Nucleoside transport in rat cerebral-cortical synaptosomes

Evidence for two types of nucleoside transporters

Chee Wee LEE* and Simon M. JARVIS††

*Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7, and †Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

The transport of [U-¹⁴C]uridine was investigated in rat cerebral-cortical synaptosomes using an inhibitor-stop filtration method. Under these conditions the rapid efflux of uridine from the synaptosomes is prevented and uridine is not significantly metabolized in the synaptosome during the first 1 min of uptake. The dose-response curve for the inhibition of uridine transport by nitrobenzylthioinosine (NBMPR) was biphasic: approx. 40% of the transport activity was inhibited with an IC₅₀ (concentration causing half-maximal inhibition) value of 0.5 nM, but the remaining activity was insensitive to concentrations as high as 1 μM. Similar biphasic dose-response curves were observed for dilazep inhibition, but both transport components were equally sensitive to dipyrindamole inhibition. Uridine influx by both components was saturable (K_m 300 ± 51 and 214 ± 23 μM, and V_{max} 12 ± 3 and 16 ± 3 pmol/s per mg of protein, for NBMPR-sensitive and NBMPR-insensitive components respectively), and inhibited by other nucleosides such as 2-chloroadenosine, adenosine, inosine, thymidine and guanosine with similar IC₅₀ values for the two components. Inhibition of uridine transport by NBMPR was associated with high-affinity binding of NBMPR to the synaptosome membrane (K_d 58 ± 15 pM). Binding of NBMPR to these sites was competitively blocked by uridine and adenosine and inhibited by dilazep and dipyrindamole, with K_i values similar to those measured for inhibiting NBMPR-sensitive uridine influx. These results demonstrate that there are two components of nucleoside transport in our rat synaptosomal preparation that differ in their sensitivity to inhibition by NBMPR. Thus conclusions regarding nucleoside transport in rat brain based only on NBMPR-binding activity must be viewed with caution.

INTRODUCTION

In the central nervous system (CNS), nucleosides have a number of important functions. First, they serve as precursors for nucleic acid synthesis. In the case of pyrimidine nucleosides (uridine and cytidine) present in the CNS these must be either derived from the re-utilization of nucleoside or transported from the periphery, since the brain lacks the complete enzymic pathways for pyrimidine synthesis (McIlwain & Bachelard, 1971). Secondly, there is a large body of evidence suggesting that the nucleoside adenosine is a neuromodulator of CNS functions (Phillis & Wu, 1981; Stone, 1981) and that transport of adenosine into surrounding cells is an important factor in terminating the action of adenosine (for a review, see Wu & Phillis, 1984). Despite the importance of nucleoside transport in the CNS, our understanding of the mechanism(s) is limited and confused.

Much of this confusion is due to the inability of earlier investigations to distinguish between transport *per se* and transport plus metabolism. For example, the transport of adenosine by rat brain synaptosomes, and the effect of a wide range of compounds on this process, have been studied by measuring the cellular accumulation of radioactivity after incubation of the tissue with radioactively labelled adenosine (Bender *et al.*, 1980, 1981a,b, 1982; Phillis *et al.*, 1981, 1983; Phillis & Wu,

1983; Wu *et al.*, 1984). However, the incubation times used in these studies (30 s up to 15 min at 37 °C) were such that the level of accumulation of cellular radioactivity reflected transport, metabolism and backflux of adenosine and metabolic products. Thus, to date, no detailed studies measuring the kinetic properties of nucleoside transport in synaptosomes have been performed.

An alternative approach for studying facilitated-diffusion nucleoside transport in brain, involves the use of [³H]NBMPR, a potent specific inhibitor of facilitated-diffusion nucleoside transport in many cell types, as a binding probe (Young & Jarvis, 1983). Site-specific high-affinity NBMPR-binding sites are present in rat brain membrane preparations (Marangos *et al.*, 1982; Wu & Phillis, 1982; Verma & Marangos, 1985; Hammond & Clanachan, 1985; Geiger *et al.*, 1985), and [³H]NBMPR has been used to localize transport sites by autoradiography (Geiger & Nagy, 1984; Bisslerbe *et al.*, 1985). Nevertheless, conclusions based on these findings concerning nucleoside transport rely on the assumptions that: (a) there is only a single facilitated-diffusion transport system that is sensitive to inhibition by NBMPR, and (b) NBMPR-binding-site occupancy results in inhibition of nucleoside transport. Such assumptions have not been tested experimentally, and the recent observations that there exist nucleoside-transport systems that are totally or partially resistant to inhibition

Abbreviations used: NBMPR, nitrobenzylthioinosine; IC₅₀, concentration causing half-maximal inhibition; CNS, central nervous system; NBTGR, nitrobenzylthioguanosine.

