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Uridine Transport in Basolateral Plasma Membrane Vesicles from Rat Liver

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Summary. The characteristics of uridine transport were studied in basolateral plasma membrane vesicles isolated from rat liver. Uridine was not metabolized under transport measurement conditions and was taken up into an osmotically active space with no significant binding of uridine to the membrane vesicles. Uridine uptake was sodium dependent, showing no significant stimulation by other monovalent cations. Kinetic analysis of the sodium-dependent component showed a single system with Michaelis-Menten kinetics. Parameter values were K_M 8.9 μM and V_{max} 0.57 pmol/mg prot/sec. Uridine transport proved to be electrogenic, since, firstly, the Hill plot of the kinetic data suggested a 1 uridine:1 Na^+ stoichiometry, secondly, valinomycin enhanced basal uridine uptake rates and, thirdly, the permeant nature of the Na^+ counterions determined uridine transport rates ($SCN^- > NO_3^- > Cl^- > SO_4^{2-}$). Other purines and pyrimidines *cis*-inhibited and *trans*-stimulated uridine uptake.

Key Words nucleoside transport · uridine · nitrobenzylthioinosine (NBMPR) · plasma membrane vesicles · rat liver

Introduction

Nucleosides have been shown to play different roles in cell physiology, most of them mediated via specific receptors [2, 4]. In liver parenchymal cells, adenosine can modulate several metabolic pathways. This nucleoside is thus able to stimulate ureagenesis [10], inhibit gluconeogenesis and even counteract the enhancement of basal gluconeogenesis triggered by glucagon [17, 21]. Interestingly, it has been suggested that these effects are not related to specific receptors but rather to transport processes. Nucleosides are taken up into mammalian cells through different transport systems. Facilitated diffusion is responsible for nucleoside uptake in many cell types. So far, it is well established that this kinetic activity can correspond to two different Na^+-independent agencies. One is sensitive to nitrobenzylthioinosine (NBMPR), dilazep and dipyridamole inhibition [1, 3, 31, 38], the other is insensitive to these compounds and has been described in a few cell systems [6, 7, 29]. Na^+-dependent saturable transport of nucleosides has been found in brush-border membrane vesicles from gut and kidney [15, 18, 36], murine splenocytes [28], freshly isolated guinea pig enterocytes [34] and cultured rat intestinal epithelial cells [13]. In a recent study using bovine renal brush-border membrane vesicles it has even been suggested that there might be two Na^+-dependent nucleoside carriers, differing in their relative specificities for purine and pyrimidine nucleosides [37]. One of these transport systems has been recently expressed in *Xenopus* oocytes after *m*RNA injection [16].

So far, little is known about nucleoside transport into liver parenchymal cells, despite the suggested role of adenosine carriers in mediating the metabolic effects of this nucleoside. Evidence in support of such a mechanism necessarily involves the prior characterization of nucleoside transport into liver parenchymal cells. An NBMPR-inhibitable carrier in plasma membrane vesicles from rat and guinea-pig liver has been suggested [39] on the basis of NBMPR binding analysis. Furthermore, Plagemann and Wohlhueter [30] described the existence of high-affinity NBMPR-binding sites in rat hepatocytes, which were associated with NBMPR-sensitive uridine transport. However, no kinetic characterization of nucleoside uptake by liver has been reported so far. Very recently, Holstege et al. [11] have addressed this specific point by using isolated perfused rat liver. In their experiments, uridine and adenosine were highly metabolized, and thus evidence for the presence of Na^+-dependent and independent carriers was indirect and no kinetic analysis could be performed. To circumvent these technical limitations we used basolateral plasma membrane vesicles isolated from rat liver to characterize, for the first time, a Na^+-dependent transport system, similar to that described in brush-border membrane vesicles from gut and kidney, which is the main agent respon-

sible for the uptake of nucleosides by liver parenchymal cells. Evidence is provided that this transport is electrogenic, might have broad specificity and shows Na^+/nucleoside stoichiometry of 1 : 1.

