Biochimica et Biophysica Acta, 981 (1989) 315–325
Elsevier

BBA 74436

Na^+-dependent and -independent transport of uridine and its phosphorylation in mouse spleen cells

Peter G.W. Plagemann and Clive Woffendin

Department of Microbiology, University of Minnesota, Minneapolis, MN (U.S.A.)

(Received 23 February 1989)

Key words: Nucleoside transport; Nucleoside salvage; Uridine; Phosphorylation; (Mouse spleen cell)

Rapid kinetic techniques were used to study the transport and salvage of uridine and other nucleosides in mouse spleen cells. Spleen cells express two nucleoside transport systems: (1) the non-concentrative, symmetrical, Na^+-independent transporter with broad substrate specificity, which has been found in all mammalian cells and is sensitive to inhibition by dipyridamole and nitrobenzylthioinosine; and (2) a Na^+-dependent nucleoside transport, which is specific for uridine and purine nucleosides and resistant to inhibition by dipyridamole and nitrobenzylthioinosine. The kinetic properties of the two transporters were determined by measuring uridine influx in ATP-depleted cells and dipyridamole-treated cells, respectively. The Michaelis-Menten constants for Na^+-independent and -dependent transport were about 40 and 200 μM, respectively, but the first-order rate constants were about the same for both transport systems. Nitrobenzylthioinosine-sensitivity of the facilitated nucleoside transporter correlated with the presence of about 10000 high-affinity ($K_d = 0.6$ nM) nitrobenzylthioinosine-binding sites per cell. The turnover number of the nitrobenzylthioinosine-sensitive nucleoside transporter was comparable to that of mouse P388 leukemia cells. The activation energy of this transporter was 20 kcal/mol. Entry of uridine via either of the transport routes was rapidly followed by its phosphorylation and conversion to UTP. The Michaelis-Menten constant for the in situ phosphorylation of uridine was about 50 μM and the first-order rate constants for phosphorylation and transport were about the same. The spleen cells also efficiently salvaged adenosine, adenine, and hypoxanthine, but not thymidine.

1. Introduction

Transport across the plasma membrane is an essential and first step in the salvage of nucleosides by mammalian cells [1–3]. In general, nucleoside transport in mammalian cells is facilitated by a symmetrical, non-concentrative carrier with broad substrate specificity [1–3]. In salvage, transport is coupled with simple, irreversible phosphorylation, which effectively activates the nucleosides and traps them intracellularly in phosphorylated form [2]. Two forms of facilitated nucleoside transport can be distinguished in mammalian cells on the basis of sensitivity to inhibition by nitrobenzylthioinosine (NBTI), but which differ little in other properties [3–5]. 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). Erythrocytes from some species express only NBTI-sensitive nucleoside transporters, whereas some cell lines express only NBTI-resistant, facilitated transporters [3–8]. But the majority of cells express both types in various proportions [3–8]. Both forms are about equally sensitive to inhibition by others inhibitors, such as dipyridamole, dilazep and lidoflazine, which are structurally unrelated to the nucleoside substrate of the transporter and to each other [5,9,10]. 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 [3,11].

In addition, choroid plexus and epithelial cells of the kidney and intestine have been shown to express a Na^+-dependent, concentrative nucleoside transport system [3,12–19]. Darnowski et al. [20] have reported that mouse splenocytes also concentratively accumulate uridine via a Na^+- and energy-dependent transporter that seems specific for uridine and perhaps purine nucleosides, and resistant to inhibition by 1 μM NBTI

Correspondence: P.G.W. Plagemann, Department of Microbiology, University of Minnesota Mayo Memorial Building, Box 196, Minneapolis, MN 55455, U.S.A.

or 10 μM dipyridamole. We have confirmed the operation of a Na^+-dependent carrier in mouse spleen cell populations but demonstrate that the apparent concentrative accumulation of uridine by these cells, observed by Darnowski et al. [20], reflects an intracellular formation of uracil nucleotides. Furthermore, we demonstrate that entry of uridine into spleen cells is mediated by both the Na^+-dependent carrier and the broadly specific, non-concentrative, facilitated nucleoside transporter that is sensitive to inhibition by dipyridamole and generally by NBTI. We have characterized both transport systems in mouse spleen cells and have assessed the contribution of each to uridine uptake by these cells.

Experimental procedures

Spleen cells. In most experiments spleens were provided as a by-product of the production and titration of lactate dehydrogenase-elevating virus (LDV) in 4–6-week-old Swiss mice [21]. However, in control experiments no significant differences in uridine transport and salvage 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 were prepared in phosphate-buffered saline (pH 7.4) containing 5 mM glucose (PBS-glucose) by gently scraping and squeezing the spleens with the rubber tip of the plunger of a 5-ml syringe on a wire mesh screen. The cells were washed once in PBS-glucose, and the cells from about 10 spleens were suspended in 0.5 ml of PBS-glucose. The erythrocytes were lyzed by mixing this suspension with 3 ml of water for 10 s and then adding 40 ml of PBS-glucose. Large cell debris and clumps were removed by passing the suspension through a wire screen. Then the cells were collected by centrifugation, washed at least once in PBS-glucose and suspended for transport assays, if not indicated otherwise, in PBS-glucose to $(3–8) \cdot 10^7$ cells/ml. For measuring Na^+-dependent and -independent nucleoside transport, the cells were washed once in 1.45 M choline chloride containing 5 mM Tris-HCl (pH 7.4, Tris-choline chloride) and suspended as indicated in 1.45 M NaCl, LiCl, KCl or choline chloride containing 5 mM Tris-HCl (pH 7.4). Typical yields varied between $5 \cdot 10^7$ and $1 \cdot 10^8$ cells/spleen and over 85% of the cells were not stained by Trypan blue.

Nucleoside uptake and transport measurements. Samples of cell suspension were supplemented with inhibitors and other substances as indicated in appropriate experiments. Then the time courses of uptake of radiolabeled nucleosides were measured at 25 °C using a dual syringe apparatus combined with manual sampling for longer time points (12–20 time points/time course) as described previously [1,3,5,22]. The concentration of radiolabeled nucleoside was kept constant in all samples of an experiment, while the specific radioactivity was altered by addition of unlabeled nucleoside. The mixing/sampling procedure involves 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). 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 or 1 M perchloric acid for rapid quenching of metabolism [23]. The acid layer was further processed and analyzed by ascending paper chromatography with a solvent composed of 30 ml 1 M ammonium acetate (pH 5) and 70 ml 95% ethanol (solvent 28) as described previously [24]. The cell-free supernatant fraction above the oil layer was immediately removed after centrifugation, mixed with trichloroacetic acid and selective samples were chromatographed with a solvent composed of 86 ml of *n*-butanol and 14 ml water (solvent 30) for the separation of uridine and uracil.

For measuring non-concentrative, facilitated uridine transport, conditions were selected that precluded the phosphorylation of a substantial proportion of the uridine during the transport assay. Either the cells were depleted beforehand of ATP by incubation in PBS or Tris-choline chloride containing 5 mM KCN and 5 mM iodoacetate at 37 °C [25], or the uptake of uridine was measured at a concentration that saturates uridine kinase and Na^+-dependent uridine transport (500 μM; see later). Under these conditions uridine equilibration across the membrane could be measured by the rapid kinetic technique described already for uptake measurements unimpeded by significant phosphorylation (see later). Transport was measured under zero-*trans* and equilibrium exchange conditions. For equilibrium exchange measurements, samples of cell suspension were incubated with an appropriate concentration of unlabeled uridine at 37 °C for 1 h and then the inward equilibration of radiolabeled uridine was measured at 25 °C. The data were evaluated by fitting appropriate integrated rate equations based on a simple carrier model assuming directional symmetry [1]. For estimation of the kinetic parameters of transport, six uridine concentrations were employed and the Michaelis-Menten parameters extracted by least-squares regression [1].

