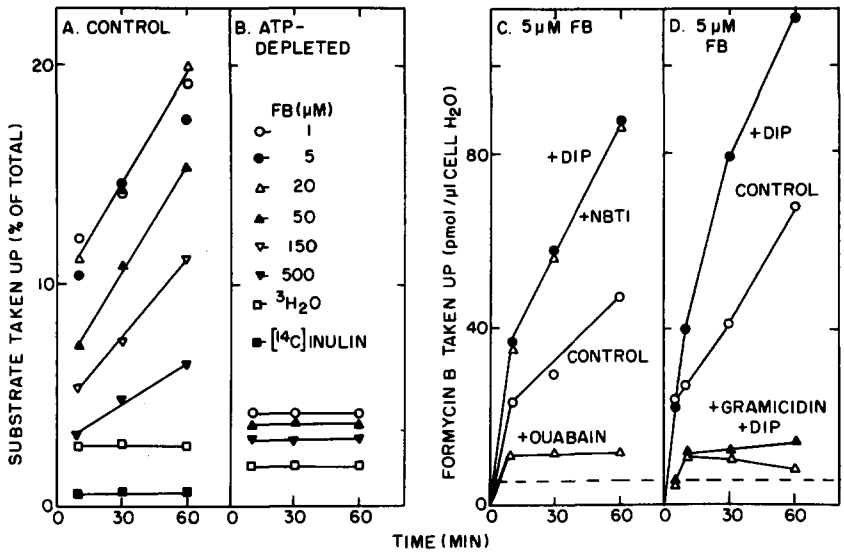


Fig. 5. Long-term $Na^+$-dependent uptake of various concentrations of formycin B by spleen cells and effects of dipyridamole, ATP depletion, gramicidin and ouabain. Spleen cells were suspended to $6.6 \cdot 10^7$ (A and B), $8.5 \cdot 10^7$ (C) or $7.5 \cdot 10^7$ (D) cells/ml in PBS containing where indicated 5 mM KCN plus 5 mM iodoacetate (ATP-depleted), 2.5 mM ouabain, 100 μg gramicidin/ml 20 μM dipyridamole (DIP) or 5 μM NBTI. After 10 or 30 min (in case of ouabain and gramicidin) incubation at 37 °C, the suspensions were equilibrated at 25 °C and supplemented with the indicated concentrations of [$^3$H]formycin B (FB; 280 cpm/μl, irrespective of concentration), $^3H_2O$ or [$^{14}$C]inulin. At various times of further incubation at 25 °C, the cells from 0.5 ml of suspension were collected by centrifugation through oil and analyzed for radioactivity. In A and B, cell-associated radioactivity was expressed as percent of the total in the suspension. In C and D, the broken lines indicate the intracellular concentration of formycin B equal to that in the medium. All points are averages of duplicate samples.