† To whom correspondence and reprint requests should be sent.

by NBMPR further emphasize the need to do so (Belt, 1983; Plagemann & Wohlhueter, 1985; Jarvis & Young, 1986). Indeed, a recent study using guinea-pig brain slices has shown that NBMPR only inhibits about 50% of the accumulation of adenosine (transport of adenosine and its subsequent metabolism), leading to the suggestion of separate NBMPR-sensitive and -insensitive adenosine-uptake sites in the CNS (Davies & Hambley, 1986). Also, studies utilizing [³H]dipyridamole, an inhibitor of facilitated-diffusion nucleoside transport, have shown that there are approx. 2-fold more high-affinity [³H]dipyridamole-binding sites (apparent K_d 10 nM) in guinea-pig brain sections and membranes than high-affinity [³H]NBMPR-binding sites (Marangos *et al.*, 1985; Bisslerbe *et al.*, 1986). NBMPR only inhibited 50% of the [³H]dipyridamole binding, but the nature of NBMPR-insensitive dipyridamole-binding sites has not been investigated.

The objectives of the present study were therefore to assess the kinetic properties of nucleoside transport in rat cerebral-cortical synaptosomes using an inhibitor-stop filtration method to measure initial rates of nucleoside transport and to determine the degree of sensitivity of transport to NBMPR inhibition. Uridine was used as the permeant because, unlike adenosine, it is poorly metabolized by cortical synaptosomes (see Table 1 below). The present results suggest that uridine transport in rat cerebral-cortical synaptosomes can be described by two components that are distinguishable by their sensitivity to inhibition by NBMPR. The properties of NBMPR binding by rat synaptosomes have also been investigated. A preliminary account of some of the work in the present study has been published in abstract form (Lee & Jarvis, 1986).

MATERIALS AND METHODS

Synaptosome preparation

Synaptosomes were prepared by the method of Gray & Whittaker (1962), as modified by White (1975). All centrifugation steps were undertaken at 4 °C. Briefly, the cerebral cortex from two male Sprague-Dawley rats (200–250 g), killed by decapitation, was removed and homogenized in 30 ml of ice-cold sucrose solution (0.32 M-sucrose/0.1 mM-EDTA/5 mM-Hepes, pH 7.5). The homogenate was centrifuged at 1000 *g* for 10 min at 4 °C and the supernatant retained. The pellet was washed once with the sucrose solution (1000 *g*, 10 min), and the supernatant was pooled with that obtained from the first centrifugation. The pooled supernatants were centrifuged at 12 000 *g* for 20 min. The resultant pellet was re-suspended in 6 ml of ice-cold sucrose solution, and 1 ml aliquots were transferred to discontinuous sucrose gradients consisting of 2 ml of 0.8 M- and 2 ml of 1.2 M-sucrose that were centrifuged at 150 000 *g* for 60 min (SW 55 Ti Beckman rotor). The synaptosomal fraction was collected from the 0.8/1.2 M-sucrose interface and diluted slowly to 30 ml with incubation medium, pH 7.5, containing (final concentrations): 120 mM-NaCl, 4.75 mM-KCl, 1.18 mM-MgSO₄, 1.2 mM-KH₂PO₄, 1.77 mM-CaCl₂, 5.5 mM-glucose, 5.8 mM-sucrose and 26 mM-NaHCO₃ (White, 1975). The synaptosomal suspension was again centrifuged at 20 000 *g* for 20 min. Finally, the pellet (synaptosomes) was suspended in ice-cold incubation medium and stored on ice. Relative to the starting homogenate the activity of 2':3'-cyclic nucleotide 3'-

phosphohydrolase (a glial-cell marker enzyme) in the synaptosome preparation was reduced 20-fold (Olafson *et al.*, 1969; Bradford, 1986).

