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Multiple sodium-dependent nucleoside transport systems in bovine renal brush-border membrane vesicles

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Na^+-dependent nucleoside transport was examined in bovine renal brush-border membrane vesicles. Two separate Na^+/nucleoside cotransporters were shown to be present: (1) a system specific for purine nucleosides and uridine, designated as the N1 carrier, and (2) an Na^+-dependent nucleoside transporter that accepts pyrimidine nucleosides, adenosine and analogues of adenosine, designated as the N2 system. Both systems exhibit a high affinity for nucleosides (apparent K_m values $\sim 10\ \mu M$), are insensitive to inhibition by facilitated-diffusion nucleoside transport inhibitors, are rheogenic and exhibit a high specificity for Na^+. Na^+ increases the affinity of the influx of guanosine and thymidine, nucleosides that serve as model permeants for the N1 and N2 nucleoside transporters respectively. The Na^+/nucleoside coupling stoichiometry is consistent with 1:1 for both carriers.

INTRODUCTION

The existence of Na^+-dependent re-absorptive uptake for nucleosides in the brush border of proximal tubules is well documented from renal clearance measurements [1] and transport studies on membrane vesicles derived from the rat [2–6]. The transport of uridine and adenosine in particular have been studied in some detail [2,4–6].

Adenosine uptake in rat renal brush-border membrane vesicles is inhibited by purine nucleosides such as inosine and guanosine with K_i values of less than 5 μM [4]. More recently, inhibition of Na^+-dependent uridine transport by rat renal brush-border membrane vesicles was investigated [6]. Pyrimidine nucleosides, adenosine and some analogues of adenosine were potent inhibitors of [^{3}H]uridine influx, suggesting that all of these nucleosides are substrates for the same system [6]. Inhibition of Na^+-dependent uridine influx by adenosine and thymidine was apparently competitive [6]. In contrast, inosine and guanosine were relatively poor inhibitors of Na^+-dependent uridine uptake, with apparent K_i values of 300 and 88 μM respectively, which failed to equate either to their K_m values for uptake or to their K_i values as inhibitors of Na^+-dependent adenosine transport (K_m and K_i values of 2–4 μM) [4,6]. These results suggest the presence of two Na^+-dependent nucleoside transporters with different but perhaps overlapping specificities in rat renal brush-border membrane vesicles. However, further studies on the detailed substrate specificity of the two proposed nucleoside carriers is required. In addition to Na^+-dependent uridine transport in rat renal brush-border membrane vesicles, K^+-dependent uridine transport, which appears to be distinct from the Na^+-coupled transport system(s), has also been demonstrated in rat renal brush-border membrane vesicles [5,6].

Relatively little work, however, has been carried out on nucleoside transport in renal brush-border membrane vesicles derived from species other than the rat. Thus in the present investigation we have extended our original observations to investigate in detail the transport of uridine, thymidine and guanosine by bovine renal brush-border membrane vesicles. In particular, the specificities of the nucleoside transport systems have been studied. Bovine vesicles can readily be prepared in large quantities, and preliminary studies indicated that they exhibit high rates of uridine transport [7]. Evidence is presented for the existence of at least two different Na^+-dependent nucleoside transport systems. Preliminary reports of some of these results have been published [7,8].

METHODS

Preparation of brush-border membrane vesicles

Fresh bovine kidneys were obtained from a local abattoir. Slices of the outer cortex were excised, finely chopped and homogenized in ice-cold 300 mM-mannitol/5 mM-EGTA/12 mM-Tris/HCl (pH 7.4) using a Polytron (setting 5) for 30 s. Homogenization was repeated four times. The brush-border membrane vesicles were then prepared by a two-step $MgCl_2$ precipitation method [9]. The purity of the preparation was assessed by measuring the specific activity of the brush-border marker enzyme, alkaline phosphatase. Alkaline phosphatase was enriched 8–12-fold with respect to the starting homogenate. The purified vesicles were suspended in 250 mM-sucrose/10 mM-Hepes/Tris (pH 7.4)/0.2 mM-$CaCl_2$, frozen in liquid N_2 and stored at −70 °C. Nucleoside transport activity in the vesicles was stable for at least 2 months. Protein determination was carried out by the method of Lowry *et al.* [10], using BSA as standard.

Nucleoside transport

The uptake of ^{3}H-labelled nucleosides (20 μCi/ml) at 22 °C was measured with a phloridizin stop-filtration method as detailed previously [5,6,11]. For inhibition studies the test compound and radiolabelled nucleoside were added simultaneously, except for the facilitated-diffusion nucleoside transport inhibitors nitrobenzylthioinosine (NBMPR), dilazep and dipyridamole. These compounds were preincubated with the brush-border membrane vesicles for 20 min before initiation of nucleoside uptake. Radioactivity retained on the filter in the absence of membrane vesicles was used as the blank value for the uptake

Abbreviations used: NBMPR, nitrobenzylthioinosine; IC_{50}, concentration causing 50% inhibition; K_{Na}, concentration of Na^+ that gave 50% of the maximum nucleoside transport rate.

