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NUCLEOSIDE TRANSPORT IN MAMMALIAN CEREBRAL CORTICAL SYNAPTOSOMES AND RAT RENAL BRUSH BORDER MEMBRANE VESICLES.

By

CHEE WEE LEE

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Nucleoside Transport in Mammalian Cerebral Cortical Synaptosomes and Rat Renal Brush Border Membrane Vesicles submitted by Chee Wee LEE in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date : Jan. 9th 1989

Dedication

To Nicole and my parents,
for their love and support.

Abstract

The transport of nucleosides was investigated in rat and guinea pig cerebral cortical synaptosomes and rat renal brush border membrane vesicles using an inhibitor-stop filtration method. Under these conditions the rapid efflux of nucleosides from the vesicles is prevented. There were two components of nucleoside transport in mammalian cerebral cortical synaptosomes that differ in their sensitivity to inhibition by nitrobenzylthioinosine (NBMPR) and other transport inhibitors. Transport affinities for NBMPR-sensitive and -insensitive components were not significantly different in rat synaptosomes (K_m's for uridine 300 ± 51 and 214 ± 23 μM with V_{max}'s 12 ± 3 and 16 ± 3 pmol/mg protein/s, respectively). In contrast, the NBMPR-sensitive component in guinea pig synaptosomes exhibits a higher transport affinity than the NBMPR-insensitive component (K_m's for adenosine 17 ± 3 and 68 ± 8 μM with V_{max}'s 2.8 ± 0.3 and 6.1 ± 0.4 pmol/mg protein/s, respectively). Inhibition of nucleoside transport by NBMPR was associated with high affinity binding of NBMPR to the synaptosomes (K_d's 58 ± 15 and 88 ± 6 pM for rat and guinea pig synaptosomes, respectively). Thus conclusions regarding nucleoside transport in mammalian brain based only on NBMPR-binding activity must be viewed with caution. The uptake of uridine into rat renal brush border membrane vesicles is mediated by Na^+- and K^+-

dependent concentrative transport processes. The amplitude of overshoot was increased by raising either Na^+ or K^+ concentration or by using more lipophilic anions (reactive order $NO_3^->SCN^->Cl^->SO_4^{2-}$). At 100 mM salt concentration, the K_m values were 6.7 ± 0.5 and 28 ± 3 μM with V_{max} values of 70 ± 1 and 15 ± 4 pmol/mg protein/s for the Na^+- and K^+-dependent systems, respectively. Both carriers are specific for nucleosides but favour adenosine and pyrimidines as substrates. The Na^+:uridine and K^+:uridine coupling stoichiometry was 1:1 and 3:2, respectively. Both systems can also be driven by an anion gradient ($K_{nitrate}$'s 42 ± 13 and 163 ± 54 mM for Na^+- and K^+-dependent systems, respectively). The physiological role of K^+-dependent nucleoside transport is not known. However it is possible that this system is involved in a secretory process.

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List of Abbreviations

3′-AMP	Adenosine 3′-monophosphate
5′-AMP	Adenosine 5′-monophosphate
5′-CMP	Cytidine 5′-monophosphate
5′-GMP	Guanosine 5′-monophosphate
5′-IMP	Inosine 5′-monophosphate
5′-UMP	Uridine 5′-monophosphate
ADP	Adenosine 5′-biphosphate
ATP	Adenosine 5′-triphosphate
ITP	Inosine 5′-triphosphate
cAMP	Adenosine 3′, 5′-cyclic-monophosphate
CHA	N^6 Cyclohexyladenosine
GABA	γ-Aminobutyric acid
L-PIA	L-N^6 Phenylisoprophyl adenosine
NECA	N-Ethylcarboxamide adenosine
NBMPR	Nitrobenzylthioinosine
NBTGR	Nitrobenzylthioguanosine

1. Introduction and Objectives

Nucleoside transport in mammalian cerebral cortical synaptosomes

Adenosine has been shown to depress neuronal firing [7, 25], inhibit release of various neurotransmitters [9, 10, 13], induce a decrease in cerebral blood flow and ischemia [5], and to have marked effects on adenylate cyclase activity [30]. It is believed that these events are terminated by the rapid removal of adenosine from the extracellular compartment via nucleoside specific transport elements in the plasma membrane [31]. Thus, drugs which inhibit the transport of nucleosides across cell membranes might be expected to potentiate the effects of adenosine. For example, it has recently been suggested that benzodiazepines exert their sedative effects by blocking the uptake of adenosine, thereby increasing the extracellular levels of adenosine and thus, decreasing neuronal excitability [22, 24, 26, 27].

Despite the importance of nucleoside transporters, few valid studies have been conducted to investigate the properties of nucleoside transport systems in brain. Most of the earlier studies [1-4, 23, 27] investigated adenosine uptake (a process that represent both transport and metabolism) rather than adenosine transport alone. An alternative approach to investigate nucleoside transport involves the measurement binding of NBMPR, a

potent inhibitor of nucleoside transport in many cell types [15, 32]. This latter approach assumes that nucleoside transport in synaptosomes is totally sensitive to NBMPR, an assumption which has not been tested experimentally.

The objectives for the first part of this thesis were therefore to establish conditions for measuring initial rates of nucleoside transport by mammalian cerebral cortical synaptosomes. Having established conditions for studying initial rates of nucleoside transport, studies were performed to investigate the kinetics, substrate specificity and inhibitor susceptibility of nucleoside transport in mammalian cerebral cortical synaptosomes. The hypothesis that the clinical doses of benzodiazepines inhibit adenosine transport by synaptosomes was also tested.

Nucleoside transport in rat renal brush border membrane vesicles

The potential usefulness of nucleosides and related substances in treatment against malignant cells, viral and parasitic infections and as a vasodilator has been demonstrated with cells in culture, with laboratory animals and in clinical tests [8, 21, 28]. However the major obstacles to the therapeutic use of these substances is toxicity to the host tissues and their

influences on many physiologic and biochemical processes in the body [12, 29]. In kidney, nephrotoxicity has been observed when nucleoside analogues, such as tubercidin and 2'-deoxycoformycin, were administered to experimental animals [6, 11, 14, 16]. Elevation of intrarenal adenosine and its analogues from either exogeneous or endogeneous sources also resulted in significant hemodynamic effects [29]. Thus, the mechanism(s) by which the kidney handles high concentrations of nucleosides in the glomerular filtrate may be an important factor in the therapeutic administration of nucleoside drugs.

Measurements of the renal clearance of adenosine have suggested that adenosine is actively reabsorbed by the proximal tubule [17]. Subsequent studies with isolated brush border preparations have demonstrated that the uptake of adenosine and a variety of purine and pyrimidine nucleosides did not proceed by facilitated diffusion, but rather by a high-affinity concentrative process which required the presence of a Na^+-gradient as a driving force [18-20]. Such ion gradients are maintained in the living cell by Na^+-K^+-ATPase. Apart from measurements of the kinetic constants of sodium cotransport of nucleosides by rat renal brush border membrane vesicles, few studies have been conducted to investigate the substrate specificity, membrane potential dependency, monovalent cation specificity, and

stoichiometry of sodium-dependent nucleoside transport in the kidney.