In one set of experiments we have also measured the efflux of uridine. In this case, samples of a suspension of about $4 \cdot 10^8$ spleen cells/ml were equilibrated with specified concentrations of radiolabeled uridine. Then efflux was measured by the rapid kinetic technique as described for influx, except that the suspension of preloaded cells was mixed at times intervals in a ratio of 1:7.4 (opposite to that in the influx assay) with PBS-glucose ('zero-*trans*' exit) or PBS-glucose containing

unlabeled uridine at the same concentration used for preloading the cells (outward equilibrium exchange). Exit measurements with this protocol are not strictly 'zero-*trans*', since the extracellular concentration of substrate at zero-time is not nil, but rather 12% of that inside the cells.

Equilibrium binding of NBTI. Equilibrium binding of NBTI was measured as described previously [5,24,26].

Materials. All radiolabeled nucleosides, nucleobases and NBTI were purchased from Moravek Biochemicals (Brea, CA) and diluted to the desired specific radioactivity with unlabeled substrates. Unlabeled nucleosides and nucleobases, lidoflazine and nifedipine were obtained from Sigma (St. Louis, MO) and unlabeled NBTI from Calbiochem (San Diego, CA). Dipyridamole (Persantin) and dilazep were gifts from Geigy Pharmaceuticals (Yonkers, NY) and Astra Werke (Frankfurt, F.R.G.), respectively.

Results

Fig. 1 illustrates representative time courses of uptake of 1 μM [^{3}H]uridine at 25°C by suspensions of mouse spleen cells that had been freed of erythrocytes. The intracellular concentration of uridine equivalents exceeded that in the medium within the first 5 s of incubation. The time course of uptake was biphasic just as observed in various lines of cultured mammalian cells with active uridine kinase [27,28]. The first phase reflected the equilibration of uridine across the plasma membrane, whereas the second linear uptake phase between 50 and 360 s reflected the accumulation of uracil nucleotides. Most of the intracellular radioactivity was associated with UTP (see inset of Fig. 1) just as is observed in cultured cell lines [27,28]. The data in Fig. 1 also illustrate the reproducibility of the uptake time courses. The S.E. of individual uptake time points was < 10% for replicate time course analyses with the same cell suspension. In addition, highly reproducible uptake time courses were obtained with cell suspensions prepared at different times from spleens harvested from either LDV-infected or uninfected mice (see subsequent figures).

The data in Fig. 2 are composed from two experiments (A and B) in which we compared the uptake of various concentrations of uridine ranging from 1 to 1500 μM. We have estimated the rates of zero-*trans* entry from the initial approximately linear portions of the uptake curves and the rates of intracellular phosphorylation from the second long-term linear uptake phases [27,28]. Michaelis-Menten analyses of the combined rates estimated in these and two other experiments yielded Michaelis-Menten constants for uridine transport and phosphorylation of 148 and 51 μM, respectively, and maximum velocities of 10 and 1.3 pmol/μl cell water per s, respectively. It is obvious that these values are rough estimates [1,2,27]. Nevertheless, these parameters fall in the range of values observed for

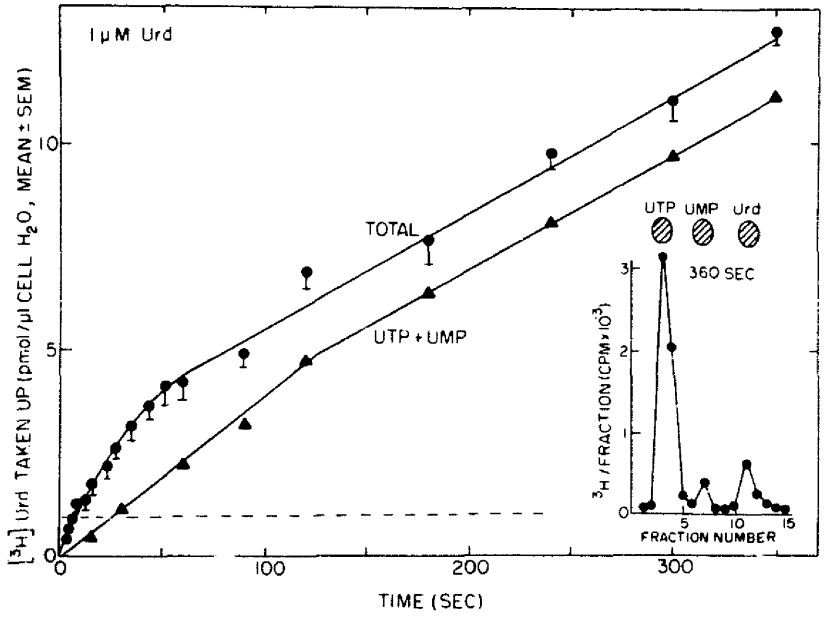

Fig. 1. Uptake and phosphorylation of uridine by mouse spleen cells. Four replicate time courses of uptake of 1 μM [^{3}H]uridine (618 cpm/μl) by a suspension of $2.1 \cdot 10^7$ cells/ml of PBS-glucose were determined at 25°C by rapid kinetic techniques as described under Experimental procedures. Each point is a mean ± S.E. ($n = 4$). The acid soluble pools were extracted with 1 M perchloric acid from replicate samples of cells and chromatographed with solvent 28. The chromatographic profile of pool components from 360 s-labeled cells is shown in the insert. The intracellular concentrations of uracil nucleotides (> 90% UTP; see inset) were calculated on the basis of the total amounts of uridine taken up and the chromatographic analyses. The broken line indicates the intracellular concentration of uridine-equivalents equal to that in the medium.

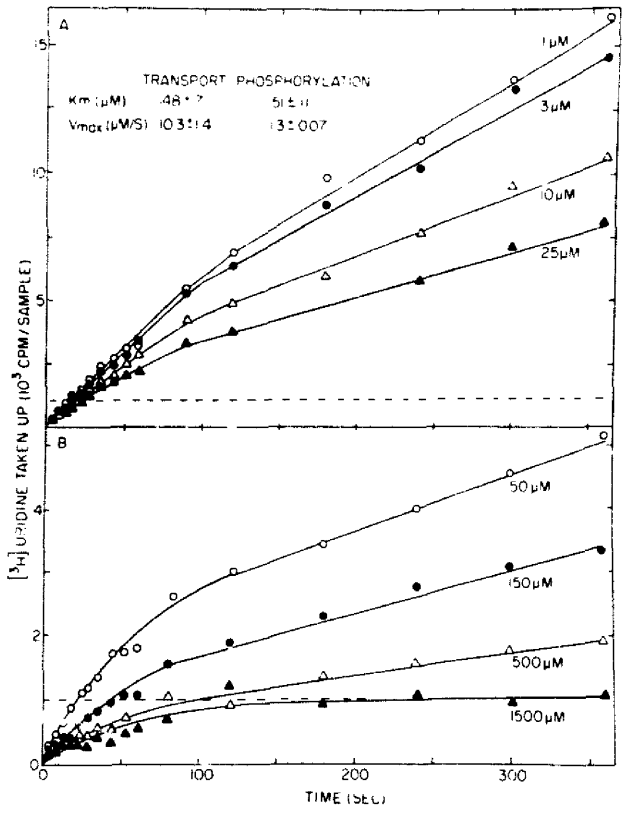

Fig. 2. Estimation of the kinetic parameters for total uridine transport and phosphorylation by untreated spleen cells. Time courses of uptake of the indicated concentrations of [^{3}H]uridine (about 550 cpm/μl, irrespective of concentration) by samples of suspensions of $2.8 \cdot 10^7$ spleen cells/ml of PBS-glucose were determined at 25°C by rapid kinetic techniques. Total transport velocities were estimated from the initial uptake time courses and rates of phosphorylation from the linear portions of the uptake curves between 100 and 360 s of incubation. The indicated kinetic parameters for transport and phosphorylation were estimated by fitting the Michaelis-Menten equation to these values. The broken lines indicate the intracellular concentration of radioactivity equal to that in the medium.

facilitated nucleoside transport and uridine phosphorylation in various types of cultured cells and the kinetic parameters for transport are similar to those measured in spleen cells incapable of uridine phosphorylation and active transport (see later).