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assay and was subtracted from measurements of nucleosides associated with the membrane vesicles in order to determine uptake rates.

Nucleoside metabolism

Metabolism of 10 μM-[^{3}H]uridine by bovine brush-border membrane vesicles was assessed by t.l.c. using the method previously described for rabbit membrane vesicles [11]. Similar procedures were used with 10 μM-[^{3}H]thymidine and [^{3}H]guanosine. For thymidine, silica-gel-coated plates (Eastman; 0.1 mm thick) impregnated with a fluorescent indicator were used, whereas guanosine metabolites were separated using cellulose-coated plates (Eastman; 0.16 mm thick) containing a fluorescent indicator. The solvent system was butan-l-ol saturated with water and glacial acetic acid/water/butan-l-ol (1:1:2, by vol.) for the silica-gel and cellulose plates respectively. The R_F values were 0.69, 0.68 and 0 for thymidine, thymine and thymidine nucleotides and 0.45, 0.35 and 0.18 for guanosine, guanine and GMP respectively. To separate thymidine from thymine, further t.l.c. was performed using the cellulose plates and the solvent 0.55 M-LiCl in 0.2% formic acid (R_F values of 0.72 and 0.82 for thymine and thymidine respectively). Radioactivity associated with these zones and with the rest of the lane (divided into 1 cm sections) was determined.

Data analysis

All transport experiments were carried out in triplicate and the errors shown in the Tables and Figures are S.D.S unless noted otherwise. Kinetic parameters for transport were determined by least-squares fits to the data, and points were weighted according to the inverse of their relative experimental errors. Analysis of inhibition studies was performed using the non-linear least-squares curve-fitting program LIGAND [12].

Chemicals

[5,6-^{3}H]Uridine (40–50 Ci/mmol) and [6-^{3}H]thymidine (25 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. [8-^{3}H]Guanosine (5 Ci/mmol) was purchased from ICN Radiochemicals, High Wycombe, Bucks., U.K. Phloridizin, valinomycin, NBMPR and dipyridamole were all from Sigma Chemical Co., Poole, Dorset, U.K. 3′-Azidothymidine, desciclovir and ganciclovir were gifts from Burroughs Wellcome, Research Triangle Park, NC, U.S.A. Dilazep was a gift from Hoffman–La Roche and Co., Basle, Switzerland. All other reagents were of analytical grade.

RESULTS

Uptake of nucleosides

The time course of the uptake of uridine, guanosine and thymidine (at 10 μM concentration) by bovine outer cortical brush-border membrane vesicles in the presence of inwardly directed electrochemical gradients of 100 mM-NaCl or -choline chloride is shown in Fig. 1. Uptake of all three nucleosides showed Na^+-gradient-dependent overshoots characteristic of Na^+-coupled transport. In the presence of inwardly directed Na^+ gradients, uptake of uridine, guanosine and thymidine was maximal between 20 and 60 s. Subsequently, accumulation of uridine and guanosine declined slowly, suggesting nucleoside efflux. After 60 min uptake of uridine and guanosine had reached equilibrium (14.3 ± 0.3 and 13.5 ± 4.6 pmol of uridine/mg of protein in the presence of NaCl and choline chloride respectively, and 14.6 ± 0.6 and 10.1 ± 0.2 pmol of guanosine/mg of protein in the presence of NaCl and choline chloride respectively). Control experiments confirmed that there was no significant metabolism of guanosine or uridine by the vesicles. In contrast, no decline in the apparent uptake of thymidine was observed (Fig. 1*c*). At 60 min the uptake of this nucleoside was still high (73.7 ± 2.6 pmol/mg of protein). Moreover, after 60 min the uptake of thymidine in the presence of choline chloride (34.5 ± 2.3 pmol/mg of protein) was also significantly above the predicted equilibrium value. These results suggest that thymidine is being metabolized to thymidine nucleotides which are unable to cross the cell membrane. T.l.c. demonstrated that this was indeed the case, with only 20% of the radioactivity comigrating with thymidine and the remaining 80% co-chromatographing with thymidine nucleotides after a 60 min incubation of bovine vesicles in the presence of NaCl. Thus the intravesicular concentration of [^{3}H]thymidine after 60 min was 14.7 ± 0.5 pmol/mg of protein, a value similar to that obtained with uridine and guanosine. Nevertheless, the rate of thymidine metabolism was low ($> 95\%$ of the radioactivity after a 10 s incubation co-chromatographed with thymidine on the t.l.c. plate). Thus thymidine metabolism did not interfere with estimates of initial rates of nucleoside uptake. Na^+-stimulated uptake of nucleosides was a linear function of time for the first 10 s of incubation. The uptake at 3–5 s was therefore taken to represent the initial rate of nucleoside influx.