The objective for the second part of this thesis was therefore, to characterize the transport properties of nucleoside transporter(s) in brush border membrane vesicles prepared from rat renal cortex in order to gain a more comprehensive view of active nucleoside transport in kidney.

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2. Literature Review

2.1. Central Nervous System

2.1.1. Presence of adenosine and its nucleotides

The ATP content of rat cerebral cortex is high, exceeding 2 mmol/kg in fresh brain [86, 108, 129, 158, 225, 228, 231]. Higher concentrations of ATP are found in gray than white matter. Although the sensorimotor cortex, entorhinal cortex, hippocampus, hypothalamus and thalamus have the highest ATP concentrations of the various brain regions that have been studied, overall, regional variability in ATP distribution is not very pronounced [89, 228, 231].

In contrast, adenosine concentrations in brain are much lower [129, 158, 225], and rise rapidly during periods of brain ischemia [86] although even under such conditions only reach levels of about 1 μM in cerebrospinal fluid [19]. Adenosine formation in ischaemic brains occurs as a result of the hydrolysis of ATP. Although the regional variability in adenosine levels is not great, the highest levels are found in the cerebral cortex, followed, in descending order of content, by the striatum, mesencephalon plus thalamus, brain stem, hypothalamus, and cerebellum [54].

2.1.2. Sources of adenosine

Animals synthesize purine derivatives from amino acids, formate and carbon dioxide *in situ*. All the enzymes involved in the synthesis of purine rings are present in mammalian cells [64]. In the mammalian central nervous system (CNS), the synthesis of purines from formate [110] and glycine [67] has also been demonstrated.

Adenosine can potentially be synthesized via a number of different pathways including: (1) by dephosphorylation of 5′-AMP; (2) from S-adenosyl-L-homocysteine formed from S-adenosyl-L-methionine in the process of methylation; (3) by dephosphorylation of 3′-AMP; (4) from adenosine end groups of ribonucleic acid; and (5) by the reaction of adenine and ribose-1-phosphate.

Of these, the dephosphorylation of 5′-AMP is the major source of adenosine. 5′-AMP may come from several sources, including turnover of the adenine nucleotide pool (from ATP and cAMP) or from the breakdown of ribonucleic acid that takes place during cellular function. The regulation of 5′-AMP dephosphorylation involves a number of enzymes, including 5′-nucleotidase as well as acid and alkaline phosphatases. Dephosphorylation of 5′-AMP occurs in both cellular and intracellular compartments [43]. Morphological and

functional chemical studies provide convincing evidence that one form of 5′-nucleotidase is localized on the outer surface of the cell membrane [51, 111]. This enzyme is important in allowing the formation and uptake of adenosine from extracellularly located 5′-AMP. Intracellular 5′-AMP is degraded to adenosine by a specific endo-5′-nucleotidase or by non-specific phosphatases. The ecto-5′-nucleotidase responsible for the production of extracellular adenosine from 5′-AMP (K_m = 220 μM) by rat glioma cells also readily hydrolyzed 5′-UMP; other mononucleotides (5′-CMP, 5′-IMP, 5′-GMP) were hydrolyzed more slowly; 2′ and 3′-nucleoside monophosphates were not attacked. ADP, ATP and ITP were inhibitory [193].

2.1.3. Effects of adenosine

The evidence for adenosine being a major modulator of CNS function has now become quite well established. At the behavioral level the sedative and hypnotic effects of adenosine and its metabolically stable analogues when administrated peripherally are well documented [32, 33, 187]. The metabolically stable adenosine agonists have sedative potencies that exceed most of the currently known CNS depressants [32]. Various pyrimidine nucleosides (uridine, cytidine, thymidine) can also depress the spontaneous activity of mice following intraperitoneal administration [91]. However the blood-

brain barrier often effectively limits the entry of adenosine into the tissues of the brain [19]. It is questionable whether these effects of peripherally administered purine nucleosides are exerted directly on the CNS or occur as a results of action elsewhere in the body. Experiments in which the responses of single neurons in the rat cerebral cortex were recorded strongly suggest that the depressant effects of intravenously administered adenosine are a consequence of the fall in blood pressure elicited by the adenosine [19, 146]. However, the behavioral effects of adenosine derivatives can also be observed when the blood-brain barrier is circumvented by direct intracerebral adminstration into the cerebral ventricles [26, 36, 66, 104, 118].

Other behavioral effects of adenosine such as evoked premature arousal in hibernating ground squirrels [209] and the facilitation of maze learning by mice [119] have also been reported.

Adenosine and its analogues have also been shown to depress the release of other neurotransmitters, such as acetylcholine, norepinephrine, dopamine, serotonin, glutamate and, to a lesser extent, GABA from brain preparations [21, 55, 63, 83, 144], and can thus play a significant role in controlling the excitability of central pathways. Adenine, inosine, guanosine, uridine, cytidine, and thymidine are inactive. The inhibitory

effects of adenosine on transmitter release are antagonized by the methylxanthines, caffeine and theophylline.

Evidence suggests that that inhibitory action of adenosine on neurotransmitter release probably results from a reduction in the calcium permeability of the nerve terminal membrane [161, 171, 233]. Less calcium will enter during action potentials, resulting in reduced transmitter release. Blockade of calcium uptake by adenosine may also affect other calcium-dependent neuronal processes [233]. However, the effects of adenosine can be reversed by raising the extracellular calcium concentration [161, 171] or blocked by theophylline and caffeine [83, 145, 147]. There is also some indication that adenosine may immobilize intracellular calcium [21, 55].

Apparently, endogenous adenosine levels (1-2 μM) [91] in the extracellular fluid of brain tissue are sufficient to modulate neurotransmitter release since treatment with adenosine deaminase (to decrease adenosine levels) enhances dopamine release, and blockade of adenosine deaminase with deoxycoformycin decreases it [122]. Treatment with the adenosine uptake inhibitor dipyridamole has also been shown to decrease GABA release [144].

Adenosine triphosphate and polyphosphates of other purines and pyrimidines can have excitatory actions on neurons in the cerebral cortex and elsewhere in the brain, being most pronounced with quiescent or slowly firing neurons [53, 72]. These excitatory effects had a short latency of onset and declined rapidly once the application had ceased. Excitation was most pronounced with the triphosphates and especially with homoadenosine triphosphate, which, lacking depressant activity, was a very effective excitant. Excitation was less pronounced with diphosphates and was infrequently observed with the monophosphates. In part, this may reflect the calcium chelating ability of these compounds [90], but there also appears to be an additional component of their excitant action which is unrelated to calcium chelation, and may be a result of stimulation of prostaglandin synthesis [23, 126]. Prostaglandins have excitatory actions on cerebral cortical neurons [139] and on the isolated amphibian spinal cord [141].

2.1.4. Inactivation of adenosine actions

2.1.4.1. Uptake

The uptake of purine and pyrimidine nucleosides has been extensively studied in various CNS tissue preparations [12-15, 75, 93, 121, 176, 238]. Such uptake mechanisms are thought to be an important factor in

modulating adenosine actions in central tissues by controlling extracellular adenosine levels [33] since enzymes which degrade adenosine occur largely intracellularly.