Table I summarizes the concentrations of radiolabeled components in the acid soluble pools of spleen cells after incubation with various concentrations of uridine for 10 min. The data confirm the saturation of uridine phosphorylation and demonstrate that the proportion of uridine entering the cells that became phosphorylated during 10 min of incubation decreased progressively with increase in extracellular uridine concentration (see $[UTP]_i/[Urd]_e$). At all concentrations, regardless of the extent of its phosphorylation, the intracellular concentration of free uridine at steady state was slightly less than that in the medium (see $[Urd]_i/[Urd]_e$: Table I). However, in this connection technical problems in extracting uracil nucleotides in intact form from spleen cells need to be mentioned. For extracting the acid-soluble pools from [^{3}H]uridine-labeled spleen cells we initially collected the cells by centrifugation into a 0.5 M trichloroacetic acid layer at room temperature, a procedure we have successfully used to rapidly quench metabolism in various cell culture lines and human erythrocytes [23]. In the case of spleen cells, we recovered greater than equilibrium concentrations of free [^{3}H]uridine in the acid extracts but only when considerable phosphorylation occurred at lower uridine concentrations (1–100 μM). This high recovery of free [^{3}H]uridine from labeled cells was found to be an artifact resulting from degradation of labeled uracil nucleotides during extraction, since it was largely abolished by sedimenting the cells into a 1 M perchloric acid layer.

The temperature during acid extraction was also a factor influencing the recovery of the uracil nucleotides from spleen cells. For example, in the experiment illustrated in Fig. 3A, about 50% of the radioactivity extracted from cells after 6 min of incubation with 50 μM [^{3}H]uridine at 37°C was recovered in free uridine and approximately the same was the case after another 6 min of incubation at 37°C. However, when the cells after 6 min of incubation at 37°C were further incubated for another 6 min at 25°C, most of the intracellular radioactivity was recovered in uracil nucleotides. When the cells were incubated with [^{3}H]uridine at 25°C for 6 min or 12 min, most of the intracellular ^{3}H was recovered in UTP and the free [^{3}H]uridine in the

TABLE I

Intracellular concentrations of radiolabeled uracil nucleotides and free uridine after incubation of spleen cells with various concentrations of [^{3}H]uridine for 10 min at 25°C

Samples of a cell suspension were incubated with various concentrations of [^{3}H]uridine as described in the legend to Fig. 2. Then replicate samples of cells were collected by centrifugation through oil and analyzed for radioactivity (total uptake) or centrifuged through oil into a cold 1 M perchloric acid-sucrose layer, which was further processed and chromatographed with solvent 28 (see inset Fig. 1). The intracellular concentrations of UTP, UMP and uridine (Urd) were calculated on the basis of the total amounts of uridine taken up by the cells and the chromatographic analyses of their acid extracts. $[Urd]_e$ and $[Urd]_i$, extracellular and intracellular concentrations of uridine, respectively. $[UTP]_i$, intracellular concentration of UTP.

$[Urd]_e$ (μM)	Intracellular concn. (μM)			$[UTP]_i$	$[Urd]_i$
	UTP	UMP	Urd	$[Urd]_e$	$[Urd]_e$
1	16	5.1	0.2	16	0.20
3	42	13	0.8	14	0.37
10	125	41	7.8	13	0.78
25	212	75	17	8.9	0.72
50	325	105	39	6.5	0.78
150	322	89	97	2.2	0.65
500	400	189	310	0.8	0.62
1500	400	190	1150	0.38	0.76

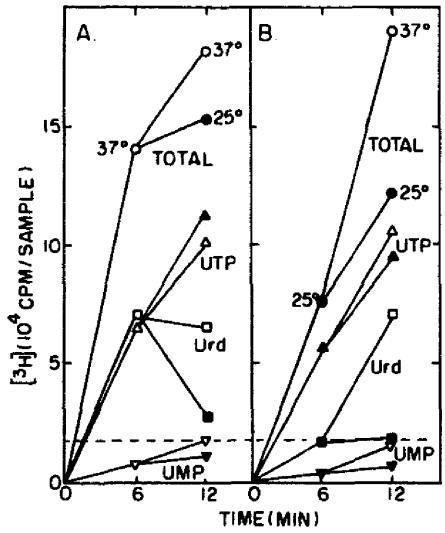

Fig. 3. Recovery of nucleotides in the acid soluble pool of spleen cells incubated with [^{3}H]uridine at 37°C and 25°C. Two samples of a suspension of $1.8 \cdot 10^8$ spleen cells/ml of PBS-glucose were supplemented with 50 μM [^{3}H]uridine (85 cpm/pmol) and first incubated for 6 min either at 37°C (A) or 25°C (B). Then each suspension was divided into two portions, which were further incubated at 37°C or 25°C for another 6 min. At 6 and 12 min of incubation, the cells from duplicate 0.5-ml of suspension were collected by centrifugation through oil and analyzed for radioactivity (total uptake; ○——○, ●——●). The cells from other replicate samples were centrifuged through oil into 1 M perchloric acid-sucrose layers (at room temperature) which were further processed and chromatographed with solvent 28 (see inset Fig. 1). The cellular radioactivity associated with UTP (△——△, ▲——▲), UMP (▽——▽, ▼——▼), and uridine (□——□, ■——■) was calculated on the basis of the total radioactivity taken up by the cells and the chromatographic analyses of their acid extracts. Empty symbols, 37°C. Solid symbols, 25°C. The broken lines indicate the intracellular radioactivity equal to that in the medium.

acid extract represented equilibrium levels (Fig. 3B). However, when, after 6 min of incubation at 25°C, the cells were incubated for another 6 min at 37°C, three times equilibrium levels of free [^{3}H]uridine were recovered in the acid extract. In later experiments, therefore, we collected labeled cells by centrifugation through oil into 1 M perchloric acid that had been cooled to 5°C (see Table I). Degradation of uracil nucleotides probably occurs during passage of the spleen cells into the acid layer, since after extraction with either trichloroacetic acid or perchloric acid the recovered uracil nucleotides were stable during storage of the acid extracts at −20°C for two weeks.

The results in Figs. 1 and 2 distinguish between rates of entry of uridine into the cells and its intracellular phosphorylation, but yield little information on the nature of uridine transporter(s) involved in salvage. The data in Fig. 4A shown that the rate of uptake of 5 μM uridine by the spleen cells was about twice as high in an isotonic NaCl medium than in isotonic LiCl, KCl or choline chloride media, whereas the rate of uptake of 500 μM uridine was about the same in all four media (Fig. 4B). The data suggest that at low uridine concentrations its entry was partially mediated by a Na^+-dependent transport system, whereas transport at 500 μM was largely Na^+-independent. The detection of the partial Na^+-dependency of uridine transport at low uridine concentration required prewashing the cells in a medium lacking Na^+, because the transporter was saturated at relatively low Na^+ concentrations. The Na^+ concentration yielding 50% of maximum Na^+-dependent velocity (K_{Na}) was 6.9 ± 1.2 mM (Fig. 4C), a value similar to that observed for active transport of formycin B in a rat epithelial cell line [16]. The data are consistent with a minimum Na^+ : uridine stoichiometry of 1 : 1. We have also analyzed the data in the Eadie-Scatchard plot of the Hill equation [29]:

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

where v_{Na} = velocity of Na^+-dependent uridine transport and n = Hill coefficient.