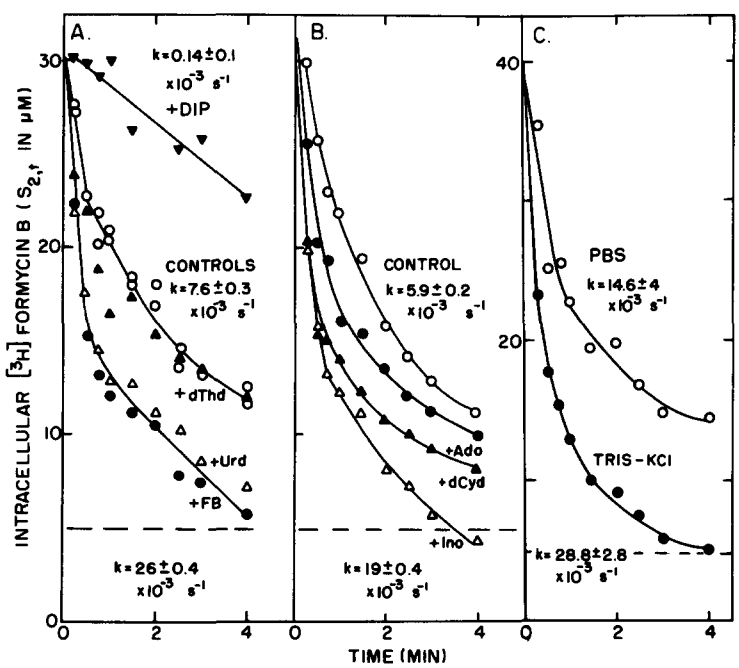


Fig. 6. Effects of dipyridamole, nucleosides and absence of $Na^+$ on the efflux of formycin B from spleen cells. Suspensions of about $6 \cdot 10^7$ cells/ml of PBS-glucose were supplemented with 5 μM [$^3$H]formycin B (20 cpm/pmol) and incubated at 37 °C for 1 h. Then samples of cells were collected by centrifugation and suspended to the original density in PBS containing, as indicated in (A) 20 μM dipyridamole (DIP; ▼———▼), or 1 mM unlabeled formycin (FB; ●———●), uridine (Urd; △———△), thymidine dThd; ▲———▲) and in (B) 1 mM adenosine (Ado; ●———●), deoxycytidine (dCyd; ▲———▲) or inosine (Ino; △———△). In (C), samples of cells were suspended in PBS (○———○) or Tris-KCl (●———●). After various times of incubation at 25 °C, the cells of samples of each suspension (removed manually) were collected by centrifugation and analyzed for radioactivity. Radioactivity/cell pellet was converted to μmolar concentration of [$^3$H]formycin B on the basis of measured intracellular $H_2O$ spaces. The following first order equation was fitted to each time course:

$$S_{2,t} = (S_{2,0} - S_{2,\infty})e^{-kt} + S_{2,\infty}$$

where $S_{2,t}$ is the intracellular concentration of [$^3$H]formycin B at time $t$ and $k$ is the first-order rate constant. Estimated values of $k$ for the control cells of three independent experiments and some treatments (±S.E. of estimate) are shown in A–C. The broken lines indicate the intracellular concentration of formycin B equal to that present in the medium during preloading.

uptake of various concentrations of formycin B (1–500 $\mu$M) by control and ATP-depleted cells and ouabain and gramicidin-treated cells in a NaCl medium over a 60-min period of incubation at 25°C (Fig. 5). The control cells accumulated formycin B to a up to 8-times higher concentration than present in the medium and the intracellular concentration was still increasing at 60 min of incubation. In contrast, in ATP-depleted, gramicidin-treated or ouabain-treated spleen cells formycin B accumulated to a maximum of only twice the extracellular concentration (Figs. 5 B–D). The results indicate that the $Na^+$-dependent transporter accumulates formycin B against a concentration gradient in an energy-dependent manner. This conclusion is in agreement with findings previously reported for other active nucleoside transporters [4–14]. The $Na^+$/energy-dependent concentrative accumulation was concentration dependent and became saturable at formycin B concentrations > 20 $\mu$M (Fig. 5A).

The presence of dipyridamole or NBTI enhanced the concentrative accumulation of formycin B by the spleen cells (Figs. 5 C and D). The latter observation has also been made for nucleoside uptake by other cell systems expressing active nucleoside transport [4,8]. Dipyridamole and NBTI probably enhance the accumulation of formycin B by inhibiting its efflux via the dipyridamole- and NBTI-sensitive facilitated transporter (see below). Treatment with gramicidin inhibited the concentrative accumulation of formycin B in both control and dipyridamole-treated cells (Fig. 5D).

We also measured the efflux of 5 $\mu$M [$^3$H]formycin B from cells that had been preloaded for 1 h in PBS-glucose (Fig. 6). Efflux in a $Na^+$-containing medium was somewhat slower than influx. The first-order rate constant for formycin B efflux at 25°C as measured in three independent experiments was $(7.7 \pm 0.3) \cdot 10^{-3}$ $s^{-1}$, $(5.9 \pm 0.2) \cdot 10^{-3}$ $s^{-1}$, and $(14.6 \pm 4) \cdot 10^{-3}$ $s^{-1}$ (Fig. 6) compared to about 30 and $11 \cdot 10^{-3}$ $s^{-1}$ for $Na^+$-dependent and $Na^+$-independent formycin B influx, respectively. Formycin B efflux was strongly inhibited by the presence of dipyridamole (Fig. 6A) and to a lesser extent of NBTI (data not shown) in the extracellular fluid. This finding indicates that efflux was largely mediated by the $Na^+$-independent, facilitated nucleoside transporter. This inhibition of efflux explains the enhanced accumulation of [$^3$H]formycin B by spleen cells illustrated in Figs. 5C and D. In contrast, formycin B efflux in Tris-KCl was about twice that in PBS (Fig. 6C), which indicates that active influx partially counteracted facilitated efflux.