In vitro CNS preparations have been used to demonstrate the existance of a high-affinity facilitated diffusion mechanism for adenosine in rat cerebral cortical synaptosomes [12-14]. This rapid uptake system has a K_m of 0.9 μM and a V_{max} of 5.26 pmol/mg protein/30 s at 37°C [13]. The uptake process is also sensitive to pH with a maximal uptake at pH 8 which dropped rapidly when the pH values were increased to 9 [14]. Over 70% of the adenosine taken up remained unchanged while 14% was metabolized to inosine. Uptake was inhibited at low temperature (Q_{10} = 1.8) and by various agents known to inhibit nucleoside transport in other tissues (hexobendine, dipyridamole, papaverine), as well as by various adenosine analogues including 2′- and 5′-deoxyadenosine, 2-chloroadenosine, inosine, theophylline and isobutylmethylxanthine. Cytidine, guanosine, 2′-deoxyguanosine, thymidine, and uridine were rather less effective at inhibiting adenosine uptake. Free bases and sugars had no inhibitory effect [12-14]. Alteration of the structure by adding phosphate group(s) on the ribose ring changes the affinity of the compound for the carrier, as shown by the decreasing potency of adenine nucleotides as uptake inhibitors, with

increasing numbers of phosphate groups at the 5' position. The potency of adenine nucleotides as inhibitors of adenosine uptake in decending order is: AMP>>ADP>ATP, which raises the possibility that inhibition of uptake exhibited by various adenine nucleotides is dependent on their degradation to adenosine [13, 37]. This finding is also consistent with the suggestion that nucleotides can not be transported across the plasma membrane [24, 42, 121, 238]. Guanine nucleotides and uracil nucleotides are very weak inhibitors of adenosine uptake. Barman [11] suggested that these nucleotides are not as readily broken down to nucleosides as are adenine nucleotides. Similarly, the metabolically stable derivatives of adenosine such as NECA, L-PIA and CHA, which have potent and long-lasting pharmacological actions, are also weak inhibitors of adenosine uptake [230]. The competitive nature of the inhibition of adenosine uptake by various purine and pyrimidine nucleosides suggests that they all interact with the same carrier. Other studies, using cultured astrocytes [69], cultured astrocytoma [105], and guinea pig brain slices [93, 176], also demonstrated that the uptake of adenosine is mediated by a high affinity system.

Unfortunately, many of the above conclusions must be viewed with caution as the majority of the studies reflect both metabolism and transport processes rather

than transport alone. For example, long incubation periods (ranging from 30 s to 15 min at 37°C) used to study the kinetics of adenosine uptake by rat brain synaptosomes [12-15, 144, 149, 150, 230] were such that the levels of accumulation of cellular radioactivity reflected transport, metabolism and backflux of adenosine and metabolic products. In addition, it has been shown that synaptosomes apparently become increasingly labile as the duration of incubation increases [218].

Recent studies also show in addition to the rapid uptake system for which equilibration is achieved within 1 min of incubation, there is another uptake system which is equilibrated after approximately 30 min of incubation of rat cerebral cortical synaptosomes [12, 13]. The slow uptake system has been characterized [12] and shown to involve 2 high affinity uptake mechanisms with apparent K_m's of 1 (high affinity A) and 5 μM (high affinity B). Both uptake processes are partially inhibited by 2,4-dinitrophenol implying the presence of active uptake and diffusional components, and are partially sensitive to the presence of external sodium and calcium ions. A 25% reduction in adenosine uptake was observed when Na^+ or Ca^{2+} was removed from the incubation media [12]. Since synaptosomal preparations are known to be contaminated with glial fragments [41], it was suggested that the high-affinity A and B uptake systems could be of separate glial and neuronal origin, respectively [12].

2.1.4.1.1. Uptake inhibitors

The best studied and most useful ligand in the study of the adenosine transport system has been NBMPR, a member of a family of S-substituted 6-thiopurine nucleosides. NBMPR is a potent inhibitor of facilitated diffusion nucleoside transport in many cell types. Kinetic studies with erythrocytes have shown that the inhibition is competitive and associated with tight, but reversible, high-affinity binding of inhibitor to functional specific sites on the cell membrane (apparent K_d 0.1-1 nM) [28, 29, 44, 45, 78, 81, 151]. Such binding is competitively inhibited by transported nucleosides [27, 81] and the occupancy of NBMPR on its sites correlates with the inhibition of transport function [28, 97]. Furthermore, in contrast to zero-trans uridine influx studies, NBMPR is an apparent non-competitive inhibitor of zero-trans uridine efflux from nucleoside-permeable sheep erythrocytes [81]. These suggested that the erythrocyte NBMPR-binding site is identical to the nucleoside-translocation site and is largely located on the outer facing conformation of the carrier.

The view that the NBMPR binding site is identical to the nucleoside translocation site in erythrocytes is further supported by radiation inactivation of uridine fluxes and NBMPR-binding studies [79], which showed that the nucleoside translocation site and NBMPR binding site

have similar molecular weights in situ of 122000. Since this value is approximately 2 fold higher than that determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (molecular weight of 45000-65000) [237], the nucleoside transporter may exist in the membrane as dimer.

NBMPR also binds to a variety of brain preparations in a saturable, reversible and specific manner (apparent K_d 0.1-1 nM) [60, 61, 77, 113, 115]. The NBMPR binding site was identified as the adenosine uptake site and not the adenosine receptor site largely on the basis of pharmacologic evidence since adenosine uptake inhibitors (dipyridamole and dilazep) have proven to be substantially more potent inhibitors of [^{3}H]NBMPR binding than adenosine receptor ligands (cyclohexyladenosine, CHA) [61, 213]. Also studies using [^{3}H]NBMPR autoradiography to localize the uptake site in rat brain and compared it directly with the localization of [^{3}H]CHA binding sites showed conclusively that the uptake and the receptor sites are two distinct entities [20].

Although NBMPR has been used extensively as a high affinity probe to study the presence of transporter for adenosine and other nucleosides in various cell types [80, 214, 221], it must be carefully qualified in CNS. Several recent studies have shown that in guinea pig brain membranes there are approximately 2-fold more high-

affinity [^{3}H]dipyridamole binding sites (apparent K_d = 10 nM) than high-affinity [^{3}H]NBMPR binding sites [112, 114]. Inhibition of [^{3}H]dipyridamole binding by NBMPR was biphasic with only 40% of the binding displaced by NBMPR concentrations as high as 10 μM. Transport studies also shown that NBMPR only inhibits about 50% of the accumulation of adenosine by guinea pig slices [39]. All these findings suggest a multiplicity of adensoine transporters in guinea pig brain.

Several coronary vasodilators such as dilazep, dipyridamole, lidoflazine, hexobendine and papavarine [12, 75, 138, 208]; antipsychotic and sedative agents such as spiroperidol and sulpiride [229]; anxiolytic, hypnotic sedative agents such as benzodiazepines [145, 149]; antidepressant such as nortriptyline [229]; and antibiotics such as puromycin and formycin [152] are also capable of inhibiting nucleoside transport.