Uptake of uridine by synaptosomes

A 10 µl portion of synaptosomes (100–200 µg of protein) and 20 µl of [¹⁴C]uridine (8 µCi/ml) were added separately on opposite sides of a polyethylene culture tube (16 mm × 100 mm) and preincubated at 22 °C for 5 min. In inhibition studies, test compounds and [¹⁴C]uridine were added simultaneously, with the exception of NBMPR, dipyridamole and dilazep, which were preincubated for 20 min with the synaptosomes. The uptake of [¹⁴C]uridine was initiated by continuous vortex-mixing such that the synaptosomes and the incubation medium came into contact and, after an appropriate time interval, the reaction was terminated by the addition of 1 ml of ice-cold stop solution (incubation medium containing 50 µM-dipyridamole). The suspension was immediately filtered through a Whatman glass-fibre filter (GF/B) under suction. The filter was subsequently washed twice with 5 ml of ice-cold stop solution, dissolved in 4 ml of Scinti-Verse Bio-HP (Fisher Scientific) after being dried, and the radioactivity determined using a LKB/Wallac 1217 liquid-scintillation counter with automatic quench correction and d.p.m. conversion. The entire stopping and washing process took less than 15 s, and from control experiments (see the Results section) no significant loss of [¹⁴C]uridine from the synaptosomes occurred during the stopping and washing procedure. 'Blank' values for uptake, attributable to non-specific binding of [¹⁴C]uridine to the glass-fibre filter and non-mediated permeation, were determined by using synaptosomes pretreated with 50 µM-dipyridamole. These blanks were subtracted from measurements of uridine uptake by synaptosomes to determine carrier-mediated transport rates. Radioactivity that became associated with synaptosomes during an uptake interval of 0 s was determined by using synaptosomes that were filtered immediately after exposure to medium containing both [¹⁴C]uridine and dipyridamole at 4 °C. This value was not significantly different from that obtained in the absence of synaptosomes. The loss of protein during filtration and washing was measured and amounted to approx. 5–10%. This loss was corrected for in calculating the transport rates. Kinetic constants of mediated transport (apparent K_m and V_{max}) were determined by non-linear least-squares fit of the equation:

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where V is the initial transport rate and $[S]$ is the permeant concentration; the computer program HYPMIC (Barlow, 1983) was used.

The intrasynaptosomal volume was determined by a centrifugation method, using ³H₂O to determine total water space and [¹⁴C]inulin for extracellular-space determination (Minn & Gayet, 1977). The mean value of the intrasynaptosomal volume was 4.5 ± 0.5 µl/mg of protein from 16 determinations, a value similar to those published previously (Marchbanks, 1975; Barberis *et al.*, 1981).

[³H]NBMPR-binding assay

Equilibrium [³H]NBMPR-binding assays were initiated by adding 10 µl aliquots of synaptosomes

(100–200 μg) to graded concentrations of [^3H]NBMPR (0.05–4 nM) in a total volume of 2 ml at 22 °C in the presence or absence of 20 μM -NBTGR, a competing non-radioactive ligand. When inhibitors of binding activity were evaluated, incubation mixtures contained both test compounds and 0.1 nM-[^3H]NBMPR. After 30 min incubation the samples were rapidly filtered (Whatman GF/B filter) and washed twice with 5 ml aliquots of ice-cold incubation medium. The filters were dried and counted for ^3H radioactivity as described above. Specific binding is defined as the difference between the membrane content of [^3H]NBMPR in the presence and in the absence of 20 μM -NBTGR.

Uridine metabolism

Synaptosomes were incubated for 10 s, 1 and 30 min with either 5 or 100 μM -[^{14}C]uridine at 22 °C. To identify the intravesicular products, the incubation was stopped with 1 ml of ice-cold stop solution and the membrane vesicles rapidly centrifuged (12000 g , 15 s). The supernatant was removed, and the pellet was immediately solubilized and deproteinized by the addition of 50 μl of ice-cold 7% (w/v) HClO_4 . The precipitate was removed by centrifugation (12000 g , 60 s). To analyse for possible extravesicular products of uridine metabolism, the synaptosomes were centrifuged at the end of the incubation period without dilution. Samples of the synaptosome-free incubation medium were diluted with an equal volume of HClO_4 . Aliquots of both types of acid extract were neutralized with 1 M- KHCO_3 and centrifuged (12000 g , 60 s) to remove the precipitate. Supernatant was chromatographed on silica-gel-coated plates impregnated with fluorescent indicator.

The following solvents were used for chromatographic analysis: (a) butan-1-ol/acetone/acetic acid/aq. 5% NH_3 /water (7:5:3:3:2, by vol.) and (b) butan-1-ol (saturated with water). The standards (uridine, uracil, UMP, UDP and UTP) were co-chromatographed with the samples. After drying, the individual standards were localized under u.v. light. Zones were scraped off the plate into scintillation vials and the remainder of the chromatogram divided into 0.5 cm horizontal strips. Radioactivity in these samples were extracted with 1 ml of water with shaking for at least 15 min before the addition of scintillation fluid.