In contrast to the inhibitory effects of the transport inhibitors, the presence of various unlabeled nucleosides enhanced the efflux of [$^3$H]formycin B but to greatly different degrees and the effects cannot be entirely explained on a mechanistic basis (Fig. 6). Unlabeled formycin B, inosine and uridine were most effective in enhancing formycin B efflux. Such trans-stimulation has only been observed for the facilitated nucleoside transporters of human, pig and mouse erythrocytes, which exhibit directional symmetry but differential mobility of nucleoside-loaded and empty carrier (Refs. 3, 19 and 33; Plagemann, unpublished data). The facilitated nucleoside transporters of all other types of cells that have been investigated move the same whether empty or loaded and do not show *trans*-stimulation [3] and the same has been found to be the case for the facilitated transporter of mouse spleen cells. These facts and the finding that uridine but not thymidine enhanced formycin B efflux might suggest that it is partly mediated by the $Na^+$-dependent transporter, but the findings that deoxycytidine greatly enhanced formycin B efflux, whereas adenosine and deoxyadenosine had relatively little effect are inconsistent with this view, since deoxycytidine had little effect on $Na^+$-dependent formycin B influx, whereas adenosine was strongly inhibitory. Clearly, further work is required to resolve the mechanism of *trans*-stimulation of formycin B efflux.

Erythrocyte-free spleen cell populations are composed to 60–70 and 30–40% of B and T lymphocytes, respectively, and some macrophages [34]. In two experiments, we have lysed the T cells by incubation of spleen cell populations with anti-CD4, anti-CD8 and anti-Thy1.2 mAbs plus complement. The treatment resulted in the loss of about 40% of the total cells and it decreased the influx of 5 $\mu$M (Fig. 7A) and 500 $\mu$M formycin B (data not shown) in PBS-glucose and the long-term concentrative accumulation of 5 $\mu$M formycin B in the presence of dipyridamole (Fig. 7B) to about the same extent. The cells remaining after the treatment with anti-T cell mAbs and complement exhibited an undiminished mitogenic response to lipopolysaccharide, but failed to respond to concanavalin A (Fig. 7C) illustrating the effectiveness of the treatment in lysing all T lymphocytes. The results indicate that B lymphocytes, and by extrapolation T lymphocytes, express both active and facilitated nucleoside transport. Mouse macrophages also exhibit considerable $Na^+$-dependent, active nucleoside transport (Plagemann and Aran, in preparation), but macrophages represent only a minor fraction of total spleen cell populations and removal of macrophages by panning on tissue culture dishes had no effect on $Na^+$-dependent uridine [15] or formycin B transport by the spleen cell population (data not shown).

It should be noted that formycin B accumulated to higher than equilibrium concentrations even in ATP-depleted and in gramicidin- or ouabain-treated spleen cells (Fig. 5). This slight concentrative accumulation of formycin B has also been observed in P388 mouse leukemia cells and human erythrocytes [24] and for other purine but not pyrimidine nucleosides [1,35–38]. It does not seem to be due to active transport, accumu-