The linearity of the plot v_{Na}/K_{Na} vs. v_{Na} (Fig. 4C) indicates $n = 1$, that is a single Na^+-binding site on the Na^+-dependent uridine carrier.

The results in Fig. 4A suggest that, at 5 μM uridine, entry into spleen cells was mediated to about 50% by a Na^+-dependent transporter and to about 50% by Na^+-independent facilitated nucleoside transport, whereas at 500 μM uridine entry was mostly by the latter. These conclusions are supported by the finding that the uptake of 5 μM uridine was inhibited only about 50% by 20 μM dipyridamole (Fig. 5A), whereas the uptake of 500 μM uridine was inhibited about 93% (Fig. 5B). Na^+-dependent nucleoside transporters have been uniformly found to be resistant to inhibition by dipyridamole and NBTI [12–19], in contrast to the inhibitor-sensitive, facilitated nucleoside transporter [3]. In Fig. 5C the initial rate of transport of 500 μM uridine is plotted as a function of concentrations of dipyridamole and NBTI. About 65% of Na^+-independent facilitated uridine transport was inhibited by 1 μM NBTI (Fig. 5C) and thus represents NBTI-sensitive transport [3,5]. The IC_{50} values for the inhibition of this transporter in spleen cells by NBTI (about 10 nM) and dipyridamole (about 800 nM) are comparable to those observed for nucleoside transport in various lines of mouse cells [3,5,11]. The uptake of 500 μM uridine was also inhibited by other substances that have been found to inhibit facilitated nucleoside transport in a variety of mammalian cells [3,9–11], namely lidoflazine, dilazep and nifedipine. The initial rate of uptake of 500 μM uridine was inhibited about 50% by 40 μM lidoflazine and 5 μM dilazep and about 80% by 80 μM nifedipine.

The results in Figs. 4 and 5 also suggest that the Michaelis-Menten constant for Na^+-dependent uridine transport must be considerably lower than that for the

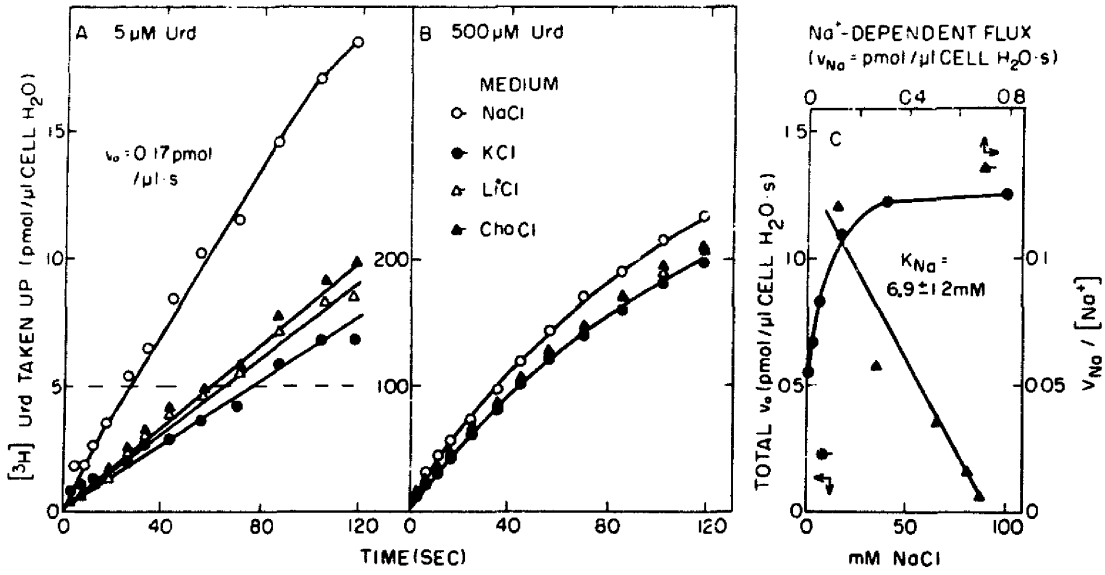

Fig. 4. Uptake of 5 μM (A) and 500 μM (B) uridine by spleen cells suspended in Tris-HCl buffered isotonic solutions of NaCl, KCl, LiCl and choline chloride and effect of the Na^+ concentration on uridine uptake (C). (A and B). Spleen cells were washed once in Tris-choline chloride and suspended to about $6 \cdot 10^7$ cells/ml in the different media. Then the uptake of 5 μM and 500 μM [^{3}H]uridine (500 cpm/μl, irrespective of concentration was measured at 25° C by rapid kinetic techniques. The broken line indicates the intracellular concentration of [^{3}H]uridine equal to that in the medium. In (C), Tris-choline chloride-washed spleen cells were suspended in Tris-KCl and samples of the suspension were mixed with Tris-NaCl to the indicated concentrations and a final density of about $6 \cdot 10^7$ cells/ml. Then the uptake of 50 μM [^{3}H]uridine (600 cpm/μl) was measured by rapid kinetic techniques. Initial velocities of uptake (v_0) were estimated graphically from the initial linear portions of the uptake curves. Na^+-dependent uridine 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 NaCl 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.

facilitated transporter. We have made use of the dipyridamole-resistance of the Na^+-dependent transport to determine its kinetic properties. Spleen cell populations were preincubated with 20 μM dipyridamole and then the uptake of 1, 2, 4, 8, 16, 32, 64 and 128 μM uridine was measured in a Na^+-containing medium at