2.1.4.2. Metabolism

In addition to the high-affinity uptake system, which removes adenosine from the extracellular compartment and hence from access to extracellular receptors [2], adenosine is also subjected to enzyme inactivation [38, 157, 183].

2.1.4.2.1. Deaminase

Adenosine deaminase plays a key role in the normal catabolic pathways for adenosine. However this enzyme appears to be primarily a cytoplasmic enzyme and there is no evidence that it is present as an ecto-enzyme. The activity of the enzyme in a sucrose dispersion of rat cerebral cortex is 115 nmol/min/g of tissue with K_m of 54-57 μM [157]. Activity is found in all regions of the brain [40, 196].

The second deaminating enzyme is adenylate deaminase, which is involved in the metabolism of adenine nucleotides [34, 107, 186]. This enzyme may play a key role, not only in the regulation of intracellular purine nucleotide metabolism, but also in the deamination of extracellular 5'-AMP. The identification of adenylate deaminase as an ecto-enzyme in muscle [111] suggests that it may also exist as an ecto-enzyme in the CNS. An ecto-adenylate deaminase in the cerebral cortex could be responsible for the relatively high levels of inosine observed in cerebral cortical perfusates [82, 148]. The presence of large amounts of inosine in the extracellular fluid cannot readily be ascribed to the activity of adenosine deaminase if this enzyme exists only as an intracellular cytoplasmic entity, unless inosine is released directly by the tissues. Any inosine from ATP probably results from the formation and subsequent

conversion of 5′-AMP to 5′-IMP and then the hydrolysis of IMP by ecto-nucleotidase to form inosine.

2.1.4.2.2. Kinases

Like adenosine deaminase, adenosine kinase, which phosphorylates adenosine to nucleotides, is a cytoplasmic enzyme [7, 93]. However, it has been suggested that the regulation of intracellular adenosine levels by adenosine kinase may provide a major driving force for the uptake processes [152, 154]. If the transport and phosphorylation of adenosine are tightly coupled, the rate of uptake should not be higher than the rate of ATP synthesis necessary for the phosphorylation [8, 176]. Any inhibition of adenosine kinase activity will, therefore, resulted in a decrease in adenosine uptake [176].

The function of these pathways appears to be the maintenance of low levels of endogenous adenosine. Because of this, intracellular levels of adenosine would appear likely to remain at 1-2 μM or less under most physiological conditions. Similarly, extracellular adenosine levels probably also never rise under normal conditions above 1-2 μM [92].

2.1.5. Psychoactive drugs and adenosine

The blockade of adenosine uptake has been postulated to be the mechanism of the sedative effects displayed by some psychotropic drugs such as benzodiazepines, neuroleptics and meprobamate and zopiclone [143].

2.1.5.1. Benzodiazepines

The benzodiazepines (such as clonazepam, flurazepam, nitrazepam, lorazepam, oxazepam and diazepam) are a widely used group of drugs with anxiolytic, anticonvulsant and muscular relaxant properties. The central nervous system mechanisms through which their actions are accomplished have remained the subject of controversy. So far, studies have proposed the existence of at least 2 biochemically distinct benzodiazepine receptors in rat brain which are responsible for the mediation of different pharmacological activities [88]. Type I receptors, which are not coupled to GABA receptors, mediate anxiolytic actions. Type II receptors are coupled to GABA receptors and mediate pharmacological effects other than anxiolytic activity such as sedation and ataxia.

Purine nucleosides such as adenosine, guanosine and inosine were suggested to be the potential endogenous ligands for benzodiazepine receptors [116, 182]. Although this interaction occurs at concentrations higher

than basal adenosine levels, it may still be physiologically relevant since under conditions of excessive energy demand, adenosine levels can increase dramatically over their basal values [223] and only a small percentage of benzodiazepine receptors must be occupied to manifest a physiological effect [116, 182]. In vivo experiments have shown that the purine nucleosides may assert their anticonvulsant effects [117] and regulate food intake [103] through interactions with benzodiazepine receptors. Furthermore, a relationship between the adenosine and benzodiazepine systems in the CNS is further suggested by the numerous experiments that have demonstrated clear antagonistic effects when methylxanthine and benzodiazepine are administered simultaneously. In animals, methylxanthines antagonize benzodiazepine effects on neuronal firing, neurotransmitter and adenosine release [142] as well as their neurophysiological and behavioral effects [155, 156]. In human, various pharmacological effects of benzodiazepine are also reversed by the administration of caffeine [49, 120, 130] and aminophylline [128, 194] at non-toxic doses. This suggests that some of the actions of the benzodiazepine may be mediated in part by the adenosine system.

Though benzodiazepine agonists or antagonists do not interact with central adenosine receptors [219, 222], it has been demonstrated that benzodiazepines anxiolytics

potentiate various effects of adenosine and other purine nucleosides, both centrally [137, 140, 184] and peripherally [30] probably through an inhibition of the adenosine reuptake mechanism [62, 227]. This effect has been demonstrated by Phillis and Wu [143] who showed that the inhibition of adenosine reuptake by various benzodiazepines was correlated with their clinical, pharmacological and biochemical potencies. Thus suggesting that the inhibition of adenosine uptake is an important factor in the central actions of benzodiazepines. Though the inhibition of adenosine reuptake takes place at concentrations higher than those required to saturate benzodiazepine receptors [184, 234], it is possible that even a 20% decrease in reuptake is sufficient to double the action of adenosine [74].

The hypothesis that adenosine reuptake inhibition mediates the sedative action of benzodiazepines, as well as that of other psychotropic drugs [143], needs to be further substantiated, especially since the specific anti-anxiety effects of benzodiazepines have been claimed to be closely associated to their sedative properties [96] and since some clinically active benzodiazepines do not inhibit adenosine uptake. The mechanism by which benzodiazepine interacts with adenosine uptake is still unknown since neither the benzodiazepine receptor [124] nor the adenosine reuptake site [137] appear to be involved in the benzodiazepine-induced inhibition of

adenosine reuptake. Though the reciprocal interactions between adenosine analogues, methylxanthines and benzodiazepines and benzodiazepine drugs clearly suggest a common site of action [232], the nature of this site is still a matter of speculation.

2.2. Kidney

2.2.1. Presence of adenosine

Tissue measurements of adenosine clearly demonstrate significant levels of adenosine in the kidney [123, 134], but the distribution of the adenosine is not known. The renal content of this nucleoside has been reported to be in the order of 6 nmol/g in the rat [123, 134] and 7.6 nmol/g in the dog [123]. That at least some of the adenosine exists in the extracellular space is suggested by measurements demonstrating adenosine in both renal plasma [132] and urine [123, 157]. Spielman and Thompson [191] computed the concentration of total tissue adenosine assuming that adenosine was distributed throughout the total tissue water (assuming 0.8 ml water/g tissue), and the concentration of adenosine came out to be 7 μM in rats and 9.5 μM in dogs. Such levels of adenosine when infused into the renal artery of dogs

resulted in autoblockade[1] to a bolus injection of exogenous adenosine [173]. This would suggest that tissue adenosine is not uniformly distributed and is not necessarily available to the vascular compartment. This leads one to consider possible tissue stores or compartments for adenosine.