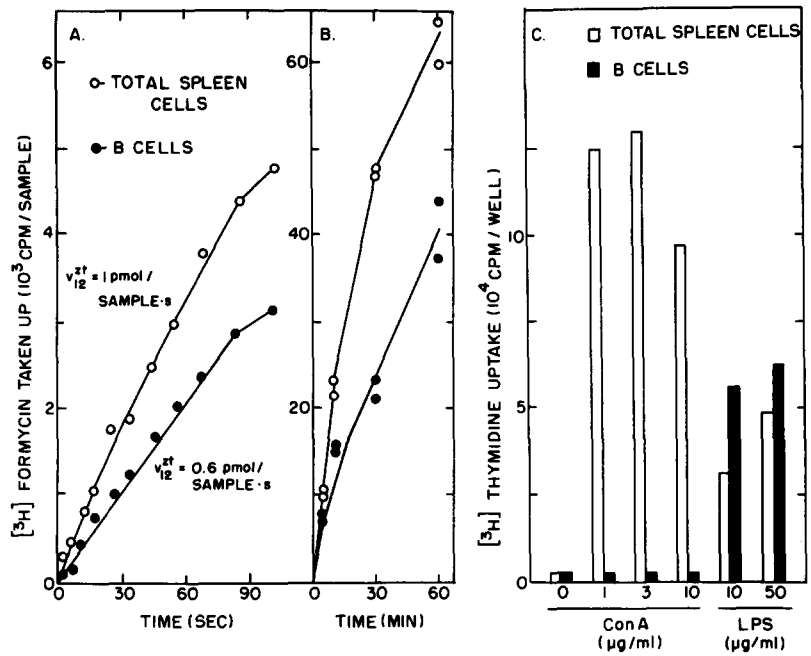


Fig. 7. Formycin B (A and B) and thymidine (C) uptake by total spleen cells and purified B cells freed of T lymphocytes by treatment with anti-T cell mAbs and complement. One-half of a suspension of spleen cells in RPMI containing BSA was freed of T cells by incubation with anti-T cell mAbs plus complement as described under Experimental procedures. These cells (●———●) and cells of an untreated portion of the suspension ((○———○) were collected by centrifugation and suspended in an identical volume of RPMI containing BSA. The suspensions contained about $4 \cdot 10^7$ total spleen cells and $2.6 \cdot 10^7$ purified B cells/ml in (A) and $7.0 \cdot 10^7$ total spleen cells and $4.1 \cdot 10^7$ purified B cells in (B). Then the short-term and long-term uptake of 5 μM [$^3$H]formycin B (50 cpm/pmol) was measured at 25 °C as described under Experimental procedures. In (A), the initial transport velocities ($v_{12}^{zt}$, in pmol/sample per s) were estimated from the initial linear portions of the uptake curves. The suspensions were also diluted 1:10–1:40 in RPMI containing 50 μM mercaptoethanol and supplemented with 10% fetal bovine serum. These suspensions were incubated with the indicated concentrations concanavalin A (ConA) or lipopolysaccharide (LPS) for 1 and 2 days at 37 °C and then the uptake of [$^3$H]thymidine (1 mCi/ml) was measured over a 6 h period at 37 °C as described under Experimental procedures. The data in frame C are for the cells whose formycin B uptake was measured in (A). Comparable results were obtained for the cells assayed for formycin B uptake in (B). All values represent means of data from three or four wells.

lation of metabolic products, or binding to cell surface components [24,36,37]. It therefore seems to reflect binding of the purine nucleosides to some intracellular component(s), perhaps enzymes involved in purine metabolism and/or nucleic acid [24].

In summary, our present study confirms the operation of the $Na^+$-dependent nucleoside transporter system in mouse spleen cells and has further characterized this transporter. The use of a metabolically inert nucleoside, formycin B, has allowed demonstration of the energy dependence of the transporter and its accumulation of the nucleoside against a concentration gradient. The specificity for uridine and purine nucleosides of the $Na^+$-dependent nucleoside transporter of spleen cells is similar to that of one of the $Na^+$-dependent transporters detected in mouse epithelial cells, but the second more broadly substrate-specific $Na^+$-dependent transporter present in the latter cells seems to be lacking in spleen cells.

The $Na^+$-dependent transporter clearly functions in conjunction with the $Na^+$-independent, facilitated nucleoside transporter found in most mammalian cells that have been investigated. T cell depletion studies have indicated that B and T lymphocytes similarly express both these nucleoside transport systems. Besides in $Na^+$- and energy-dependence, the two transport systems differ in kinetic properties. Both the $K_m$ and $V_{max}$ values of the facilitated transporter are at least 5–10-fold higher than those of the $Na^+$-dependent carrier. It follows that at high concentrations ($> K_m$ for both systems) nucleoside entry is primarily via the facilitated transporter. At low physiological concentrations ($\ll K_m$ for both systems), on the other hand, nucleosides enter the spleen cells at least as rapidly via the active than via the facilitated transport system. However, the physiological significance of the presence of the active carrier in lymphocytes is unclear, since even at low concentrations nucleosides enter the cells at a significant rate via the facilitated transport system. Furthermore, although a physiological function for an active nucleoside transporter can be readily envisioned for epithelial cells of the kidney and intestine, it is not apparent what role or advantage such carrier may have for lymphocytes.

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