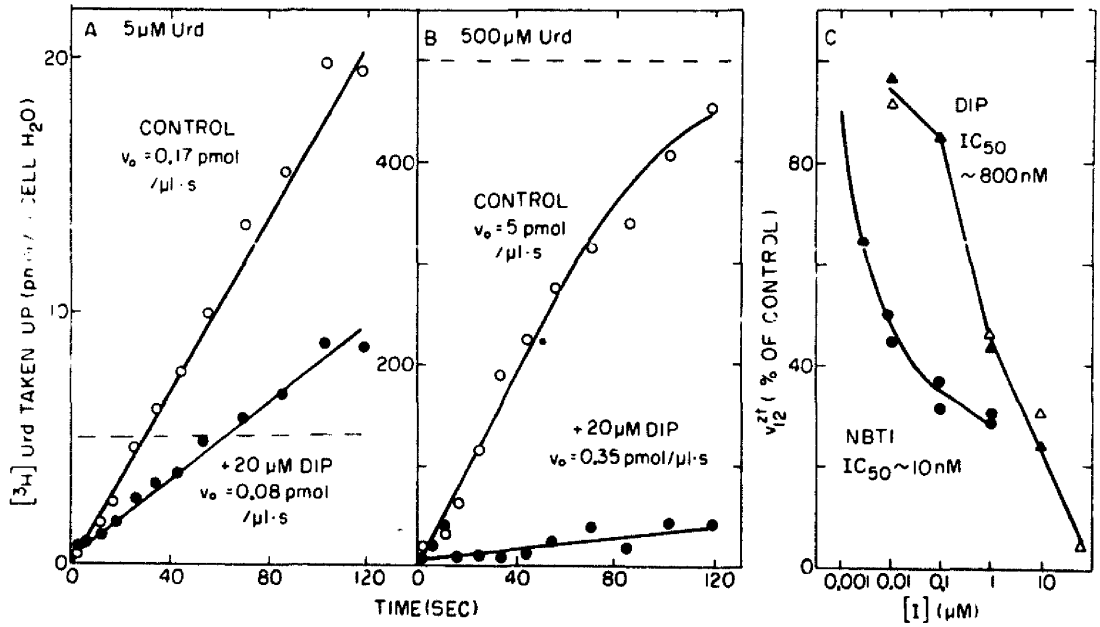

Fig. 5. Effects of dipyridamole and NBTI on uridine uptake by spleen cells. Samples of suspensions of about $5 \cdot 10^7$ cells/ml of PBS-glucose were supplemented with the indicated concentrations of dipyridamole or NBTI and incubated for at least 5 min at 25° C. Then the uptake of 5 μM (A) or 500 μM (B and C) [^{3}H]uridine (500 cpm/μl) was measured by rapid kinetic techniques in samples of the suspensions. Initial uptake velocities in A and B (v_0) and in C (v_{12}^{zt}) were calculated graphically and by integrated rate analysis, respectively. In A and B, the broken lines indicate the intracellular concentration of [^{3}H]uridine equal to that in the medium. The data in C are from several independent experiments, in one of which the cells were suspended in a LiCl (▲———▲) rather than a NaCl medium (△———△).

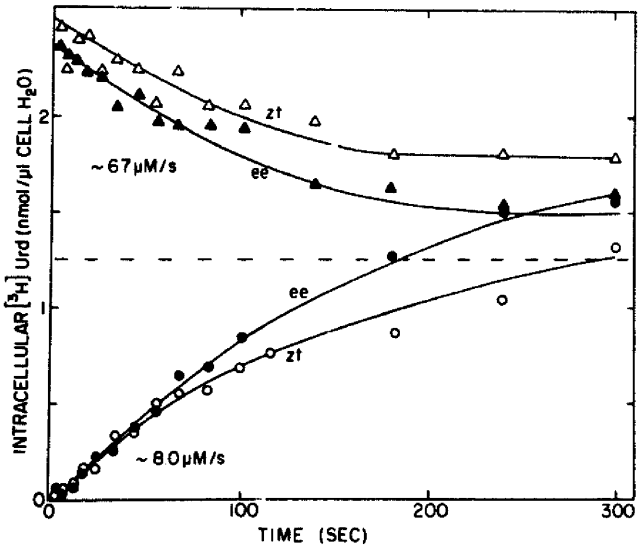

Fig. 6. Comparison of the zero-*trans* (zt) influx and efflux and inward and outward equilibrium exchange (ee) of uridine in mouse spleen cells at 25°C. The trans-membrane equilibration of 1.25 mM [^{3}H]uridine was measured in the four experimental protocols in samples of a suspension of $6.6 \cdot 10^7$ spleen cells/ml of PBS-glucose as described under Experimental procedures. The broken line indicates the intracellular concentration of uridine equivalents equal to that in the medium.

25°C. The initial velocities were estimated graphically from the initial linear portions of the uptake curves (see Fig. 5A). Fits of the Michaelis-Menten equation to the data yielded K_m and V_{max} values of 35 ± 5 µM and 1.46 ± 0.08 pmol/µl cell water per s, respectively, in one experiment, and 37 ± 2.3 µM and 0.9 ± 0.02 pmol/µl cell water per s, respectively, in another experiment. In both experiments the first order rate constant for Na^+-dependent transport was about 0.03 min^{-1}.

For assessing the general kinetic properties of the Na^+-independent, facilitated nucleoside transporter of spleen cells, we have compared the zero-*trans* influx and efflux and inward and outward equilibrium exchange of 1.25 mM uridine (Fig. 6). The results indicate that within experimental errors zero-*trans* influx and efflux were the same, which is consistent with directional symmetry of the carrier as is typical for this transporter in all cells that have been investigated [1,3]. Second, zero-*trans* flux and equilibrium exchange flux did not differ significantly, which indicates equal mobility of uridine-loaded and empty carrier [1]. This property is characteristic for the facilitated nucleoside transporters of cultured cells but not that of human and pig erythrocytes [1,3,30]. The time courses of uridine equilibration under equilibrium exchange and zero-*trans* conditions diverged at later time points (Fig. 6), but we have documented previously [31,32] that this behavior is entirely predictable by the simple carrier model for a carrier with these properties and at substrate concentrations approaching K_m and higher. The results depicted in Fig. 6 have been confirmed in a second experiment. After preloading the cells with [^{3}H]uridine (1 h, 37°C) for efflux measurements the intracellular concentration of uridine equivalents was about twice that in the medium (Fig. 6). This was due to phosphorylation and thus intracellular trapping of the uridine during preloading; 40–50% of the intracellular radioactivity of the preloaded cells was associated with uracil nucleotides (data not shown).

For direct measurements of facilitated uridine transport we depleted the spleen cells of ATP by incubation in PBS containing 5 mM KCN and 5 mM iodoacetate [25]. Spleen cells that had been preincubated in the presence of deoxycoformycin with 0.05 µM [^{3}H]adenosine at 37°C for 10 min, lost close to 90% of their labeled ATP during 30 min of incubation in this medium and after 1 h of incubation most of the resulting AMP had also become degraded to adenosine, which was mainly recovered in the culture fluid (data not shown). A 1-h treatment had no significant effect on the exclusion of trypan blue by the cells, but the volume of the treated cells was generally 10–30% larger than that of the untreated cells and the treatment caused considerable inactivation of the nucleoside transporter and perhaps an increase in general permeability of the plasma membrane. Inactivation of the transporter could have resulted from an interaction of the iodoacetate with essential sulfhydryl groups of the carrier [33].

We found, however, that a 10-min incubation at 37°C in the KCN-iodoacetate medium was sufficient to reduce the phosphorylation of 50 µM uridine > 90% and to abolish Na^+-dependent transport (data not shown) without affecting significantly the volume and membrane permeability of the cells and without apparent effect on facilitated uridine transport. [^{3}H]-Uridine accumulated in these cells only to equilibrium with the extracellular concentration (data not shown). Fig. 7 illustrates typical time courses of uptake of six concentrations of uridine (40–1280 µM) by 10-min-treated spleen cells. Uptake was clearly saturable and an integrated rate analysis of the pooled data yielded a Michaelis-Menten constant and maximum velocity of 187 ± 13 µM and 5.3 ± 1.3 pmol/µl cell water per s, respectively. After 3 min of incubation < 10% of the intracellular radioactivity was associated with uracil nucleotides, even at the lower uridine concentrations (data not shown). Kinetic parameters similar to those derived from the data in Fig. 7 were obtained in a second comparable experiment ($K = 207 \pm 25$ µM; $V = 6.5 \pm 0.3$ pmol/µl cell water per s). They fall within the ranges of values observed for facilitated uridine transport in various types of cultured cells [1,3,5]. The first-order rate constant for facilitated uridine (V/K) transport in the spleen cells (0.03 min^{-1}) was about the same as that for Na^+-dependent uridine transport.