Chemicals

[U- ^{14}C]Uridine (sp. radioactivity 453 mCi/mmol) and [^{14}C]inulin (sp. radioactivity 5 mCi/mmol) were purchased from Amersham International, Oakville, Ontario, Canada. [^3H]NBMPR (sp. radioactivity 23 Ci/mmol) and $^3\text{H}_2\text{O}$ (sp. radioactivity 100 mCi/ml) were obtained from Moravsek Biochemicals, Brea, CA, U.S.A., and ICN, Irvine, CA, U.S.A., respectively. NBTGR and dilazep were generously given by Professor A. R. P. Paterson, Cancer Research Group, University of Alberta, and Hoffman-La Roche and Co., Basle, Switzerland, respectively. Dipyridamole was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

The rapid filtration technique used in the present study for measuring nucleoside transport by cerebral-cortical synaptosomes is only valid if all transport ceases after the addition of the stop solution and no loss of radioactivity

Fig. 1. Effect of various stop solutions on uridine uptake by rat cerebral-cortical synaptosomes

Cortical synaptosomes were incubated with [^{14}C]uridine (100 μM) at 22 °C for 30 s, and transport was terminated by the addition of 1 ml of stop solution composed of incubation medium at 22 °C (O) or ice-cold incubation medium (●) or ice-cold incubation medium containing 50 μM -dipyridamole (■). The synaptosomes were then either filtered immediately (0 s) or left in stop solution for the times indicated and then filtered. The filters were washed twice with 5 ml of the respective stop solution. The time interval between termination and the completion of washing was less than 15 s. The radioactivity retained by the vesicles is expressed as a percentage of the radioactivity retained at 0 s when ice-cold stop solution plus 50 μM -dipyridamole was used.

from the synaptosomes occurs during the filtration. In preliminary experiments the effect of different stop solutions on the retention of [^{14}C]uridine by the synaptosome vesicles was tested. Synaptosomes were incubated with [^{14}C]uridine for 30 s and then subsequently diluted 1:33 into various stop solutions (see Fig. 1). These diluted vesicles were then filtered immediately or after a 5–90 s delay. Fig. 1 shows that, when an ice-cold stop solution containing 50 μM -dipyridamole, a nucleoside transport inhibitor, was used, less than 6% of the radioactivity in the synaptosomes was lost after a 20 s delay, the time required to complete the washing and filtration (see the Materials and methods section). By contrast, stop solution at either 4 or 22 °C in the absence of dipyridamole resulted in a rapid loss of radioactivity from synaptosomes. These results suggest that no significant loss of ^{14}C label is apparent with the ice-cold stop solution containing 50 μM -dipyridamole, and thus this solution was routinely used in the present study.

The data of Fig. 1 also indicate that approx. 40% of the radiolabel is retained by the synaptosome after dilution at 22 °C. Calculation of the intravesicular concentration of [^{14}C]uridine yields a value of 4 μM , which is close to that of the new extravesicular uridine

Table 1. Metabolism of [14 C]uridine by rat cerebral-cortical synaptosomes

Two concentrations of [14 C]uridine (5 and 100 μ M) were incubated with synaptosomes for 10 s, 60 s and 30 min at 22 $^{\circ}$ C. Extraction and identification of the intravesicular and extravesicular products were performed as described in the text. Values are shown as a percentage distribution of total radioactivity analysed from intravesicular extracts. Results for extravesicular extracts were not included, since no product other than uridine was detected. Abbreviation: ND, not detected.

[14 C]Uridine concn. (μ M)	Incubation period	Distribution (% of total radioactivity recovered)		
		Uridine	Uracil	Nucleotides
100	10 s	100	ND	ND
	60 s	100	ND	ND
	30 min	88	3	9
5	10 s	100	ND	ND
	60 s	100	ND	ND
	30 min	79	5	16

Fig. 2. Time course of uridine uptake by rat cerebral-cortical synaptosomes

Synaptosomes were preincubated with buffer (■), 20 μ M-NBMPR (●) or 50 μ M-dipyridamole (○) for 20 min before the addition of [14 C]uridine (final extracellular concentration 100 μ M). Values are the averages of triplicate estimates.

concentration after dilution (3 μ M), suggesting both that equilibration of uridine across the synaptosomal membrane has occurred and that uridine is also not metabolized. The results of Table 1 confirm that no significant metabolism of uridine occurs within the first 1 min of uptake, and > 95% of the radioactivity co-chromatographed with uridine. The starting [14 C]uridine

Fig. 3. Effect of NBMPR, dilazep and dipyridamole on uridine influx by rat cerebral-cortical synaptosomes

Synaptosomes (0.1–0.2 mg) were preincubated with various concentrations of NBMPR (●), dilazep (■) or dipyridamole (○). Transport of 100 μ M-[14 C]uridine was determined as described in the text, and the results are expressed as a percentage of the control influx rate versus the inhibitor concentration. The blank value for uptake was determined in the presence of 50 μ M-dilazep. In the case of NBMPR, [3 H]NBMPR in the concentration range 0–30 nM was used in parallel experiments to determine the final free concentration of the inhibitor.

was 97% radiochemically pure. After 30 min incubation of [14 C]uridine with synaptosomes, 10–20% of the radioactivity was recovered in the uridine nucleotide fractions. No metabolites of uridine were detected in the extracellular medium (results not shown).