In contrast with Na^+, inwardly directed gradients of choline chloride and KCl failed to stimulate the uptake of uridine, guanosine and thymidine, and no overshoot was observed (Figs. 1 and 2). Other cations were also completely ineffective

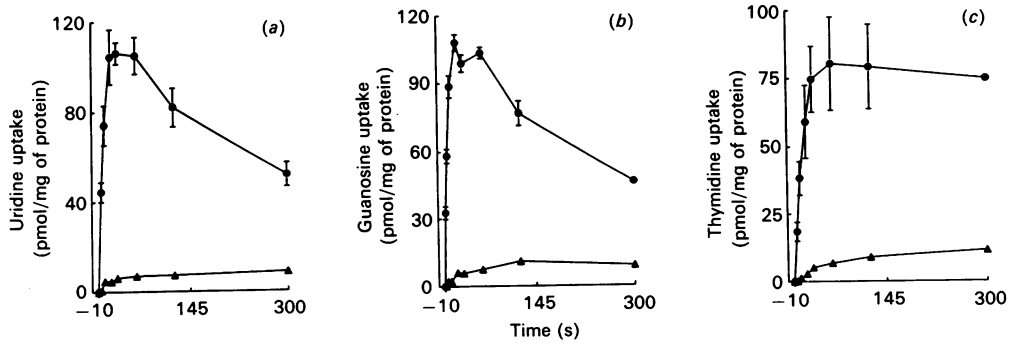

Fig. 1. Time course of the uptake of (*a*) uridine, (*b*) guanosine and (*c*) thymidine into bovine renal brush-border membrane vesicles

Vesicles were incubated in sucrose medium with a final concentration of 10 μM ^{3}H-labelled nucleoside in buffer containing either 100 mM-NaCl (●) or 100 mM-choline chloride (▲).

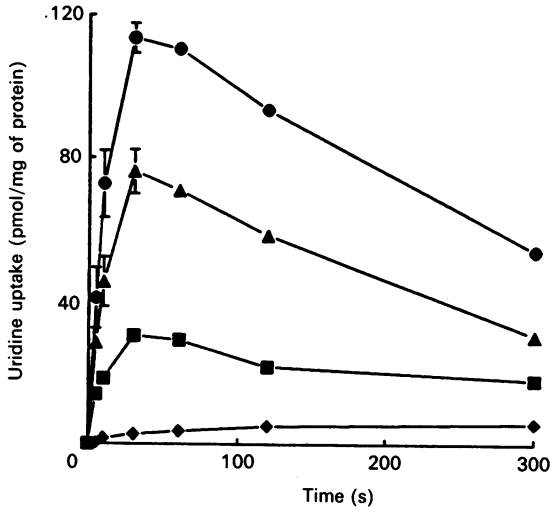

Fig. 2. Effect of anions on Na^+-dependent uridine uptake

The transport of 10 μM-[^{3}H]uridine was determined in the presence of inwardly directed gradients of 100 mM-NaCNS (●), 100 mM-$NaNO_3$ (▲), 50 mM-Na_2SO_4 (■) or 100 mM-KCl (◆).

(8.3±0.58, 0.27±0.03, 0.21±0.04, 0.27±0.02 and 0.37±0.04 pmol/s per mg of protein for 100 mM concentrations of chloride salts of Na^+, K^+, Li^+, Rb^+ and choline respectively at 10 μM-uridine). In further experiments the Na^+-dependent component of nucleoside transport was calculated as that in the presence of NaCl minus that in the presence of choline chloride.

The effect of anions on the rates of nucleoside uptake was investigated. Fig. 2 shows that the maximum rate of uridine transport (both the initial rate and magnitude of the transient overshoot) was obtained in the presence of thiocyanate. Nitrate and sulphate gave progressively lower rates, in agreement with the magnitude of their permeability coefficients [13]. Similar results were obtained for guanosine and thymidine transport (results not shown). These results confirm the electrogenicity of Na^+-dependent nucleoside transport, but differ from earlier findings for uridine transport by rat renal brush-border membrane vesicles, which had shown that nitrate exerted the maximum stimulatory effect on uridine transport [5,6].

Kinetics of nucleoside transport

The kinetic parameters of Na^+-dependent nucleoside influx were measured at different Na^+ concentrations and at nucleoside concentrations from 0 to 100 μM. The Na^+-independent rate (i.e. that estimated in the presence of choline chloride) was found to be linear. In contrast, the Na^+-dependent rate was saturable and conformed to simple Michaelis–Menten kinetics, and evidence of only a single component could be discerned by this method. Table 1(*a*) shows the K_m and $V_{max.}$ values obtained for uridine, guanosine and thymidine. The K_m values were on average 2-fold higher than those previously determined in rat kidney brush-border membrane vesicles [3,4,6]. However, the $V_{max.}$ values showed considerable variation compared with those obtained in rat kidney brush-border membrane vesicles. For uridine and thymidine the $V_{max.}$ values were 5–10-fold higher in the rat but, interestingly, for guanosine the $V_{max.}$ values were up to 3-fold higher in bovine vesicles [3,4,6]. The $V_{max.}$ values were variable from one batch of membranes to another. Table 1(*a*) also shows that as the Na^+ concentration was increased from 2 to 100 mM, the $V_{max.}$ for all three nucleosides remained relatively constant but the apparent K_m decreased by about 4-fold.