Materials and Methods

Preparation of Plasma Membrane Vesicles from Rat Liver

Plasma membrane vesicles were isolated from livers of adult Wistar rats fed a standard diet (UAR A04, Panlab, Barcelona, Spain). Animals had been kept under controlled conditions of temperature (22 ± 2°C), humidity (40–60%) and light (12 hr light-dark cycle). The method for plasma membrane isolation was that described by Pastor-Anglada et al. [23]. This protocol has been used before in our laboratory, and the characteristics of the plasma membrane vesicles have been published elsewhere [5, 8, 23, 32, 33]. The isolated vesicles were resuspended in 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM HEPES/KOH (pH 7.4), frozen in liquid nitrogen and stored at −40° until use. Uridine transport activity in these vesicles was stable for up to 2 months (*not shown*). The protein content of the plasma membrane preparations was measured according to Peterson et al. [24].

Uridine Transport

The transport of ^{3}H-uridine in plasma membrane vesicles was measured using a method adapted from Van Amelsvoort et al. [35] and described in Pastor-Anglada et al. [23] with some modifications. Briefly, plasma membrane preparations were mixed with transport media to give the following final concentrations: 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM HEPES/KOH (pH 7.4), either Na^+ or K^+ salts (both at 100 mM), except when sulphate salts were used (50 mM), and ^{3}H-uridine at the desired concentrations. The reaction was stopped at the indicated times by adding 1 ml of an ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, 0.2 mM $CaCl_2$, 10 mM HEPES/KOH at pH 7.4). Then, the whole content was quickly filtered through a nitrocellulose membrane (0.45 μm pore size) (Schleicher & Schuell, Germany) and washed with 4 ml of the ice-cold stop solution. As phloridzin has sometimes been used in stop buffers as an inhibitor of nucleoside efflux [18], previous experiments were carried out to test whether this compound should be used in our conditions. No significant efflux of uridine was detected without phloridzin for up to 1 min (*not shown*). Therefore, phloridzin was no longer added to our stop solutions.

For inhibition studies, the compound tested and radiolabeled uridine were added simultaneously, except for NBMPR and dipyridamole. These compounds were dissolved in dimethyl sulfoxide (DMSO) and preincubated with vesicles for 30 min before the uptake studies. The final concentration of DMSO in the incubation medium was 2% (vol/vol). Equivalent volumes of DMSO were added to control incubations. Kinetic studies were carried out at 3 sec, near initial velocity conditions, in the presence of a transmembrane gradient of either NaSCN or KSCN (both at 100 mM). All experiments were performed at 22°C. Subsequent modifications of the condition used are given in the legends to the figures and tables.

Uridine Metabolism

Membrane vesicles were incubated with 1 μM ^{3}H-uridine, 100 mM NaSCN, 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM HEPES/KOH, pH 7.4, for different times (3 sec, 15 sec, 6 min, 60 min) at room temperature. The whole content of the tube was centrifuged at 12,000 × *g* for 30 sec and the supernatant was discarded. The pellet was treated with 200 μl of 0.5% Triton-X100 and deproteinized with acetone. The tube was again centrifuged (12,000 × *g* for 5 min) and the protein-free extract was run on Whatman 3MM with *n*-butanol : acetone : NH_3 : H_2O (110 : 30 : 7.5 : 52.5) as eluent. The whole lane was divided into several sectors which were counted for radioactivity. More than 95% tritium was recovered in a single peak at an R_f value corresponding to uridine in the standard lane. An identical pattern was obtained when commercial ^{3}H-uridine was run.

Data Analysis

All transport experiments were carried out in triplicate in at least four different vesicle preparations. The analysis of sodium-dependent uridine uptake was carried out using the Enzfitter program (Elsevier, Biosoft, Cambridge). All data are expressed as mean ± SEM and statistical analysis was performed using the Student's *t* test.

Chemicals

(5,6-^{3}H) Uridine (40–50 Ci/mmol) was purchased from Amersham (Bucks, UK). NBMPR, valinomycin, dipyridamole and phloridzin were obtained from Sigma (St. Louis, MO). Percoll was purchased from Pharmacia (Milton Keynes, UK). All other reagents were of analytical grade.