The time course of [14 C]uridine uptake (100 μ M) by rat cerebral-cortical synaptosomes in the presence of 20 μ M-NBMPR or 50 μ M-dipyridamole is shown in Fig. 2. The transport of [14 C]uridine by synaptosomes was linear for at least 10 s at 22 $^{\circ}$ C. Therefore, in subsequent kinetic experiments, initial rates of uridine uptake were determined by measuring the cellular content of uridine after incubation intervals of 10 s at 22 $^{\circ}$ C. Under these conditions, the maximum intravesicular uridine levels did not exceed 10% of the extravesicular concentrations under most circumstances. Dipyridamole was a potent inhibitor of uridine transport and decreased the rate of influx by 75%. Higher concentrations of dipyridamole (100 μ M) produced no further inhibition of uridine uptake (results not shown). In contrast, 20 μ M-NBMPR, a concentration 10^5 – 10^6 -fold greater than the apparent dissociation constant for high-affinity NBMPR binding to rat brain membranes (Wu & Phillis, 1982; Geiger *et al.*, 1985; Hammond & Clanachan, 1985; Verma &

Fig. 4. Concentration-dependence of uridine transport by control and NBMPR-treated rat cerebral-cortical synaptosomes

[¹⁴C]Uridine transport by synaptosomes in the presence (○) or absence (●) of 500 nM-NBMPR or in the presence of 50 μM-dipyridamole (■) or 50 μM-dipyridamole plus 20 μM-NBMPR (□) was determined as described in the Materials and methods section. The synaptosomes were preincubated for 20 min with inhibitors before addition of [¹⁴C]uridine. Kinetic constants of saturable uridine transport were determined by non-linear least-squares fit of the Michaelis-Menten equation and gave *K_m* values of 135 ± 36 and 180 ± 81 μM, with *V_{max}* estimates of 12 ± 1 and 17 ± 2.6 pmol/s per mg of protein, for NBMPR-sensitive and -insensitive transport respectively.

Marangos, 1985; the present study), only partially inhibited uridine influx.

To investigate further the effect of NBMPR and dipyridamole on uridine influx by rat cortical synaptosomes, the dose-response curves for inhibition of uridine transport by these compounds were compared (Fig. 3). In contrast with that for dipyridamole, the inhibition curve for NBMPR was biphasic; about 30% of the transport activity was inhibited by NBMPR, with an *IC₅₀* value of 0.5 nM. No further inhibition was observed until the NBMPR concentration exceeded 1 μM. The wide plateau in the inhibition curve suggests that the inhibition observed at > 1 μM-NBMPR occurs by a mechanism different from that involved at lower concentrations of NBMPR. Furthermore, the NBMPR inhibition profile suggests that there may be two mechanisms for uridine transport by rat cerebral-cortical synaptosomes that can be distinguished on the basis of their sensitivity to inhibition by NBMPR. Fig. 3 also demonstrates that the dose-response curve for inhibition of uridine transport by dilazep was also biphasic (approximate *IC₅₀* values for the two phases of 0.02 and 2 μM), but not so marked as that observed with NBMPR. Further characterization of these two transport components was performed using synaptosomes pretreated or not with 500 nM-NBMPR. Transport remaining in the presence of 500 nM-NBMPR was defined as 'NBMPR-insensitive', whereas the difference between transport

Table 2. Effect of nucleosides on uridine influx by NBMPR-sensitive and NBMPR-insensitive components in rat cerebral-cortical synaptosomes

The uptake was initiated by addition of [¹⁴C]uridine (final concn. 100 μM) (in the simultaneous presence or absence of various nucleosides) to the synaptosomes treated or not with NBMPR (500 nM). After 10 s at 22 °C uridine uptake was terminated as described in the Materials and methods section. *IC₅₀* values were obtained by extrapolation from the dose-response curve. *K_i* values were calculated from the equation: $K_i = IC_{50}/(1 + L/K_m)$

where *K_m* values for NBMPR-sensitive and -insensitive uridine influx were taken as 360 and 225 μM respectively and *L* is the uridine concentration. The values are averages for at least three separate experiments.