The Na^+-dependence of nucleoside (Figs. 1 and 2) was explored further by measuring the Na^+ activation curves at a fixed nucleoside concentration. Nucleoside uptake (10 μM) as a function of the extravesicular Na^+ concentration increased in a hyperbolic manner with Hill coefficients not significantly different from 1, consistent with a Na^+/nucleoside stoichiometry of 1:1. Clamping the membrane potential at zero with K^+ and valinomycin also resulted in a hyperbolic relationship between uridine influx and Na^+, with a Hill coefficient of 1.13 (Table 1*b*). The concentration of Na^+ that gave 50% of the maximum nucleoside transport rate (K_{Na}) was between 3 and 11 mM (see Table 1*b*).

Table 1. Kinetic constants of nucleoside transport

(*a*) Concentration-dependence of Na^+-dependent nucleoside influx was determined by incubating membrane vesicles with [^{3}H]-nucleoside (0–100 μM) in the presence of various concentrations of NaCl (0, 2, 5 and 100 mM). Choline chloride replaced NaCl isoosmotically to obtain the various Na^+ concentrations studied. Initial rates were calculated from the uptake at 3 s. The Na^+-dependent flux was taken as the rate in the presence of NaCl minus that in the presence of choline chloride for each substrate concentration. A similar experimental protocol was used to determine the effect of Na^+ on nucleoside influx (10 μM; *b*). In one experiment the membrane potential was clamped to zero by suspending the membrane vesicles in 300 mM-mannitol/100 mM-KSCN/0.2 mM-$CaCl_2$/10 mM-Hepes/Tris, pH 7.4, and preincubated with valinomycin at 12.5 μg/mg of protein for 1 h. The incubation medium contained (final concentrations) 100 mM-mannitol, 100 mM-KSCN, 10 mM-Hepes/Tris, pH 7.4, and various proportions of NaCl/choline chloride (final 100 mM). Kinetic parameters (K_m, $V_{max.}$, K_{Na} and Hill coefficient; means±S.E.M.) were determined by least-squares analysis.

(*a*)

Substrate	Expt. no.	Na^+ concn. (mM)	K_m (μM)	$V_{max.}$ (pmol/s per mg of protein)
Uridine	1	100	13±4	8.1±0.9
	1	5	49±11	11.6±2.0
	2	100	10±4	24.2±2.4
Guanosine	3	100	11±3	25.2±2.4
	3	5	24±5	25.8±2.4
	3	2	43±11	19.8±2.6
Thymidine	4	100	8±2	5.8±0.9
	4	5	22±9	5.9±1.2
	4	2	40±12	5.7±1.1
	5	100	5±1	10.4±1.2

(*b*)

Substrate	K_{Na} (mM)	Hill coefficient
Uridine	11.4±4.8	0.97
	2.9±0.5	1.02
	9.1±6.1*	1.13*
Guanosine	3.2±0.5	1.08
Thymidine	9.4±3.1	1.13

* In this experiment, the membrane potential was clamped at zero (see the legend).

Inhibition of uridine transport

Concentration-dependencies for inhibition of uridine transport by inosine, adenosine, thymidine, deoxyuridine and glucose are presented in Fig. 3. Adenosine and deoxyuridine inhibited uridine uptake in a simple monophasic manner with IC_{50} values (concentrations causing 50% inhibition) of 26 and 58 μM respectively. In contrast, inosine and thymidine gave biphasic inhibition profiles, indicating that about 50% of Na^+-dependent