Results

Time-Course of Uridine Influx

The time-course of 1 μM uridine uptake into plasma membrane vesicles from rat liver is shown in Fig. 1. Uridine was taken up by vesicles in a sodium-dependent manner, showing a clear overshoot over equilibrium values in the presence of an inward gradient of 100 mM NaSCN. The uptake was linear for 5 sec and, thus, further studies were performed at 3 sec. Equilibrium values in the presence of a transmembrane gradient of either 100 mM NaSCN or 100 mM KSCN were obtained at 6 min (about 0.3 pmol/mg prot).

Effect of Extravesicular Osmolarity on Uridine Uptake

Vesicles were incubated for 6 min in media of increasing osmolarities (from 0.41 to 1.54 osmoles), generated by adding increasing amounts of sucrose.

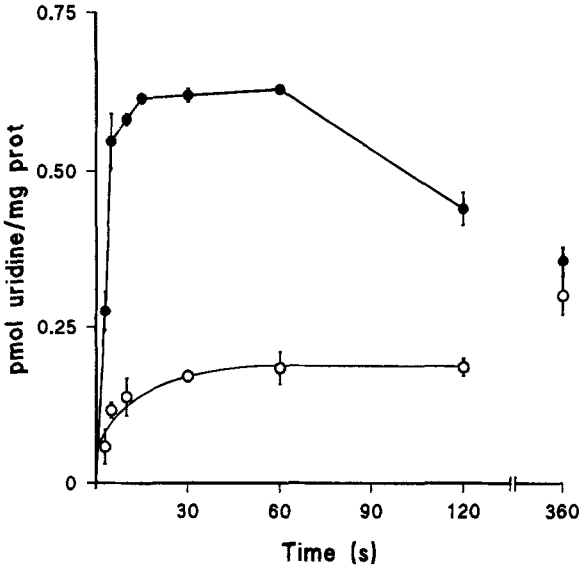

Fig. 1. Time-course of uridine uptake into plasma membrane vesicles from rat liver. Plasma membrane vesicles were incubated, as indicated in Materials and Methods, either with 100 mM NaSCN (●) or 100 mM KSCN (○) and 1 μM uridine. Uridine content was then monitored as a function of time. Results are the mean ± SEM of three observations made on pooled fractions from four independent plasma membrane preparations.

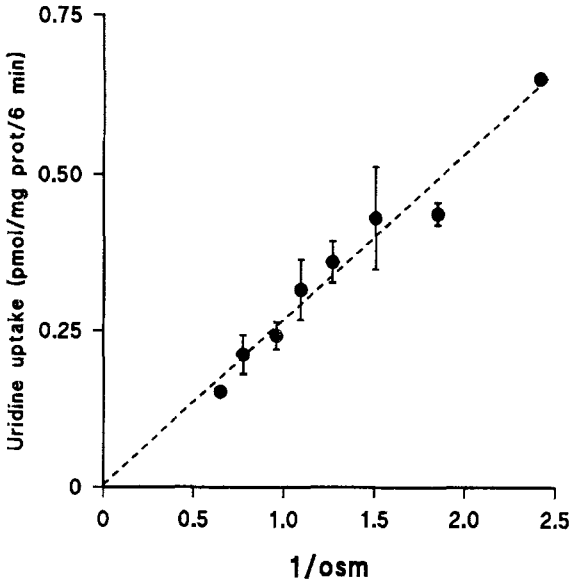

Fig. 2. Effect of the osmolarity of the medium on the uridine content of plasma membrane vesicles at equilibrium. Plasma membrane vesicles were incubated with NaSCN and 1 μM uridine for 6 min in the presence of increasing osmolarities (*see* Materials and Methods for details). Uridine content of the vesicles was then measured and plotted against the inverse of the osmolarity. The results are the mean ± SEM of three observations on pooled fractions from four independent plasma membrane preparations.

The uridine content at equilibrium was monitored and is shown in Fig. 2. As expected, the uridine content was inversely proportional to the extravesicular osmolarity. No significant binding of uridine to the membrane surface was detected, as suggested by the intercept to the vertical axis. These results show that uridine is retained in an osmotically sensitive space.