Nucleoside	NBMPR-sensitive		NBMPR-insensitive	
	IC ₅₀ (μM)	K _i (μM)	IC ₅₀ (μM)	K _i (μM)
2-Chloro-adenosine	26 ± 5	21 ± 4	53 ± 7	39 ± 6
Adenosine	79 ± 5	62 ± 5	77 ± 22	54 ± 16
Thymidine	250 ± 80	200 ± 65	370 ± 43	260 ± 31
Inosine	440 ± 71	350 ± 61	390 ± 32	270 ± 28
Guanosine	450 ± 95	330 ± 70	590 ± 92	430 ± 53

rates determined in the presence of 500 nM-NBMPR and the total uptake rates represents 'NBMPR-sensitive' transport.

The concentration-dependence of uridine influx in the presence or absence of 500 nM-NBMPR is shown in Fig. 4. Uptake of [¹⁴C]uridine in the presence of 50 μM-dipyridamole was linear with concentration and was similar for NBMPR-treated and non-treated synaptosomes (15 pmol/s per mg of protein at 0.5 mM). Identical results were obtained when 50 μM-dilazep was used instead of 50 μM-dipyridamole (results not shown). By contrast, uridine transport by untreated and NBMPR-treated synaptosomes was resolved into two components: (i) a linear component and (ii) a saturable component. To determine the kinetic constants for the saturable NBMPR-insensitive transport component, the linear component of transport estimated in the presence of dipyridamole was subtracted from the data obtained with NBMPR-treated synaptosomes. The saturable component obeyed simple Michaelis-Menten kinetics consistent with the existence of a single NBMPR-insensitive uridine transporter (*K_m* 180 ± 81 μM; *V_{max}* 17 ± 2.6 pmol/s per mg of protein). The saturable NBMPR-sensitive component of Fig. 4 was calculated from the total flux by subtracting the total NBMPR-insensitive flux using NBMPR-treated synaptosomes. The data fitted well to the Michaelis-Menten equation, suggesting a single NBMPR-sensitive uridine transport site (*K_m* 135 ± 36 μM; *V_{max}* 12 ± 1 pmol/s per mg of protein). The mean apparent *K_m* values for uridine influx for the NBMPR-sensitive and NBMPR-insensitive components from four separate experiments were 300 ± 51 and 214 ± 23 μM respectively, with *V_{max}* values of 12 ± 3 and 16 ± 3 pmol/s per mg of protein respectively (means ± S.E.M.).

The ability of other nucleosides to interact with the

Fig. 5. Concentration-dependence of [^3H]NBMPR binding to rat cerebral-cortical synaptosomes

Synaptosomes were incubated with [^3H]NBMPR (0.1 nM) of [^3H]NBMPR for 30 min at 22 °C in the absence (■) and presence (●) of 20 μM -NBTGR. Specific binding of NBMPR represents the difference between membrane-associated NBMPR in the presence and absence of NBTGR, and the binding constants for the data shown are K_d 74 $\pm$ 19 pM and B_{max} 580 $\pm$ 34 fmol/mg of protein.

Fig. 6. Inhibition of site-specific binding of NBMPR to rat cerebral-cortical synaptosomes

Synaptosomes were incubated with [^3H]NBMPR (0.1 nM) for 30 min at 22 °C in the presence of dipyridamole (○), dilazep (●), adenosine (■) and uridine (□). Results are plotted as a percentage of site-specific binding of NBMPR in the absence of inhibitor (480 fmol/mg of protein). Values are the means of triplicate estimates.

uridine-transport mechanisms in rat cortical synaptosomes was studied by investigating the effect of adenosine, inosine, thymidine, 2-chloroadenosine and guanosine on uridine influx by untreated and NBMPR-treated synaptosomes. Table 2 shows that in both cases 2-

chloroadenosine was the most effective inhibitor. The apparent K_i values estimated from the IC_{50} values for inhibition of uridine influx by the NBMPR-sensitive and NBMPR-insensitive transport mechanisms were respectively 21 and 31 μM for 2-chloroadenosine, 62 and 54 μM for adenosine, 200 and 270 μM for thymidine, 350 and 270 μM for inosine and 330 and 430 μM for guanosine (mean values from three experiments). These results, together with the determination of the K_m values for uridine transport, suggest that the NBMPR-sensitive and NBMPR-insensitive components of nucleoside transport in rat cerebral-cortical synaptosomes exhibit similar affinities.

In the preceding sections the [^{14}C]uridine associated with the synaptosome has been assumed to be due to transport into the intrasynaptosomal space. It is possible, although highly unlikely, that the radiolabel is only binding to the synaptosome. Thus the uptake of [^{14}C]uridine at equilibrium (5 min incubation) as a function of medium osmolarity was determined (results not shown). This uptake was quite sensitive to changes in medium osmolarity, sharply decreasing with increases in osmolarity. When extrapolated to infinite medium osmolarity, uptake was negligible and not significantly different from zero. This indicates uptake into an osmotically active intravesicular space without appreciable binding to the surface of the synaptosomes.