2.2.2. Sources of adenosine

2.2.2.1. Tissue stores of adenosine

One possible source of this compartmentalized adenosine may be an adenosine-binding protein. Such a protein has been isolated from renal tissue [132, 210]. The identity of this protein in kidney has not been elucidated, but it is probable that it is the enzyme S-adenosyl-L-homocysteine hydrolase [45, 50, 68, 210]. The calculated values for adenosine binding represent nearly 30% of the total renal content of adenosine [191]. Thus, the interpretation of total tissue content of adenosine must be viewed with caution. Furthermore, the levels of adenosine found in the urine and plasma [123, 134, 160] may contribute significantly to the total renal adenosine values.

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[1] Adenosine autoblockade is a phenomenon in which the constant infusion of adenosine to the kidney prevents the vasoconstrictor response to a bolus injection of adenosine [65, 173].

2.2.2.2. Adenosine production

The second possible source of adenosine in the kidney is through net adenosine production. The total tissue content has been shown to increase during periods of ischemia in dogs, cats and rats [123, 134]. That this adenosine can enter the extracellular spaces is shown by an increase in urine concentration and excretion of adenosine in the dog following renal ischemia [123]. However, there was no detectable increase in the renal plasma levels of adenosine in the above study. This latter result suggested it may be due to a localized production of adenosine preferentially into the tubular space or alternatively rapid uptake and/or degradation of adenosine in renal venous blood [123].

There are several possible precursor pools for adenosine formation in the kidney. Adenosine may be formed from 5'-AMP by the action of the enzyme 5'-nucleotidase, or it may be formed from S-adenosyl-L-homocysteine by S-adenosyl-L-homocysteine hydrolase [45, 123, 169, 172]. Although the production of adenosine from S-adenosyl-L-homocysteine has not been demonstrated in renal tissues, it has been shown that there is significant activity in the pathway leading to S-adenosyl-L-homocysteine formation from S-adenosyl-L-methionine [195].

Little is known about the localization of S-adenosyl-L-homocysteine hydrolase, but 5'-nucleotidase has been reported to be associated with the basolateral membranes of proximal tubular cells [123]. These studies do not rule out other possible sites of adenosine formation, but suggest that during ischemia the relatively high levels of adenosine in urine may be the result of adenosine formed in the proximal tubule and released directly into the tubular fluid. Although the concentration of adenosine formed in any specific segment of the renal tubule can not be determined from these studies, the relatively high concentration (1-5 μM) found in the urine during mannitol diuresis [123] is well above the threshold of activity for exogenously administered adenosine (10^{-8} M). This suggests that adenosine levels within the tubules may exert a potent physiological effect on renal function.

2.2.3. Effects of adenosine

Adenosine is generally known to be a potent smooth muscle relaxant; it dilates most vascular beds [57, 65] and relaxes smooth muscle of the small intestine [1] and isolated renal artery strips [215]. In contrast, when injected as a bolus directly into the renal artery, adenosine produces a prompt and dose-related reduction of renal blood flow in a variety of species [65, 125, 133, 135, 163, 174, 189, 199]. The renal vasoconstrictive

response to adenosine is viewed as energy sparing rather than counterproductive [132]. This is based on the understanding that lower solute and fluid filtration rates resulting from vasocontriction will cause a decrease in solute delivered to the tubular epithelium and, hence, diminish renal energy utilization through the reduction of solute and fluid reabsorption.

However, the continuous infusion of adenosine into the renal artery has been generally reported to produce a biphasic response in renal blood flow: a transient decrease in renal blood flow that reverses rapidly and returns to, or rises above, the preinfusion flow rate [136, 192, 197]. Despite the return of blood flow to normal levels during the continued infusion of adenosine, glomerular filtration rate (GFR) remains depressed [136]. The elevation of intrarenal adenosine from endcgenous sources also results in significant hemodynamic effects. For example, the intrarenal infusion of dipyridamole or maleic acid into sodium-depleted dogs elevates the renal venous and urinary concentrations of adenosine. This elevation of intrarenal adenosine is accompanied by a fall in the GRF which can be reversed by the intrarenal infusion of theophylline [4, 131]. Studies directed at the mechanism of the fall in GFR suggest that it results from the fall in glomerular hydrostatic pressure as a result of both preglomerular vasoconstriction and postglomerular dilation [136]. This renal hemodynamic

action of adenosine seems to be relatively unique; the adenine nucleotides, ATP and ADP, both produce a vasodilation of the renal vasculature. Likewise, the metabolic products of adenosine, i.e. inosine, hypoxanthine, xanthine and uric acid, are relatively inactive on the renal vasculature. On the other hand, a renal blood flow response very similar to that produced by adenosine is seen with the intrarenal infusion of 5'-AMP [57]. It is possible that the renal effects of 5'-AMP are due to its conversion to adenosine by ecto-5'-nucleotidase.

The mechanism by which adenosine produces its intrarenal hemodynamic effects is unclear. In other tissues adenosine has been reported to antagonize calcium influx during membrane depolarization [3] as well as to stimulate cAMP by activating adenylate cyclase [25]. Both calcium antagonism as well as increased cAMP are generally associated with decreased contraction of vascular smooth muscle [212], and, therefore, make explaining the renal vasoconstriction action of adenosine difficult. An attractive alternative hypothesis is that the vasoconstrictive action of adenosine in the kidney is due to the release or formation of another vasoconstrictive substance that mediates the response [131, 132, 190]. A possible renal vasoconstrictor is angiotensin II. Measurements of renin release and the angiotensin II concentration in renal lymph during the

infusion of adenosine have demonstrated that renal adenosine infusion decreases the formation of angiotensin II by the inhibition of renin release [188]. This adenosine-induced transient vasoconstriction and the fall in GFR can be blocked by angiotensin antagonists [131, 132, 190] suggesting that adenosine's vasoconstrictive action is likely being mediated by the renin-angiotensin system.

2.2.4. Inactivation of adenosine actions

It is widely held that through tissue washout, cellular adenosine uptake and/or degradation may control the effective adenosine concentration at the site of action in renal tissue.

2.2.4.1. Uptake

In renal tissues, extracellular adenosine has been reported to enter both the vascular and tubular compartments by a dipyridamole-sensitive uptake mechanism [4]. It is not known what the nature of the carrier is, but it is possible that it is an important point of controlling extracellular adenosine levels. It has been demonstrated that changes in uptake can affect the action of adenosine by infusing the nucleoside uptake inhibitors dipyridamole and dilazep and observing a potentiation of the effects of adenosine [4, 132, 164].

Recent studies have shown that nucleosides are actively accumulated in renal proximal tubular cells [94, 200]. The accumulation of nucleosides is likely to be a consequence of reabsorption processes rather than termination of adenosine actions. The accumulation of nucleosides by renal proximal tubular cells will be discussed in Section 2.2.5.1.1.

2.2.4.2. Metabolism

Once in the cell, adenosine may be either degraded to inosine by the enzyme adenosine deaminase or rephosphorylated to 5'-AMP by adenosine kinase. Both enzymes have been shown to exist in the kidney [76]. Inosine, an immediate degradation product of adenosine deamination [123, 134, 160], is further degraded to hypoxanthine by nucleoside phosphorylase [160]. The ultimate end product of adenosine degradation is uric acid [169].