Cation Dependence of Uridine Uptake

Uridine (1 μM) uptake was studied at 3 sec in the presence of an inward gradient (100 mM) of the chloride salt of various monovalent cations. When K^+, Cs^+, Li^+, or choline were used, the transport rates were lower than with Na^+ (0.072 ± 0.009, 0.048 ± 0.008, 0.049 ± 0.008, 0.072 ± 0.007 and 0.126 ± 0.004 pmol/mg prot/3 sec, respectively). No significant differences were seen between the uptake rates measured using cations other than Na^+, so, Na^+-independent transport measurements were carried out with K^+ salts.

Kinetics of Uridine Transport

The concentration dependence of uridine uptake was studied by varying the extravesicular uridine concentration (0.25–50 μM) in the presence of a gradient of either 100 mM NaSCN or 100 mM KSCN. Figure 3 shows that uridine Na^+-dependent uptake was saturable following Michaelis-Menten kinetics. The uptake of uridine in the presence of KSCN appeared to be linear (r = 0.999) and could be attributed to a diffusion process with a first order rate constant value (k) of 0.078 sec^{-1}. The kinetic analysis by nonlinear regression of the data shown in Fig. 3 yielded a K_M value for the saturable component of uridine uptake of 8.9 μM and a V_{max} of 0.57 pmol/mg prot/sec. The Na^+-dependence of uridine uptake was further analyzed by measuring the sodium activation curve at a fixed uridine concentration (1 μM). Increases in the extravesicular NaSCN concentration resulted in higher transport rates following a hyperbolic pattern (Fig. 4*a*) and giving a K_{Na} of 22.5 mM. Isoosmolarity was maintained with choline chloride in all cases. When these data were analyzed by Hill plotting (Fig. 4*b*) a coefficient of 1.01 was obtained. This value is consistent with a stoichiometry of the Na^+-uridine coupled transport of 1 : 1.

Dependence on Membrane Potential

Assuming the stoichiometry indicated above, Na^+-nucleoside coupled transport would involve a net uptake of one positive charge into plasma membrane

vesicles, thus, uridine influx must be electrogenic. This possibility was assessed in two different ways. First, we performed a set of experiments in the presence of different Na^+ salts, in an attempt to determine to what extent the permeability of the anion tested could modify the Na^+-dependent uridine uptake. The results are shown in Table 1. Uptake rates decreased according to the following order $SCN^- > NO_3^- > Cl^- > SO_4^{2-}$, which is in agreement with the relative permeabilities of the anions through the plasma membrane. The uridine influx in the presence of a similar gradient of potassium salts was not modified in any case. In another set of experiments, 1 μM uridine transport was studied in the presence of a transmembrane gradient of either NaCl or KCl (both at 100 mM) in media supplemented with valinomycin (10 or 100 μM). The results obtained in the Na^+ media were 0.139 ± 0.025, 0.211 ± 0.006 ($P < 0.05$) and 0.211 ± 0.003 pmol/mg prot/3 sec ($P < 0.05$)

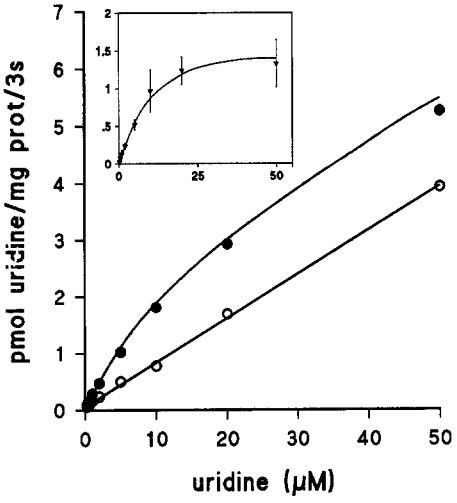

Fig. 3. Concentration dependence of uridine uptake. Plasma membrane vesicles were incubated in the presence of increasing uridine concentrations (0.25–50 μM), either with 100 mM NaSCN (●) or 100 mM KSCN (○). Uptake rates were then measured in initial velocity conditions (3 sec). The Na^+-dependent component of transport proved to be saturable and is shown in the box. The results are the mean ± SEM of the estimations in triplicate on four separate individual preparations.