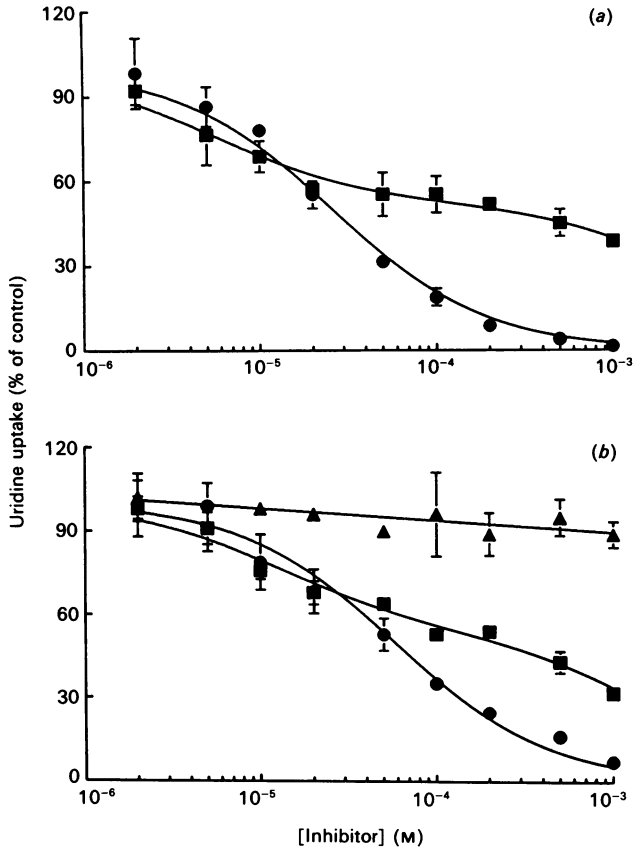

Fig. 3. Effect of nucleosides and glucose on Na^+-dependent uridine influx

Bovine brush-border membrane vesicles were incubated in sucrose medium containing (final concentrations) 100 mM-NaCl, 10 μM-[^{3}H]uridine and various concentrations of nucleoside or glucose for 5 s. Results are expressed as a percentage of the control flux (uptake in the presence of NaCl in the absence of inhibitor minus that in the presence of choline chloride). (*a*) ●, Adenosine; ■, inosine. (*b*) ●, Deoxyuridine; ■, thymidine; ▲, glucose.

uridine transport was of low sensitivity to inhibition by either of these two nucleosides. Guanosine, deoxyguanosine, deoxyinosine, cytidine and deoxycytidine also inhibited Na^+-dependent uridine transport in a biphasic manner (Table 2). However, deoxyadenosine and uridine totally inhibited [^{3}H]uridine transport (Table 2). D-Glucose produced little inhibition of uridine transport (Fig. 3), suggesting that the observed inhibition by nucleosides is not a consequence of dissipation of the inwardly directed Na^+ gradient. One possible interpretation of these data is that more than one Na^+-dependent nucleoside transport system is present in bovine renal brush-border membrane vesicles.

Further evidence that Na^+-dependent uridine transport is composed of at least two transport systems is demonstrated in Fig. 4. Uridine transport in the presence of 100 μM-thymidine plus increasing concentrations of cytidine failed to inhibit transport beyond the inhibition observed with 100 μM-thymidine alone. Replacement of cytidine with guanosine resulted in total inhibition of uridine transport (Fig. 4). Conversely, addition of guanosine (100 μM) to 100 μM-inosine resulted in no further inhibition of uridine transport beyond that observed in the absence of guanosine (results not shown). However, addition of cytidine (100 μM) together with 100 μM-inosine blocked uridine transport by 100% (results not shown). Similar additive inhibition experiments (results not shown) suggested that the nucleosides guanosine, deoxyguanosine, inosine and deoxyinosine are permeants for a common Na^+-dependent nucleoside carrier, and cytidine, deoxycytidine and thymidine are substrates for a separate Na^+-dependent nucleoside transporter. Adenosine, deoxyadenosine, uridine and deoxyuridine appear to be transported by both systems.

Inhibition of guanosine and thymidine transport

To explore further the substrate specificity of the two Na^+-dependent nucleoside transporters suggested to be present by the above inhibition experiments, we have compared the potency of a variety of nucleosides to inhibit guanosine and thymidine transport (Fig. 5). Uridine and adenosine were potent inhibitors of guanosine influx (IC_{50} values of 30 and 11 μM), whereas 2-chloroadenosine exhibited a low potency (IC_{50} 480 μM) (Fig. 5*a*). As predicted, cytidine failed to inhibit guanosine transport. In contrast, deoxycytidine inhibited thymidine influx (Fig. 5*b*). Adenosine and 2-chloroadenosine also blocked thymidine transport and, interestingly, 2-chloroadenosine exhibited a 10-fold greater potency as an inhibitor of thymidine uptake as compared with guanosine uptake. No inhibition of thymidine transport by guanosine was observed (Fig. 5*b*). The apparent K_i values determined from the inhibition profiles for these nucleosides and others are listed in Table 2. In general, purine nucleosides were potent inhibitors of guanosine influx, with the notable exception of 2-chloroadenosine and tubercidin, whereas pyrimidine nucleosides inhibited thymidine transport. Uridine, deoxyuridine and adenosine were equally effective at inhibiting guanosine and thymidine influx. Neither guanosine nor thymidine influx was inhibited by the nucleoside drugs 3′-azidothymidine, desciclovir and ganciclovir at concentrations of $\leqslant$ 500 μM. The facilitated-diffusion nucleoside transport inhibitors [14,15] NBMPR, dilazep and dipyridamole, at concentrations of $\leqslant$ 20 μM, were also without effect on Na^+-dependent nucleoside uptake.