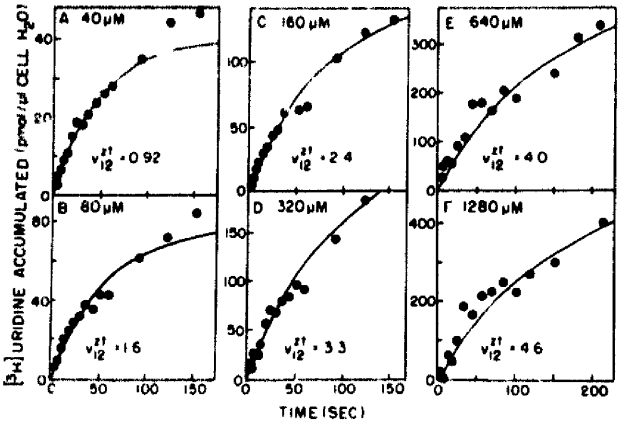

Fig. 7. Representative time course of *zero-trans* entry of uridine in ATP-depleted mouse spleen cells at 25°C. Spleen cells were suspended to $6 \cdot 10^7$ cells/ml in PBS containing 5 mM KCN and 5 mM iodoacetate. After 10 min of incubation at 37°C, the suspension was equilibrated at 25°C for 5 min and the zero-*trans* influx of uridine was measured at six [^{3}H]uridine concentrations ranging from 40 to 1280 μM (602 cpm/μl, irrespective of concentration) by rapid kinetic techniques. The pooled data were subjected to integrated rate analysis (Ref. 1; for kinetic parameters see text). The initial transport velocities (v_{12}^{zt}) shown were calculated from the fitted parameters for the given substrate concentrations as the slopes of the theoretical curves at $t = 0$ and are expressed as pmol/μl cell water per s.

To rule out potential effects of the ATP depletion treatment, we have assessed the substrate specificity and temperature characteristics of facilitated uridine transport and its sensitivity to inhibitors in untreated spleen cells using a high uridine concentration (500 μM). At this concentration, uridine phosphorylation is relatively slow compared to the rate of transport (Fig. 2, Table I). Furthermore, at this concentration uridine transport was largely Na^+-independent (Fig. 4B).

As discussed already (Fig. 5C), facilitated uridine transport in spleen cells measured at 500 μM uridine was as sensitive to inhibition by dipyridamole and NBTI as nucleoside transport in various mouse cell lines. Consistent with the uridine transport inhibition by nanomolar concentrations of NBTI, the spleen cells possessed high-affinity NBTI binding sites.

The mean $K_d \pm$ S.E. for NBTI binding by the spleen cells of 0.58 ± 0.14 nM calculated from results of six independent experiments was comparable to that for P388 mouse leukemia cells (0.49 ± 0.09 nM), which was analyzed in the same experiments. The mean number of NBTI binding sites/cell ± S.E. were 10000 ± 2000 and $2.4 \cdot 10^5$ for spleen cells and P388 cells, respectively. The total number of NBTI binding sites of different cell types cannot be directly compared if the size of the cells differs greatly as in the case of P388 and spleen cells. More appropriate would be to compare the surface density of NBTI binding sites, but such a comparison is made difficult by the lack of knowledge of the surface areas of the cells. Nevertheless, we have derived a rough estimate for mouse spleen cells and P388 leukemia cells by assuming both to represent smooth spheres with volumes of 1.3 μl (mean observed in the present experiments; $n = 6$) and 10 μl/10^7 cells [5], respectively, and $1 \cdot 10^4$ and $2 \cdot 10^5$ NBTI binding sites/cell, respectively. The calculation yielded $7 \cdot 10^{11}$ NBTI binding sites/mm^2 for spleen cells and $3.8 \cdot 10^{12}$/mm^2 for P388 cells. Considering the uncertainties involved in this calculation, this difference between P388 and spleen cells in surface density of NBTI sites agrees with a 5-fold greater maximum velocity of NBTI-sensitive, facilitated uridine transport of P388 than spleen cells (Ref. 5, and unpublished data) and suggests that the turnover numbers for the Na^+-independent, NBTI-sensitive, facilitated nucleoside carriers of the mouse lymphocytes and leukemia cells are comparable.

Facilitated uridine transport in spleen cells was highly temperature-dependent. An Arrhenius plot of the initial velocities of uptake of 500 μM uridine obtained at 5, 15, 25 and 35°C yielded an activation energy of about 20 kcal/mol, which is comparable to those reported for facilitated nucleoside transport in other types of cells [1,3,32,34].

The following experiments deal with the substrate specificities of the Na^+-dependent and -independent nucleoside carriers of spleen cells. The Na^+-independent transport of 100 μM [^{3}H]uridine was measured in KCl medium in the presence of various unlabeled nucleosides and nucleobases all at a concentration of 1 mM (Table II). All ribo- and deoxyribo-nucleosides strongly inhibited the uptake of radioactivity from 100 μM [^{3}H]uridine, but purine nucleosides were more effective than pyrimidine nucleosides. Purine nucleosides are also more efficient substrates than pyrimidine nucleosides for the facilitated nucleoside transporters of all other types of cells that have been investigated [1,3,10,32]. That adenosine, deoxycytidine, and thymidine inhibited uridine uptake at the level of influx and not phosphorylation is indicated by the fact that all four nucleosides are phosphorylated by different kinases [2]. In contrast to the nucleosides, adenine and hypoxanthine had no effect on uridine uptake in these spleen cells (Table II). The lack of effect of hypoxanthine is characteristic for facilitated nucleoside transport in various mouse cell lines, but contrasts with the finding that in some rat and hamster cell lines uridine and hypoxanthine seem to be transported by the same carrier [3,35].

The effect of various nucleosides and nucleobases on Na^+-dependent uridine transport was assessed in spleen cell populations that had been preincubated with 20 μM dipyridamole in order to block Na^+-independent facilitated nucleoside transport (see Fig. 5). The results in Table II show that the uptake of 5 μM [^{3}H]uridine by these dipyridamole-treated cells was strongly inhibited by 100 μM unlabeled uridine and all purine nucleosides

TABLE II

Effects of various nucleosides and nucleobases on Na^+-dependent and -independent transport of uridine

For measuring Na^+-dependent uridine transport, suspensions of about $5 \cdot 10^7$ spleen cells/ml of Tris-NaCl were supplemented with 20 μM dipyridamole and, after at least 2 min, the uptake of 5 μM [^{3}H]uridine (56 cpm/pmol) was measured in samples of the suspension by rapid kinetic techniques as illustrated in Fig. 5A. The indicated nucleosides and nucleobases were added to a final concentration of 100 μM simultaneously with the [^{3}H]uridine. For measuring Na^+-independent transport, spleen cells were washed in Tris-choline chloride and suspended to about $5 \cdot 10^7$ cells/ml in Tris-KCl. Then the uptake of 100 μM [^{3}H]uridine (2.5 cpm/pmol) was measured by rapid kinetic techniques in samples of the suspension as illustrated in Fig. 4A, in the presence of the indicated nucleosides and nucleobases at a final concentration of 1 mM. In each case the values are from two independent experiments (1 and 2). Initial uptake velocities were estimated graphically from the initial linear portions of the uptake curves. n.d., not determined.