Table 1 Effect of anions on uridine uptake

	Uridine uptake (pmol/mg prot/3 sec)	
	Na^+	K^+
SCN^-	0.239 ± 0.023	0.051 ± 0.003
NO_3^-	0.175 ± 0.010[a]	0.060 ± 0.006
Cl^-	0.140 ± 0.018[a]	0.054 ± 0.012
SO_4^{2-}	0.096 ± 0.015[b]	0.065 ± 0.015

Liver plasma membrane vesicles were incubated, as indicated in Materials and Methods, with 100 mM of different Na^+ and K^+ salts. Uridine (1 μM) uptake was monitored and the results are given as the mean ± SEM of four observations on pooled fractions from four independent plasma membrane vesicle preparations. Statistical comparisons versus the uptake rates determined in the SCN^- media were performed using the Student's t test. [a] $P < 0.05$; [b] $P < 0.01$.

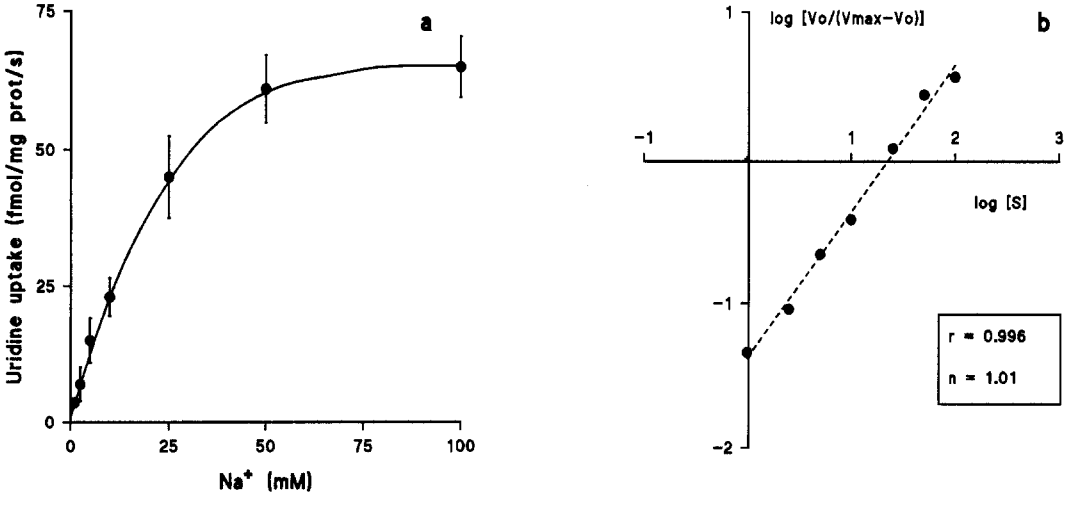

Fig. 4. Dependence of uridine uptake on extravesicular Na^+. Uridine (1 μM) uptake was monitored, in initial velocity conditions (3 sec), as a function of increasing concentrations of extravesicular Na^+ (SCN^- as counterion) (*a*). A Hill plot of these data is also shown in (*b*). The results are the mean ± SEM of four observations on pooled fractions from four independent plasma membrane vesicle preparations.

for 0, 10, and 100 μM valinomycin, respectively ($n = 4$). The values in the presence of KCl were 0.072 ± 0.007, 0.068 ± 0.013 and 0.079 ± 0.006 pmol/mg prot/3 sec for the same conditions indicated above ($n = 4$). These results, taken together, support the view that Na^+-dependent uridine transport is an electrogenic process, involving the net transfer of a positive charge.