2.2.5. Physiological roles of kidney

The kidneys perform two major functions: first, they excrete most of the end-products of bodily metabolism, and second, they control the concentrations of most of the constituents of body fluids and reabsorb substances of nutritional value to the body.

2.2.5.1. Reabsorption in renal proximal tubule

The proximal tubule of the mammalian kidney is the nephron site where the major portion (2/3 to 3/4) of the filtered salts (Na^+ 144 mM, K^+ 4 mM, Ca^{2+} 2.5 mM, Mg^{2+} 1.5 mM, Cl^- 114 mM, HCO_3^- 30 mM, PO_4^{2-} 2 mM, SO_4^{2-} 1 mM and organic acids 5 mM) is actively reabsorbed [99, 217]. This energy-consuming reabsorption process subsequently provides the main driving force for transepithelial water movement, thus maintaining an adequate and constant extracellular fluid and plasma volume.

In addition to the reabsorption of a very large fraction of the filtered salts and fluid, proximal tubular sodium transport also plays a key role in the reabsorption of some electrolytes and non-electrolytes, among them sugars, L-amino acids, phosphate, sulphate, as well as mono- and dicarboylic acids [162, 211]. It appears that downhill sodium movement from the lumen to the cell compartments across the luminal brush border membranes of proximal tubule cells provides the driving force for "uphill" (or concentrative) transport of these solutes into the cell by what has been termed "secondary active transport". The movement of sodium is downhill because the activity of the sodium pump (ie. ouabain-sensitive Na^+-K^+-ATPase) in the basolateral membrane maintains a low intracellular sodium concentration. The term "primary active transport" has been reserved for

this latter type of transport which is directly coupled to ATP hydrolysis [31].

2.2.5.1.1. Renal reabsorption of glucose and amino acids

Over 99% of the filtered glucose is actively reabsorbed by the proximal tubule. The reabsorption of glucose is coupled to the reabsorption of Na^+. Specifically, carrier-mediated transport of glucose across the brush border is coupled to passive entry of Na^+ across the brush border. The electrochemical gradient for Na^+ entry therefore provides the energy for the transport of glucose. In fact, in isolated brush border preparations glucose can be transported against a concentration gradient by a Na^+ concentration gradient [6, 70, 204-206]. The exact mechanism whereby the Na^+ electrochemical gradient facilitates the transport of glucose is not known. Nor has the glucose carrier been fully identified, although it can be selectively inhibited by phloridzin [207].

There are significant differences in glucose transport between early and late segments of the proximal tubule. Studies of isolated perfused rabbit tubules demonstrate that active glucose reabsorption occurs by a low-affinity, high-capacity process (K_m 1.6 mM, V_{max} 83 pmol/mm/min) in early proximal segments, and by a higher-affinity, lower-capacity process (K_m 0.35-0.70 mM, V_{max}

8-13 pmol/mm/min) in later segments [10]. Studies of isolated brush border membranes also reveal a similar low-affinity, high-capacity transport system (K_m 6 mM, V_{max} 10 nmol/mg protein/min at 40 mM NaCl at 17°C) which is strongly inhibited by phloridzin (K_i = 1.63 μM) in vesicles prepared from rat outer cortex (early proximal tubule), and a high-affinity, low-capacity transport system (K_m 0.35 mM, V_{max} 4 nmol/mg protein/min under the same experimental conditions) which is less sensitive to phloridzin inhibition (K_i = 51 μM) in vesicles from outer medulla (late proximal tubule) [201]. Also, the Na^+-glucose cotransport stoichiometry is 1:1 for the early proximal tubular system and 2:1 for the late proximal tubular system [202, 203]. The higher the Na^+-solute stoichiometry of a cotransport process, the steeper is the concentration gradient against which the Na^+-coupled reabsorption of the solute can proceed [5]. Thus, the high-affinity glucose transport system of the late proximal tubule is well suited for reclaiming the last traces of filtered glucose remaining after bulk reabsorption of glucose has occurred via the low-affinity system of the early proximal tubule.

The proximal tubule also reabsorbs over 95% of the filtered amino acids when plasma amino acid levels are normal [17, 22, 46, 177, 198, 235]. Like glucose reabsorption, the reabsorption of amino acids by the proximal tubule is also coupled to Na^+ reaborption [47,

48, 58, 162, 211]. Several proximal tubular amino acid transport systems have been identified, although the exact number of transport systems is not known. These transport systems include one for neutral amino acids (phenylalanine, histidine), one for basic amino acids (lysine, ornithine, arginine), one for acidic amino acids (aspartic acid, glutamic acid), one for -amino acids and the iminoglycine system (proline, glycine) [48, 52, 58, 59, 71, 166, 167, 170, 175]

Although glucose and amino acids entering kidney epithelial cells by crossing the brush border can probably be used by the kidney for metabolic fuel and for protein synthesis, most exit across the basolateral membrane to maintain the plasma constituents. The exit of metabolites from the epithelium across the renal basolateral membrane could be slowed and may well be the rate-limiting step in the overall reabsorption of metabolites from the glomerular filtrate [9]. Despite the importance of the basolateral membrane in the process of reabsorption, little is known about these transport pathways in mammalian renal epithelia. It is generally believed that metabolites accumulated within cells simply move down their concentration gradient across the basolateral membranes into the interstitial fluid before reaching the blood via a facilitated transport system.

Studies have shown that glucose is transported across the basolateral membrane by a Na^+-independent carrier-mediated process [84, 87, 106, 205, 211]. The transport is more rapid for D-glucose pyranose ring conformation [180], and seems to possess substrate specificity that is different from Na^+-dependent systems. The K_m for basolateral D-glucose uptake (150 mM) [106] is much higher than for brush border Na^+-dependent processes [10, 201]. This basolateral D-glucose uptake is inhibited by phloretin and cytochalasin B but it is not inhibited by phloridzin [178, 179]. Thus, the renal basolateral membrane transport system for glucose resembles the facilitated glucose transport system seen in a variety of cells including adipocyte, muscle, and erythrocyte [16, 56, 98, 220].

2.2.5.1.2. Renal reabsorption of adenosine

Studies have shown adenosine is reabsorbed by the mammalian kidney [94, 200]. Adenosine concentration in the plasma is normally in the 10-100 nM range [3], while the renal intracellular concentration is in the 10-20 μM range [123]. Therefore, to explain net reabsorption one would have to invoke either active adenosine transport from the tubular lumen into the cell or intracellular adenosine compartmentation. Loss of fluid from the proximal tubule could result in a rise in luminal concentration, but this would be insufficient to cause

movement of adenosine against a concentration gradient. Thus, a driving force is required for concentrative uptake of adenosine across renal brush border membrane.