To elucidate the characteristics of this inhibition by nucleosides, the effect of cytidine on thymidine uptake was examined (Fig. 6). The results, expressed in the form of a Dixon plot, demonstrate that cytidine is a competitive inhibitor of thymidine influx, with an apparent K_i value of 11 μM. Similar experiments showed that uridine is a competitive inhibitor of thymidine and guanosine transport (apparent K_i values of 4 and 13 μM respectively), and inosine also inhibited guanosine influx in a competitive manner (apparent K_i 5 μM).

DISCUSSION

The results presented in this paper have identified the presence of Na^+-dependent nucleoside transport in bovine renal brush-border membrane vesicles. Moreover, the results are consistent with the presence of at least two active nucleoside transporters in our preparation with distinct, but overlapping, substrate specificities.

The first system appears to have a preference for purine nucleosides, but this is not absolute. Thus [^{3}H]guanosine influx is inhibited by adenosine, deoxyguanosine, inosine, deoxyinosine and the C nucleoside analogue of inosine, formycin B. Inhibition by inosine is competitive, with an apparent K_i value 2-fold higher than the apparent K_m value for Na^+-dependent inosine influx in rat renal brush-border membrane vesicles [4]. The pyrimidine nucleosides thymidine, cytidine, deoxycytidine and 5-iodo-2′-deoxyuridine have little inhibitory effect on [^{3}H]guanosine influx. In contrast, uridine and deoxyuridine, and to a lesser extent 5-fluorouridine, are relatively potent inhibitors of [^{3}H]guanosine transport. Furthermore, the K_i for uridine inhibition of guanosine uptake and the K_m for uridine uptake are similar. We designate this Na^+-dependent transporter as the 'N1' system, using the

Table 2. Inhibition of nucleoside transport by nucleosides and other compounds

Values are the means ± S.E.M. of at least three separate experiments. Influx of [^{3}H]nucleoside was initiated by the addition of brush-border membrane vesicles to medium containing (final concentrations) 10 μM-[^{3}H]nucleoside, 100 mM-NaCl or -choline chloride and test compound at 20 °C. For dilazep, NBMPR and dipyridamole, vesicles were preincubated with these inhibitors before addition of [^{3}H]nucleoside. Initial rates of nucleoside influx were taken at 3–5 s. IC_{50} values were determined using the non-linear least-squares curve-fitting program LIGAND. K_i values were calculated from the equation $K_i = IC_{50}/(1 + L/K_m)$, where L is the concentration of [^{3}H]nucleoside and K_m values for uridine, guanosine and thymidine influx were taken as 11, 10 and 7.5 μM. N.D., not determined. [a]Inhibition curves were biphasic and consistent with two components; [b]no inhibition at 20 μM inhibitor concentration; [c]no inhibition at 500 μM inhibitor concentration.

Inhibition	Apparent K_i (μM)		
	Uridine influx	Thymidine influx	Guanosine influx
Adenosine	14±0.2	12±4	15±4
Deoxyadenosine	32±11	16±8	44±11
Guanosine	9±2; > 1000[a]	0[c]	N.D.
Deoxyguanosine	9±1; > 1000[a]	0[c]	23±2
Inosine	11±3; > 1000[a]	0[c]	7±3
Deoxyinosine	13±4; > 1000[a]	0[c]	12±2
2-Chloroadenosine	N.D.	18±5	180±20
Tubercidin	N.D.	23±16	0[c]
Formycin B	N.D.	0[c]	36±12
Uridine	18±2	22±7	23±6
Deoxyuridine	33±2	30±8	31±6
Thymidine	20±6; > 1000[a]	13±2	0[c]
Cytidine	8±3; > 1000[a]	9±1	0[c]
Deoxycytidine	4±1.5; > 1000[a]	4±1	0[c]
5-Fluorouridine	28(n = 1)	33±8	100±6
5-Iodo-2′-deoxyuridine	N.D.	14±6	0[c]
NBMPR	0[b]	0[b]	0[b]
Dipyridamole	0[b]	0[b]	0[b]
Dilazep	0[b]	0[b]	0[b]
3′-Azidothymidine	N.D.	0[c]	0[c]
Desciclovir	N.D.	0[c]	0[c]
Ganciclovir	N.D.	0[c]	0[c]

capital letter format adopted for amino acid carriers to indicate Na^+-dependency.

The second system accepts pyrimidine nucleosides, adenosine and analogues of adenosine, and we refer to it as the 'N2' transporter. 2-Chloroadenosine and tubercidin show at least a 10-fold higher affinity for the N2 transporter than for the N1 carrier (K_i values of 18±5 and 180±20 for 2-chloroadenosine, and of 23±16% and less than 10% inhibition at 500 μM for tubercidin inhibition of [^{3}H]thymidine and [^{3}H]guanosine influx respectively). Inhibition of [^{3}H]thymidine influx by cytidine and uridine is competitive.