Inhibition of Uridine Transport

To characterize uridine transport, inhibition by a variety of compounds (at 100 μM) of 1 μM uridine uptake was studied (Table 2). Adenosine, inosine and uridine totally inhibited the sodium-dependent uridine uptake into plasma membrane vesicles. Thymidine and cytidine inhibited about 60–70% of uridine uptake and some analogues like 2-deoxyuridine and Br-deoxy-uridine also inhibited around 70–90%. NBMPR and dipyridamole, inhibitors of facilitated diffusion nucleoside transport [29, 31] were unable to inhibit uridine Na^+-dependent transport in liver plasma membrane vesicles, even at high concentrations. Although nucleosides inhibit glucose transport into erythrocytes [14, 19], no effect on uridine transport in liver was produced either by glucose itself or by phloridzin, a Na^+-dependent glucose transport inhibitor. No significant effect of the inhibitors was found when uridine uptake was measured in the KSCN medium.

Table 2. Inhibition of uridine uptake

Inhibitor	Uridine uptake (pmol/mg prot/3 sec)	
	NaSCN	KSCN
None	0.196 ± 0.019	0.081 ± 0.021
Uridine	0.052 ± 0.014[c]	0.066 ± 0.012
Adenosine	0.058 ± 0.020[c]	0.060 ± 0.014
Thymidine	0.124 ± 0.025[a]	0.092 ± 0.020
Cytidine	0.129 ± 0.032	0.088 ± 0.021
Inosine	0.057 ± 0.025[c]	0.075 ± 0.003
2-deoxyuridine	0.114 ± 0.031[a]	0.066 ± 0.013
Br-deoxyuridine	0.101 ± 0.012[b]	0.087 ± 0.009
NBMPR	0.198 ± 0.021	0.092 ± 0.010
Dipyridamole	0.170 ± 0.020	0.085 ± 0.007
Phloridzin	0.181 ± 0.024	0.083 ± 0.006
Glucose	0.184 ± 0.018	0.079 ± 0.009

Uridine (1 μM) uptake into liver plasma membrane vesicles was monitored in a medium containing either 100 mM NaSCN or 100 mM KSCN. Potential inhibitors of uridine transport were added simultaneously at a concentration of 100 μM, except for NBMPR and dipyridamole, which were preincubated with vesicles for 30 min prior to the uptake measurements. Results are the mean ± SEM of 5–11 observations made on two sets of pooled fractions from three independent preparations. The statistical significance of the inhibition exerted by every compound on the uridine uptake rates in the absence of inhibitors, was determined by the Student's t test. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$.

Trans-Stimulation of Uridine Uptake by Other Nucleosides

In order to test whether the inhibitory effect of adenosine, cytidine and 2-deoxy-uridine, was caused by sharing the same carrier, *trans*-stimulation studies were performed. Vesicles were loaded with nucleosides by preincubating 72 μl of the plasma membrane preparations with 3 μl of the nucleoside solutions for 5 min, giving a final concentration of 5 μM. Then, 10 μl of the preloaded vesicles was mixed with 90 μl of the incubation medium containing 1 μM uridine and uptake was monitored at 3 sec. As shown in Table 3, there was clear stimulation of Na^+-dependent uridine influx when the compounds tested were inside the intravesicular space. Following approximately the same pattern of *cis*-inhibition studies, adenosine was the most potent agent causing *trans*-stimulation, 2-deoxy-uridine ranking second and cytidine, third. This is consistent with the hypothesis that all these nucleosides might be translocated by the same carrier system. No *trans*-stimulation was observed in the presence of 100 mM KSCN instead of 100 mM NaSCN, thus suggesting that no Na^+-independent carrier system is involved in nucleoside transport across the plasma membrane of the hepatocyte.

Table 3. *Trans*-stimulation of uridine uptake

	Uridine uptake (% of control)	
	NaSCN	KSCN
Control	100	100
Uridine	157 ± 15[b]	115 ± 34
Adenosine	150 ± 20[a]	90 ± 14
Cytidine	116 ± 4[a]	107 ± 30
2-deoxyuridine	136 ± 10[a]	109 ± 16

Liver plasma membrane vesicles were preloaded with different nucleosides at a concentration of 5 μM (*see* Results for details). Then, 1 μM uridine uptake was monitored, in either a NaSCN or a KSCN medium. Results are given as a percentage of the control uptake rates (0.267 ± 0.013 and 0.100 ± 0.048 pmol/mg prot/3 sec for the NaSCN and the KSCN media, respectively) and are the mean ± SEM of four observations on pooled fractions from four independent plasma membrane preparations. The statistical significance of the *trans* effects induced by the nucleosides was determined by the Student's t test. $^aP < 0.05$; $^bP < 0.01$.