With buffers identical with those of the transport assays, Fig. 5 shows the concentration-dependence of [^3H]NBMPR binding to rat synaptosomes, where membrane-associated binding is plotted against the equilibrium free concentration of the inhibitor. [^3H]NBMPR binding was resolved into two components, one of which was saturable with an apparent K_d of 74 $\pm$ 19 pM and a B_{max} (maximal binding) of 580 $\pm$ 34 fmol/mg of protein, whereas the other was proportional to the free [^3H]NBMPR concentration. NBTGR (20 μM) eliminated the saturable binding, but had no effect on the linear component, and hence specific binding is defined as the difference in membrane content of [^3H]NBMPR in the presence and absence of 20 μM -NBTGR. Scatchard plots of the specific binding data from three different rat cortical synaptosome preparations revealed a linear relationship, indicating a single population of high-affinity [^3H]NBMPR-binding sites with an apparent K_d of 58 $\pm$ 15 μM and a B_{max} of 470 $\pm$ 26 fmol/mg of protein.

Various compounds, including nucleosides and nucleoside-transport inhibitors, were tested for their ability to inhibit the specific binding of [^3H]NBMPR to rat synaptosomes. Fig. 6 shows that dilazep was a more effective inhibitor than dipyridamole, with calculated K_i values of 4 and 400 nM for dilazep and dipyridamole respectively, values similar to those calculated for inhibiting NBMPR-sensitive uridine transport (see Fig. 3). Adenosine was a more effective inhibitor than uridine, IC_{50} values being 60 and 700 μM respectively, a result consistent with the relative affinities of these two nucleosides for NBMPR-sensitive nucleoside transport (see Fig. 4 and Table 2). Addition of deoxycofomycin (0.1 μM), an inhibitor of adenosine deaminase, did not affect the IC_{50} value for adenosine inhibition of [^3H]NBMPR binding (results not shown). In other experiments, the nature of the inhibition by adenosine and uridine was explored by using various concentrations of both nucleoside and [^3H]NBMPR (Fig. 7). In both cases the inhibition profiles were consistent with a

Fig. 7. Effect of adenosine and uridine on [^3H]NBMPR binding to rat cerebral-cortical synaptosomes

The reciprocals of [^3H]NBMPR bound to specific sites in the presence of graded concentrations of adenosine (a) and uridine (b) at 22 °C are plotted against the respective reciprocals of the free equilibrium concentrations of [^3H]NBMPR. (a) Initial concentrations (μM) of adenosine were 0 ($\square$), 50 ($\blacksquare$), 100 ($\circ$) and 200 ($\bullet$). (b) Initial concentrations (μM) of uridine were 0 ($\square$), 250 ($\blacksquare$), 500 ($\circ$) and 1000 ($\bullet$). Apparent K_i values for adenosine and uridine were 15 and 207 μM respectively.

competitive type of inhibition, with apparent K_i values of 15 and 207 μM for adenosine and uridine respectively.

DISCUSSION

We present here the results of a series of experiments that examined the properties of uridine transport by rat cerebral-cortical synaptosomes. In contrast with earlier studies on adenosine uptake (Bender *et al.*, 1980, 1981a,b, 1982; Wu *et al.*, 1984), we found it necessary to use an ice-cold stop solution containing 50 μM -dipyridamole for the rapid filtration transport assays. The earlier studies employed a stop solution composed of buffer at room temperature. Fig. 1 clearly demonstrates that this stop solution was unsatisfactory for quantitative uridine uptake by rat cortical synaptosomes. In addition, these earlier studies also used incubation times that resulted in substantial metabolism of adenosine, thereby complicating the interpretation of the data. The present results were obtained under conditions where initial rates of transport were measured and no metabolism of radioactively labelled uridine was detected. The rates of carrier-mediated uridine transport were calculated after subtraction of the flux measured in the presence of 50 μM -dipyridamole. This concentration of dipyridamole caused total inhibition of transport activity (see Fig. 3). Moreover, the concentration-dependence of uridine influx in the presence of 50 μM -dipyridamole was linear, and no further inhibition occurred after the addition of 20 μM -NBMPR (Fig. 4). The magnitude of this linear flux component was not significantly different when 50 mM-dilazep was used instead of dipyridamole. Dilazep was approx. 10-fold more potent an inhibitor of uridine transport by rat synaptosomes than dipyridamole. These data demonstrate that 50 μM -dipyridamole blocks carrier-mediated uridine transport by rat cerebral-cortical synaptosomes, and therefore it is valid to use this concentration of dipyridamole to correct for non-mediated uridine uptake.