Indeed, nucleoside transport in brush border membrane vesicles prepared from rat kidney proximal tubules has been shown to occur by a high-affinity sodium-dependent active transport process [100-102]. This transport system is very similar to that reported to exist for glucose [70, 73] and amino acids [211, 224, 226] in various epithelial cells by the ability to transport uphill in the presence of an inwardly directed gradient of Na^+ (Section 2.2.5.1.1). In cell types where nucleosides are taken up by facilitated diffusion, the rate of uptake of a given nucleoside is determined by its intracellular metabolism [152, 154]. The coupling to the transport of sodium at the luminal membrane of the proximal tubule makes the uptake of nucleosides largely independent of intracellular metabolism. Thus, the main function of the Na^+-dependent nucleoside uptake in renal brush border membrane is reabsorption from the glomerular filtrate rather than fueling intracellular metabolism.

The high affinity for nucleosides is also a remarkable feature of the nucleoside carrier in the renal brush border membrane when compared to other nucleoside carriers. The K_m's in the presence of 100 mM Na^+ have been found to be in the range of 1 to 9 μM for adenosine,

inosine, guanosine, 2'-deoxyadenosine, cytidine, uridine and thymidine [100-102], with adenosine appearing to have the highest affinity for transport (K_m 1.1 μM). These K_m values are one or two orders of magnitude lower than most of the K_m's which were found with other cell types [152, 236], but are in the range of the concentrations found in the serum [85, 94, 109, 160, 168, 185].

Studies on the substrate specificity of Na^+-dependent nucleoside transport in rat renal brush border membrane vesicles showed the K_i values for guanosine, inosine and 2'-deoxyadenosine when used as inhibitors of adenosine transport are close to the K_m's for the transport of these three compounds [101]. This suggested that these purine nucleosides might be the substrates for the same transport mechanism in rat kidney brush border membranes. However, the effect of pyrimidine nucleosides on this transport system was not identified.

In addition to the high affinity and the ability to transport nucleosides uphill, the Na^+-dependent nucleoside transport in kidney brush border membrane vesicles is also insensitive to NBMPR inhibition. All these observations sharply contrast with the mechanisms responsible for the facilitated diffusion of nucleosides studied in many cell types [18, 152, 153, 236].

In contrast to the transport process across the renal brush border membrane, little is known about the transport of adenosine across the basolateral membrane. One report had shown that the transport of adenosine into basolateral membrane was not saturable at adenosine concentrations as high as 800 μM [200], and was little affected by temperature (0°C vs 26°C) and dipyridamole (at 20 μM).

2.2.5.2. Renal excretion/secretion of glucose and amino acids

It is important to distinguish between secretion and excretion. Tubular secretion is the process whereby substances in the peritubular capillaries are transported across the tubular epithelium into the tubular lumen. Whereas excretion is the process where substances that filtered at the glomerulus are not totally reabsorbed by the nephron, thus be excreted.

Although glucose and amino acids are neither secreted nor excreted by the kidney under normal conditions [Section 2.2.5.1.1], the presence of excess glucose (glucourias) or amino acids (aminoacidurias) in the urine may occur as a result of disordered metabolism, defective transport, or a combination of the two.

Metabolic disorders account for a large percentage of glucourias and aminoacidurias. Under such conditions,

the plasma glucose or amino acid levels may exceed their renal threshold[2]. When the transport system is saturated by the filtered load, the excess load will be excreted.

In contrast, certain inherited disorders (eg. Fanconi's syndrome) in which renal tubular transport mechanisms are defective, glucourias and aminoacidurias occur without elevation of plasma glucose and amino acid levels, respectively. There are few potential sites where transport disorders can occur: (1) reabsorption site in brush border membrane, (2) exit site in basolateral membrane, and (3) the availability of Na^+ driving force. The latter can be caused by a reduction in number of Na^+ pumps at the basolateral membrane. Since the Na^+ gradient depends primarily on the low intracellular Na^+ concentration maintained by outward Na^+ pumping at the basolateral membrane, impaired Na^+ transport could explain all features of the tubular dyfunction. For the first two instances, the defect can be stereospecific. However in the last instance, lack of specificity is expected.

2.2.5.3. Renal excretion/secretion of adenosine

It has been shown that 2'-deoxyadenosine and its presumed nonmetabolizable analogue, 2'-deoxytubercidin,

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[2] Renal threshold represents that plasma concentration at which the filtered load exactly saturates the transport system.

are secreted by the kidney [94, 95]. The secretion of these nucleosides is probably real since the excess purine nucleoside present in urine was not due to "ion-trapping" in the acidic urine (pK_a's 3.8 and 5.3 for 2'-deoxyadenosine [48] and 2'-deoxytubercidin [95], respectively) nor to synthesis by the kidney tissue [94].

The mechanism for renal secretion of nucleosides is unknown. Studies using mouse kidney slices [95, 127] have suggested that the secretory process for 2'-deoxytubercidin and its sugar-modified analogues (3'-deoxytubercidin, arabinosyltubercidin and xylosyl-tubercidin) is probably identical with the organic cation secretory system known to secrete tetraethylammonium ion (TEA) and a wide variety of basic, organic compounds [159]. 2'-Deoxyadenosine, however, failed to meet the requisite criteria expected of a substrate for the organic cation secretory system. That is, 2'-deoxyadenosine did not inhibit TEA uptake [95] and cimetidine, a selective inhibitor of organic cation secretory [216] blocked TEA and 2'-deoxytubercidin but not 2'-deoxyadenosine secretion [127]. This suggested that the mechanism for the renal secretion of 2'-deoxyadenosine may be different and independent of the organic cation secretory system.

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3. Study 1 : Uridine transport in rat cerebral cortical synaptosomes[1]

3.1. 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 derived either from the reutilization of nucleosides already present or transported from the periphery since the brain lacks the complete enzymatic pathways for pyrimidine synthesis [25]. Secondly, there is a large body of evidence suggesting that the nucleoside adenosine is a neuromodulator of CNS functions [29, 34], and that transport of adenosine into surrounding cells is an important factor in terminating the action of adenosine [39]. 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 measure transport without the complication of metabolism. For example, the transport of adenosine by rat brain synaptosomes, and the effect of

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[1] A version of this section has been published. Lee, C.W., and Jarvis, S.M. (1988). Biochem. J. 249:557-564.

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 [4-7, 30-32, 40]. 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 to study the nucleoside transport in brain involves the use of [^{3}H]NBMPR, a potent specific inhibitor of facilitated-diffusion nucleoside transport in many cell types, as a binding probe [19, 41]. Site-specific, high-affinity NBMPR binding sites are present in rat brain membrane preparations [15, 17, 22, 35, 38], and [^{3}H]NBMPR has been used to localize transport sites by autoradiography [8, 14]. Nevertheless, conclusions based on these findings concerning nucleoside transport rely on the assumptions that: (a) there is only a single facilitated diffusion transport system which 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 by NBMPR further emphasizes the need to do so [3, 20, 33]. 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 [11]. Also, studies utilizing [^{3}H]dipyridamole, an inhibitor of facilitated diffusion nucleoside transport, have shown that there are approximately 2-fold more high-affinity [^{3}H]dipyridamole binding sites (apparent K_d 10 nM) in guinea pig brain sections and membranes than high-affinity [^{3}H]NBMPR binding sites [9, 23]. NBMPR only inhibited 50% of the [^{3}H]dipyridamole binding, but the nature of NBMPR-insensitive dipyridamole binding sites has not been investigated.