Unlabeled competitor	Na^+-dependent transport			Na^+-independent transport		
	pmol/μl cell H_2O per s		inhibition (%)	pmol/μl cell H_2O per s		inhibition (%)
	Expt. 1	Expt. 2		Expt. 1	Expt. 2	
None	0.15	0.14	-	1.6	1.1	-
Uridine	0.049	0.065	61	0.4	0.22	77
Thymidine	0.15	n.d.	0	0.8	n.d.	50
Deoxycytidine	0.15	n.d.	0	n.d.	0.66	40
Cytidine	n.d.	0.14	0	n.d.	0.66	40
Formycin B	0.049	n.d.	67	n.d.	0.39	65
Adenosine	0.010	n.d.	93	0.2	n.d.	97
Inosine	n.d.	0.029	80	n.d.	0.18	84
Deoxyadenosine	n.d.	0.065	53	n.d.	n.d.	n.d.
Guanosine	n.d.	0.029	80	n.d.	0.22	80
Chloroadenosine	n.d.	0.029	80	n.d.	n.d.	n.d.
Hypoxanthine	0.15	n.d.	0	1.6	n.d.	0
Adenine	n.d.	0.14	0	n.d.	1.1	0

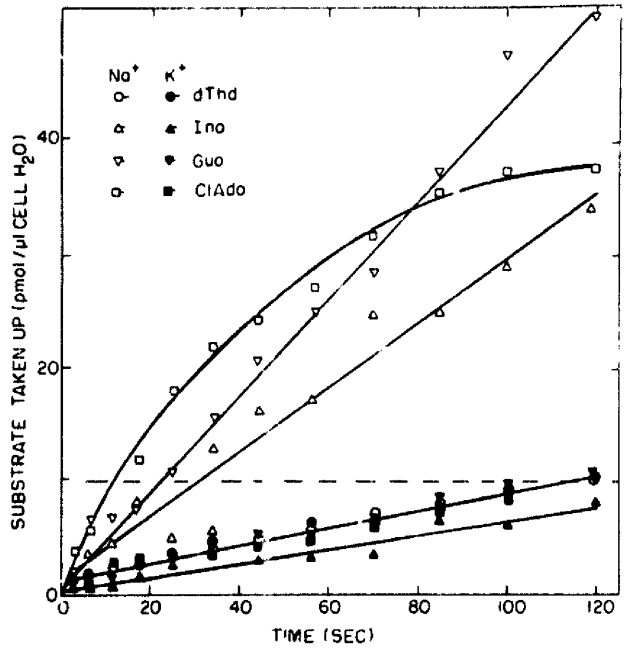

Fig. 8. Uptake of thymidine, inosine, guanosine and 2-chloroadenosine by mouse spleen cells in NaCl and KCl media. The uptake of 10 μM [^{3}H]thymidine (dThd; 13 cpm/pmol), [^{3}H]inosine (Ino; 38 cpm/pmol), [^{3}H]guanosine (Guo; 20 cpm/pmol) and [^{3}H]2-chloroadenosine (ClAdo; 36 cpm/pmol) was determined by rapid kinetic techniques in samples of suspensions of $3.3 \cdot 10^7$ Tris-choline-chloride-washed spleen cells/ml of the appropriate medium. The broken line indicates the intracellular concentration of substrate equal to that in the medium.

tested, but not be 100 μM unlabeled thymidine, deoxycytidine, or cytidine, or by 100 μM hypoxanthine or adenine. Darnowski et al. [20] observed that the uptake of 50 μM [^{3}H]uridine by spleen cells was inhibited to some extent by 500 μM thymidine, deoxyuridine, and cytidine, but these inhibitions were probably of the Na^+-independent carrier rather than the Na^+-dependent carrier, since total uptake was measured in these experiments.

The results in Table II suggest that uridine and purine nucleosides but not other pyrimidine nucleosides are substrates for the Na^+-dependent nucleoside transporter of spleen cells. This conclusion is supported by the data in Fig 8. The uptake of 5 μM inosine, guanosine and 2-chloroadenosine was several times more rapid in isotonic NaCl than KCl media, whereas the rate of uptake of 5 μM thymidine was equally low in both media. The uptake of 5 μM of 2'-deoxycytidine was also low and Na^+-independent (data not shown).

Discussion

We have confirmed the operation in mouse spleen cell populations of a Na^+-dependent nucleoside transport system that is specific for uridine and all purine ribo- and deoxyribonucleosides we have tested. However, we find that the Na^+-dependent uridine transporter in spleen cells functions in conjunction with

Na^+-independent, facilitated nucleoside transport. The facilitated nucleoside transporter of spleen cells exhibits kinetic and other properties comparable to the facilitated nucleoside transporter found in all mammalian cells that have been investigated, whereas Na^+-dependent, active nucleoside transporters have so far only been detected in renal and intestinal epithelial cells of various species, in rabbit choroid plexus, and in mouse spleen cells [12–20]. The substrate specificities of most of the active nucleoside transport systems have not been fully explored. They seem to be broad but more restricted than that of the facilitated transporter and there is evidence that two or more active nucleoside transporters with different substrate specificities exist [19]. The Na^+-dependent transporter of spleen cells seems to be identical in substrate specificity to one of the active nucleoside transporters described for mouse intestinal epithelial cells, which is specific for uridine and purine nucleosides [19]. The other active transporter observed in the latter cells, which also transports thymidine, seems not to be present in mouse spleen cells.

All active nucleoside transporters have in common that they are resistant to inhibition by dipyridamole and NBTI [12–20], two potent inhibitors of the facilitated nucleoside transporter [1,3]. This fact has been made use of in the present study to specifically measure the Na^+-dependent uridine transport in cell populations that exhibit both kinds of transport activity. Both the Michaelis-Menten constant and maximum velocity of the Na^+-dependent nucleoside transporter are only 1/5 of those of the facilitated nucleoside transporter. The fact that the first order rate constants for Na^+-dependent and -independent uridine transport in mouse spleen cells are about the same (0.03 min^{-1}) indicates that at low uridine concentrations ($\ll K_m$ for both systems) uridine entry into the cells occurs about equally via both routes. In contrast, at high uridine concentrations ($> K_m$) facilitated uridine transport is the primary route of entry.

The concentrative nature and energy dependence of Na^+-dependent uridine transport in spleen cells is difficult to assess at present, because of the rapid phosphorylation of low concentrations of uridine in these cells (Figs. 1 and 2). ATP depletion of the cells blocks uridine phosphorylation but it is difficult to evaluate to what extent reduced accumulation of uridine in form of UTP reflects decreased phosphorylation or inhibition of Na^+-dependent, concentrative accumulation. However, energy dependence of Na^+-dependent uridine transport is indicated by the lack of Na^+-dependence of uridine uptake in ATP-depleted cells and by the inhibition of Na^+-dependent uridine transport by incubation of the cells with ouabain [20].

When present at low concentrations, the uridine entering spleen cells via either Na^+-dependent or -independent transport becomes rapidly phosphorylated and trapped intracellularly mainly as UTP. The K_m for the in situ phosphorylation by the spleen cells is similar to the values reported for various mammalian cell lines [1,2]. As in the cell lines, the first order rate constant for in situ uridine phosphorylation by spleen cells is similar to those of the nucleoside transporters assuring efficient salvage of the substrate and trapping in form of nucleotides. The non-stimulated spleen cells also efficiently salvage adenosine, adenine and hypoxanthine, but not thymidine. Darnowski et al. [20] did not detect any phosphorylation of uridine in mouse spleen cells and concluded that it is concentratively accumulated by the cells. Since the overall time courses of uridine uptake by spleen cells we have observed (Figs. 1 and 2) are comparable to those reported by Darnowski et al. [20], we suggest that their failure to detect uridine phosphorylation may be an experimental artifact, perhaps related to the tendency of uracil nucleotides to become degraded to uridine during extraction from spleen cells, especially at higher temperatures. In the study of Darnowski et al. [20], the spleen cells were incubated with [^{3}H]uridine at 37 °C, then pelleted and homogenized before extraction with 1 M trioctylamine in Freon. No mention was made whether and at what stage the cells were extracted with acid and at what temperature [20]. We find that for a high recovery of uracil nucleotides from spleen cells, the cells needed to be pelleted directly into 1 M perchloric acid at lower temperatures. Centrifugation of the cells into 0.5 M trichloroacetic acid at room temperatures, especially from suspensions incubated with [^{3}H]uridine at 37 °C resulted in extensive degradation of the accumulated uracil nucleotides. The reason for this apparent instability of uracil nucleotides during extraction from spleen cells is unclear, but it could reflect a high nucleotidase activity in the cells.