Discussion

Our results clearly demonstrate that in basolateral plasma membranes from rat liver, uridine is mainly translocated by an electrogenic Na^+-dependent transport system, showing a stoichiometry of 1 Na^+ : 1 uridine. The dependence of uridine uptake on Na^+ and its corresponding K_{Na} have previously been reported in cultured cells [6, 13, 27, 29] and plasma membrane vesicles from cells of different origin [15, 18]. Furthermore, the K_M value for uridine uptake into our plasma membrane preparations is in the range of those reported elsewhere for other cell systems [15, 36] and close to the K_M reported for Na^+-dependent uridine uptake into brush border membrane vesicles from renal outer cortex [37].

From our data we cannot fully exclude the existence of a Na^+-independent saturable system for uridine uptake into plasma membrane vesicles from rat liver. Indeed, in the recent study by Holstege et al. [11], this agency has been reported, although the high compartmentation of the isolated rat liver and the measurement of nucleoside catabolites to determine uptake rates complicate the understanding of the NBMPR-sensitive occurrence. However, K_M for equilibrative nucleoside transport is around 300 μM [3, 31], which is much higher than the maximal substrate concentration we used (50 μM). This feature does not explain why NBMPR does not inhibit Na^+-independent uridine uptake by liver plasma membrane vesicles. Plagemann and Wohlhueter [30] described high-affinity binding of NBMPR to isolated rat hepatocytes causing significant inhibition of uridine transport. There is, however, a possibility that NBMPR-sensitive and insensitive components could be lost after membrane isolation, which should not, theoretically, affect the possibility of NBMPR binding to liver plasma membrane vesicles, as reported by others [39]. In any case, the Na^+-dependent agency should be the main site of nucleoside uptake by liver, because uridine and adenosine blood concentrations are around 1 μM [9, 12, 20, 22], which is much closer to the K_M reported in this study.

The inhibition pattern of the active uridine transport in basolateral plasma membrane vesicles from rat liver closely resembles that found in other tissues and species [15, 28, 36]. Very recently two different Na^+-dependent nucleoside transport systems have been reported, known as N1 (purine-preferring) and N2 (pyrimidine-preferring) [37]. Both systems seem to be able to translocate both uridine and adenosine. Our data do not exclude the possibility of either agency being present in plasma membrane vesicles from rat liver. The occurrence of a common pathway(s) for nucleoside transport in our cell system is suggested by the *trans*-stimulation studies, where we show that those nucleosides which *cis*-inhibit Na^+-dependent uridine uptake are equally able to stimulate uridine influx. In this context, Plagemann [25] recently reported that the low degree of inhibition of concentrative formycin B transport by other nucleosides is not associated with a direct uptake of the inhibitors. However, strong inhibitors like 2-deoxyadenosine are good substrates for the carrier. The lack of inhibition of Na^+-dependent uridine transport by either glucose or phloridzin supports our decision not to use the latter compound in the stop buffer solutions and does not agree with the evidence found in other cell types that glucose might share a common transport pathway with uridine [14, 19, 26, 31].

An important feature of our contribution is that we show that uridine, and probably most nucleosides, enter the liver parenchymal cells mainly by a Na^+-dependent NBMPR-insensitive mechanism. This raises the question as to whether the choice of NBMPR as an inhibitor of nucleoside transport to study the mechanism of nucleoside action on liver metabolism is propitious. Another feature of special interest is the probable physiological role of this carrier activity. So far, nothing is known about the modulation of this transport system, but we speculate that this agency could play a role in providing nucleosides for liver cells when the organ shows hyperplasia, as it does during development, pregnancy or regeneration after partial hepatectomy.

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