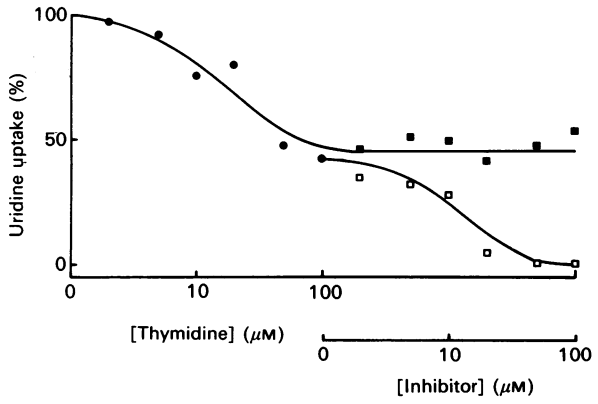

Fig. 4. Inhibition of Na^+-dependent uridine influx

Na^+-dependent uridine influx (final concentration 10 μM; 3 s flux) was measured in the presence of increasing concentrations of thymidine (●). Thymidine at 100 μM was further supplemented with increasing concentrations of guanosine (□) or cytidine (■). Results are expressed as a percentage of the control flux (uptake in the presence of 100 mM-NaCl minus that in the presence of 100 mM-choline chloride.

The above inhibition studies suggest that uridine and adenosine are permeants for both active nucleoside transport systems. Concentration-dependence analysis of uridine transport revealed only a single system, but inhibition studies (Figs. 3 and 4 and Table 2) demonstrated the presence of two Na^+-dependent uridine uptake mechanisms with substrate specificities identical with that revealed by the transport studies with [^{3}H]guanosine and [^{3}H]thymidine. Thus all of the inhibition data are entirely consistent with the presence of both N1 and N2 transporters in bovine renal brush-border membrane vesicles. It should be noted that the definitions of the substrate specificities of the two transporters are based mainly on inhibition studies. An earlier study [16] reported that, although tubercidin inhibited Na^+-dependent thymidine transport in mouse intestinal cells, no Na^+-dependent tubercidin (1 μM) influx was apparent in these cells. Thus it is possible that some of the present inhibition observed with nucleosides is via a non-competitive mechanism. However, in all cases examined the inhibition profiles were competitive. Furthermore, recent studies with rat renal membrane vesicles have also suggested the existence of separate carriers for purine and pyrimidine nucleosides based on trans-stimulation experiments, although the discrimination between the two classes of nucleosides is not absolute [17].

The substrate specificities of the active nucleoside transporters

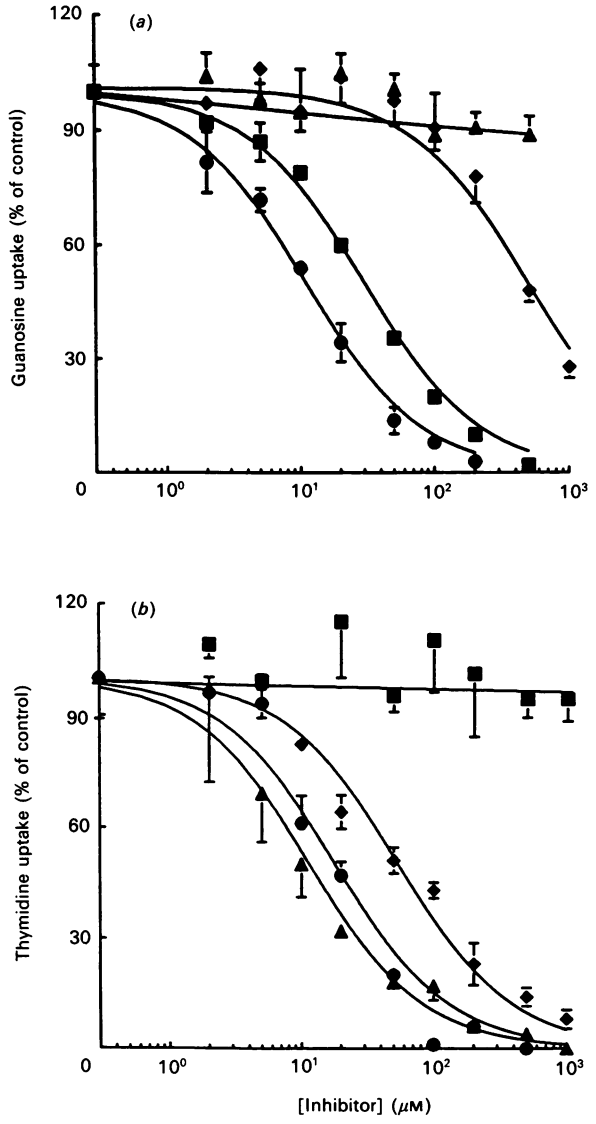

Fig. 5. Inhibition of Na^+-dependent guanosine and thymidine uptake

(*a*) The initial rate of guanosine uptake (10 μM final concentration; 3 s flux) in the presence of cytidine (▲), 2-chloroadenosine (◆), uridine (■) and adenosine (●) was determined in sucrose medium containing 100 mM-NaCl. Na^+-independent guanosine uptake in the absence of inhibitor was subtracted for each inhibitor concentration and the results are expressed as a percentage of the Na^+-dependent control flux. (*b*) Thymidine uptake (10 μM final concentration) in the presence of guanosine (■), 2-chloroadenosine (◆), deoxycytidine (▲) and adenosine (●). All other experimental details were as in (*a*).