Other potential factors are less likely to be responsible for the discrepancies between our results and those of Darnowski et al. [20]. For example, the presence of erythrocytes in the spleen cell suspension analyzed by Darnowski et al. [20] was probably not a factor, since we found uridine uptake by crude spleen cell suspensions to be indistinguishable from that observed with spleen cell suspensions that had been freed of erythrocytes (data not shown).

In this connection the heterogeneity of spleen cells needs to be considered. Spleen cell populations are composed mainly of B and T lymphocytes. T cells exceed B cells by about 50% and consist of distinct subpopulations [36]. The transport activity of spleen cell suspensions, therefore, must represent an average of those of these different cell types. However, the finding that the spleen cells behave kinetically with respect to facilitated uridine transport as a single population (Fig. 7) suggests that B and T lymphocytes possess comparable facilitated nucleoside transporters. This might be

expected, since it is the case for all cultured cell lines that have been investigated regardless of tissue origin [1,3,5]. Activated lymphocytes may differ in transport activity from quiescent cells, but did not play a significant role in our studies as indicated by the lack of thymidine salvage by our spleen cell populations (Fig. 8). The contribution of macrophages was also insignificant, since their removal by a 4-h absorption to tissue culture plates had no effect on the uridine uptake by our spleen cell suspension (data not shown).

On the other hand, whether NBTI-resistant and sensitive facilitated nucleoside transporters and the Na^+-dependent nucleoside transporter are preferentially or solely associated with a specific type of cell in the spleen cell population is unclear. Both NBTI-resistant and sensitive facilitated nucleoside transporters as well as Na^+-dependent and -independent transporters have previously been found to coexist in populations of cells of a single type and most likely in single cells [3,19]. However, this finding does not rule out that the Na^+-dependent transporter might not be restricted to a specific cell type, since active, Na^+-dependent transport systems have generally been found to be limited to certain cell types.

One approach to further access the cell distribution of the Na^+-dependent nucleoside transporter in spleen cell populations and its concentrative nature is to fractionate the cells into specific subpopulations and examine the uptake of a non-metabolizable nucleoside, such as formycin B, which is an effective substrate for the facilitated nucleoside transporter of mouse P388 cells and human erythrocytes [37] as well as for the active nucleoside transporter of intestinal epithelial cells [16,19].

Acknowledgments

The authors thank Laurie Erickson and John Erbe for excellent technical assistance, Robert M. Wohlhueter for editorial comments, and Dana Clark for typing the manuscript. This work was supported by USPHS research grant GM 24468 and training grant Ca 09138 (C.W.).

References

1 Plagemann, P.G.W. and Wohlhueter, R.M. (1980) Current Topics Membr. Transport 14, 225–330.
2 Wohlhueter, R.M. and Plagemann, P.G.W. (1980) Int. Rev. Cytol. 64, 171–240.
3 Plagemann, P.G.W., Wohlhueter, R.M. and Woffendin, C. (1988) Biochim. Biophys. Acta 947, 405–443.
4 Belt, J.A. (1983) Mol. Pharmacol. 24, 479–484.
5 Plagemann, P.G.W. and Wohlhueter, R.M. (1984) Biochim. Biophys Acta 773, 39–52.
6 Plagemann, P.G.W. and Wohlhueter, R.M. (1985) Biochim. Biophys. Acta 816, 387–395.
7 Woffendin, C. and Plagemann, P.G.W. (1987) Biochim. Biophys. Acta 903, 18–30.
8 Jarvis, S.M. and Young, J.D. (1986) J. Membr. Biol. 93, 1–10.
9 Plagemann, P.G.W. and Woffendin, C. (1987) Biochim. Biophys. Acta 928, 243–250.
10 Plagemann, P.G.W. and Kraupp, M. (1986) Biochem. Pharmacol. 35, 2559–2567.
11 Plagemann, P.G.W. and Woffendin, C. (1988) Biochim. Biophys. Acta 969, 1–8.
12 Spector, R. (1982) Arch. Biochem. Biophys. 216, 693–703.
13 Spector, R. and Huntoon, S. (1984) J. Neurochem. 42, 1048–1052.
14 LeHir, M. and Dubach, U.C. (1985) Pflüger's Arch. 404, 238–243.
15 LeHir, M. and Dubach, U.C. (1985) Eur. J. Clin. Invest. 15, 121–127.
16 Jacobs, E.S. and Paterson, A.R.P. (1986) Biochem. Biophys. Res. Commun. 14, 1028–1035.
17 Jarvis, S.M. (1987) in Topics and Perspectives in Adenosine Research (Gerlach, E. and Becker, B.F., eds.), pp. 102–117, Springer-Verlag, Berlin.
18 Schwenk, M., Hegazy, E. and Lopez del Piño, V. (1984) Biochim. Biophys. Acta 805, 370–374.
19 Vijayalakshmi, D. and Belt, J. (1988) J. Biol. Chem. 263, 19419–19423.
20 Darnowski, J.W., Holdridge, C. and Handschuhmacher, R.F. (1987) Cancer Res. 47, 2614–2619.
21 Cafruny, W.A., Chan, S.P.K., Harty, J.T., Yousefi, S., Kowalchyk, K., McDonald, D., Forman, B., Budweg, G. and Plagemann, P.G.W. (1986) Virus Res. 5, 357–375.
22 Wohlhueter, R.M., Marz, R., Graff, J.C. and Plagemann, P.G.W. (1978) Methods Cell Biol. 16, 211–236.
23 Plagemann, P.G.W. and Wohlhueter, R.M. (1983) J. Cell. Physiol. 116, 236–246.
24 Plagemann, P.G.W. (1971) J. Cell. Physiol. 77, 213–240.
25 Plagemann, P.G.W., Marz, R. and Erbe, J. (1976) J. Cell. Physiol. 89, 1–18.
26 Wohlhueter, R.M., Brown, W.E. and Plagemann, P.G.W. (1983) Biochim. Biophys. Acta 731, 168–176.
27 Plagemann, P.F.W. and Wohlhueter, R.M. (1983) in Regulatory Function of Adenosine (Berne, R.M., Rall, T.W. and Rubio, R., eds.), pp. 179–201, Martinus Nijhoff Publishers, Boston.
28 Plagemann, P.G.W., Marz, R. and Wohlhueter, R.M. (1978) J. Cell. Physiol. 97, 49–72.
29 Segel, I.H. (1975) in Enzyme Kinetics, pp. 360–369, John Wiley and Sons, New York.
30 Plagemann, P.G.W., Wohlhueter, R.M. and Erbe, J. (1982) J. Biol. Chem. 257, 12069–12074.
31 Plagemann, P.G.W. and Wohlhueter, R.M. (1982) Biochim. Biophys. Acta 688, 505–514.
32 Abidi, T.F., [illegible]mann, P.G.W., Woffendin, C. and Stollar, V. (1987) Biochim. Biophys. Acta 897, 431–444.
33 Plagemann, P.G.W., Wohlhueter, R.M. and Kraupp, M. (1985) Biochim. Biophys. Acta 817, 51–60.
34 Plagemann, P.G.W. and Wohlhueter, R.M. (1984) J. Biol. Chem. 259, 9024–9027.
35 Plagemann, P.G.W. and Wohlhueter, R.M. (1984) J. Membr. Biol. 81, 255–262.
36 Sell S. (1987) in Basic Immunology, Elsevier Science Publ. Co., New York.
37 Plagemann, P.G.W. and Woffendin, C. (1989) Biochim. Biophys. Acta 1010, 7–15.