Uptake of uridine by our rat cerebral-cortical synaptosome preparation was found to be into an osmotically active space. Uridine transport was inhibited in a biphasic manner by NBMPR, suggesting two components of nucleoside transport by rat synaptosomes distinguishable on the basis of their sensitivity to inhibition by certain nucleoside-transport inhibitors. The first transport component, which we define as 'NBMPR-sensitive', was inhibited by nanomolar concentrations of NBMPR with an estimated apparent K_i value of approx. 0.35 nM and represented approx. 35–55% of the total uridine flux at 100 μM extracellular uridine. Similarly, dilazep was also a potent inhibitor of this transport component (apparent $K_i \approx 14$ nM). Inhibition of uridine transport by NBMPR was associated with high-affinity binding of [^3H]NBMPR to the synaptosomes. The apparent dissociation constant for [^3H]NBMPR binding, 58 ± 15 pM, was similar to values reported previously in studies using either crude P_2 fractions or synaptosome membranes (range 0.05–0.18 nM) (Marangos *et al.*, 1982; Wu & Phillis, 1982; Geiger *et al.*, 1985; Hammond & Clanachan, 1985). Moreover, the similarity of the K_i value for [^3H]NBMPR binding to the K_i value of NBMPR inhibition of NBMPR-sensitive transport indicates that NBMPR is binding to a region of the transporter that is essential for transporter function. In addition, the K_i values for inhibition of NBMPR-sensitive uridine transport by adenosine, uridine and dilazep (62 and 360 μM and 14 nM respectively) are similar to the inhibitor constants for inhibiting high-affinity [^3H]NBMPR binding (15 and 210 μM , and 4 nM respectively), providing evidence to suggest that NBMPR competes with nucleosides for the permeation site of the NBMPR-sensitive carrier. Adenosine and uridine blocked [^3H]NBMPR binding in a competitive manner. These data contrast with those in earlier reports by Phillis and colleagues that reported major discrepancies between (1) the dissociation constant for [^3H]NBMPR binding in rat brain (0.05 nM; Wu & Phillis, 1982) and its K_i value for

inhibition of adenosine uptake ($30\ \mu\text{M}$; Bender *et al.*, 1981b) and (2) the IC_{50} values for inhibition of [^3H]NBMPR binding and adenosine uptake into synaptosomes by nucleosides (Bender *et al.*, 1981b; Wu & Phillis, 1982). As stated above, the adenosine-uptake studies by Phillis and colleagues failed to measure initial rates of adenosine transport. The present NBMPR-binding and -transport data also do not support the conclusion of Geiger *et al.* (1985) that [^3H]NBMPR binds to adenosine-preferring transporter sites in rat brain that do not have a broad specificity for nucleosides. However, it does appear from our results that adenosine and 2-chloroadenosine have the highest affinity of the nucleosides tested for the NBMPR-sensitive transporter.

The second uridine-transport component, defined as 'NBMPR-insensitive', was not inhibited by concentrations of NBMPR as high as $1\ \mu\text{M}$. Similarly, dilazep was also a much less potent inhibitor of this flux component (apparent K_i $1.5\ \mu\text{M}$) compared with inhibition of the NBMPR-sensitive uridine transport by rat cortical synaptosomes. In other respects the two transport components are quite similar. Both mechanisms are facilitated-diffusion systems with similar apparent K_m values for uridine ($\sim 200\ \mu\text{M}$). Inhibition experiments also suggest that there are no significant differences in the specificity of the two transport components for a variety of nucleosides and that the systems have a broad specificity for both purine and pyrimidine nucleosides. The affinity of the nucleosides, as determined from the inhibition of uridine transport, is similar to that observed in other cells, with adenosine and 2-chloroadenosine having the highest affinity (Young & Jarvis, 1983). Also, both transporters in rat cerebral-cortical synaptosomes appear to be equally sensitive to inhibition by dipyrindamole, as judged from the monophasic curve presented in Fig. 3. It is unlikely that one of the transport components that we observe in the present study might be of glial origin, because contamination of glial-cell fragments in the synaptosomal fraction, estimated by activities of the glial-cell-specific enzyme 2':3'-cyclic nucleotide 3'-phosphohydrolase, is only 5%.

In conclusion, the present results suggest that there are two components of facilitated-diffusion nucleoside transport present in our preparation of rat cerebral-cortical synaptosomes. These two components have similar substrate affinities, but differ in their susceptibility to inhibition by NBMPR. These two transport components may represent the products of separate genes, or alternatively two conformations of a single protein that differ in their ability to bind NBMPR with high affinity.

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