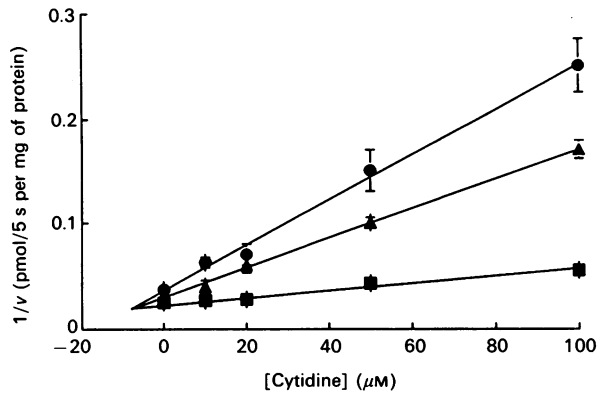

Fig. 6. Dixon plot for inhibition of thymidine influx in bovine renal brush-border membrane vesicles by cytidine

The reciprocals of the initial rate of [^{3}H]thymidine influx at 32 μM (■), 12 μM (▲) and 6 μM (●) are plotted against the respective concentration of inhibitor (cytidine). Data shown have been corrected for Na^+-independent thymidine influx. The apparent K_i value is 11 μM.

in bovine renal brush-border membrane vesicles show similarities to and differences from those in other tissues and species. Mouse intestinal epithelial cells appear to possess two Na^+-dependent nucleoside transporters with substrate specificities similar to those determined in the present study [16]. Na^+-dependent uridine transport in guinea-pig enterocytes and rabbit intestinal brush-border membrane vesicles is inhibited by purine and pyrimidine nucleosides [18,19]. In contrast, Na^+-dependent uridine transport by murine splenocytes is inhibited by purine nucleosides, but not by other pyrimidine nucleosides [20,21]. Furthermore, no Na^+-dependent thymidine or deoxycytidine uptake is detected [20,21]. These results suggest that murine splenocytes only possess the N1 nucleoside transporter. However, one possible difference between the splenocyte and renal N1 transporter is the finding that 2-chloroadenosine appears to be as potent as inosine and guanosine in inhibiting Na^+-dependent uridine transport in mouse splenocytes, although a full range of inhibitor concentrations was not tested [21]. In renal vesicles, 2-chloroadenosine is a more potent inhibitor of the N2 system. One property that all active nucleoside transporters have in common is their resistance to inhibition by NBMPR, dipyridamole and to a lesser extent dilazep (Table 2) [3,6,16,19–22], which are potent inhibitors of facilitated-diffusion nucleoside transport [14,15].

The kinetic properties of the two Na^+-dependent nucleoside transporters in bovine renal membrane vesicles, as revealed from the influx of [^{3}H]guanosine and [^{3}H]thymidine used as model permeants for the N1 and N2 carriers respectively, show many similarities. Both systems obey Michaelis–Menten kinetics, with K_m values for influx at 100 mM-Na^+ and 22 °C of approx. 10 μM, a value 10–20-fold lower than the K_m values for the facilitated-diffusion nucleoside transporters [14,15]. Increasing the external Na^+ concentration results in an increase in the affinity of the nucleoside for influx in both systems. These data suggest that Na^+ binds first to increase the affinity of the transporter for the nucleoside. The Na^+/nucleoside coupling stoichiometry for both transporters, determined by the activation method, was consistent with a ratio of 1:1, with a K_{Na} of 3–11 mM. Similar K_{Na} values have been reported for Na^+-dependent uridine and formycin B influx in mouse spleen cells and rat cultured IEC-6 intestinal cells respectively [21,22]. These apparent K_{Na} values for Na^+ activation of nucleoside transport are much lower than those observed for other Na^+-dependent systems, e.g. 35 and 57 mM for the rabbit intestinal and outer medullary renal glucose transporter respectively, and 21 mM for the rabbit renal proline carrier [23–25]. Finally, nucleoside transport by both systems is stimulated by lipid-soluble anions, consistent with the view that transport is rheogenic, involving a net transfer of positive charge. No evidence for a direct effect of anions on nucleoside influx was obtained, as has been previously reported for uridine transport by rat renal brush-border membrane vesicles [6].

In summary, the present results strongly indicate the presence of two distinct Na^+-dependent nucleoside transporters in bovine renal brush-border membrane vesicles. These systems, the N1 and N2 nucleoside cotransporters, also appear to be present in other selected tissues, either separately or together [16,20,21]. The physiological significance of why adenosine and uridine are

permeants for both active nucleoside transporters is unknown. In the case of adenosine, this may relate to the multitude of functions ascribed to this nucleoside [26].

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