The objectives of this 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 it is poorly metabolized by rat cerebral cortical synaptosomes (see Table 3.2).

3.2. Methods

Synaptosome preparation

Snaptosomes were prepared by the method of Gray and Whittaker [16] as modified by White [36]. The cerebral cortex from two male Sprague-Dawley rats (200-250 g), sacrified by decapitation, was removed and homogenized in 30 ml of ice-cold sucrose solution containing: 0.32 M sucrose, 0.1 mM EDTA and 5 mM Hepes at pH 7.5. The homogenate was centrifuged at 1000 x g for 10 min at 4°C and the supernatant retained. The pellet (P_1) was washed once with the sucrose solution (1000 x g, 10 min) and the supernatant was pooled with that obtained from the first centrifugation. The pooled supernatants were centrifuged at 12000 x g for 20 min. The resultant pellet (P_2) was resuspended 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 (pH 7.5) that were centrifuged at 150000 x 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 containing: 120 mM NaCl, 4.75 mM KCl, 1.18 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.77 mM $CaCl_2$, 5.5 mM D-glucose, 5.8 mM sucrose and 26 mM $NaHCO_3$ at pH 7.5 [36]. The synaptosomal suspension was again centrifuged at 20000 x g for 20 min. Finally, the pellet (synaptosomes)

was suspended in ice-cold incubation medium and stored on ice pending assay on the same day. Protein was determined according to Lowry et al. [21] with bovine serum albumin as standard.

Enzyme assays

The purity of the synaptosomal preparations was determined using the following marker enzymes assayed in a Philips Pye Unican Spectrophotometer (model PU 8600): NADPH:cytochrome C oxidoreductase [28], rotenone-insensitive NADH:cytochrome C oxidoreductase [13], cytochrome C oxidase [10], 2′, 3′-cyclic nucleotide 3′-phosphohydrolase [27], and Na^+-K^+-ATPase [18].

The Na^+-K^+-ATPase activity was further assayed using synaptosomes treated with 0.1% deoxycholic acid to determine the membrane orientation.

Uridine transport by synaptosomes

A 10-µl aliquot of synaptosomes (100-200 µg protein) and 20 µl of [^{14}C]uridine (8 µCi/ml) were added separately on opposite sides of a polyethylene culture tube (16x100 mm) and preincubated at 22°C for 5 min. In inhibition studies, test compounds and [^{14}C]uridine were added simultaneously, with the exception of NBMPR, dipyridamole and dilazep, which were preincubated for 20 min with the synaptosomes. Test concentrations of NBMPR

and dipyridamole were diluted from 10 mM dimethyl sulfoxide stock. The transport of [^{14}C]uridine was initiated by continuous mixing such that the synaptosomes and the incubation medium came in contact. 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 fiber filter (GF/B) under suction. The filter was subsequently washed twice with 5 ml of ice-cold stop solution and dissolved in 4 ml of Scinti Verse Bio-HP (Fisher Scientific) after being dried. The radioactivity was determined by a LKB/Wallac 1217 liquid scintillation counter with automatic quench correction and disintegration per minute conversion. The entire stopping and washing process took less than 15 s, and from control experiments (see Fig. 3.1) no significant loss of [^{14}C]uridine from the synaptosomes occurred during the stopping and washing procedure. 'Blank' values for transport, attributable to non-specific binding of [^{14}C]uridine to the glass fiber filter and non-mediated permeation, were determined by using synaptosomes pretreated with 50 μM dipyridamole. These blanks were subtracted from measurements of uridine transport by synaptosomes to determine carrier-mediated transport rates. Radioactivity that became associated with synaptosomes during a transport interval of 0 s was

determined by using synaptosomes that were filtered immediately after exposure to medium containing both [^{14}C]uridine and dipyridamole (50 μM) at 4^{o}C. This value was not significantly different from that obtained in the absence of synaptosomes ($P<.01$). The loss of protein during filtration and washing was measured and amounted to approximately 5-10%. This loss was corrected for in calculating the transport rates. Kinetic constants for mediated transport (apparent K_m and V_{max}) were determined by nonlinear least squares fit of the equation:

$$v = V_{max} \cdot [S]/K_m + [S]$$

where v is the initial transport rate and [S] is the permeant concentration; the computer program "HYPMIC" [2] was used. Student's t-test was used to determine statistical significance.

The intrasynaptosomal volume was determined by a centrifugation method, using $^{3}H_2O$ for total water space and [^{14}C]inulin for extrasynaptosomal space determination [26]. The mean intrasynaptosomal volume from 16 determinations was 4.5 $\pm$ 0.5 μl/mg protein (mean $\pm$ S.E.), a value similar to those published previously [1, 24].

[^{3}H]NBMPR binding assay

Equilibrium [^{3}H]NBMPR binding assays were initiated by adding 10-μl aliquots of synaptosomes (100-200 μg protein) to graded concentrations of [^{3}H]NBMPR (0.05-

2 nM) in a total volume of 2 ml at 22°C in the presence or absence of 20 μM NBTGR, a competitive nonradioactive ligand. When inhibitors of binding activity were evaluated, incubation mixtures contained both test compounds and 0.1 nM [^{3}H]NBMPR. After 30-min incubation the samples were rapidly filtered (Whatman GF/B) and washed twice with 5 ml aliquots of ice-cold incubation medium. The filters were dried and counted for [^{3}H] radioactivity as described above. Specific binding was defined as the difference between the membrane content of [^{3}H]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 intrasynaptosomal products, the incubation was stopped with 1 ml of ice-cold stop solution and the synaptosomes were rapidly centrifuged (12000 x g, 15 s). The supernatant was removed and the pellet was immediately solubilized and deproteinized by the addition of 50 μl of ice-cold perchloric acid (7%, w/v). The precipitate was removed by centrifugation (12000 x g, 60 s). To analyze for possible extrasynaptosomal 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 perchloric acid. Aliquots of both types of acid extract were neutralized with 1 M $KHCO_3$ and centrifuged (12000 x 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 the chromatographic analysis: (a) 1-butanol/acetone/acetic acid/5% ammonia hydroxide/water (7:5:3:3:2, v/v) and (b) 1-butanol (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 ultraviolet light (R_f values for solvent (a) were 0.75, 0.8, 0.5, 0.2 and 0.1, and for solvent (b) were 0.6, 0.7, 0.05, 0 and 0 for uridine, uracil, UMP, UDP,and UTP, respectively). 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 was extracted with 1 ml of water by shaking for at least 15 min before the addition of scintillation fluid.

3.3. Results

Purity of synaptosomes

A selection of putative marker enzymes for various subcellular contituents such as mitochondrial inner and outer membranes, microsomes, myelin and glial, and neural plasma membranes were assayed in the synaptosomal preparations. The amount of contamination of synaptosomes by various nonsynaptosomal particles was calculated by taking the ratio of the specific activity of marker enzymes measured in the synaptosomal fraction to that measured in the initial homogenate. This value is expressed as a percentage (Table 3.1).

The microsomal contamination of the synaptosomal fractions was assessed by NADPH:cytochrome C oxidoreductase activity, and it amounted to approximately 7.5%. A 3 and 8% contamination was indicated for the mitochondrial inner (cytochrome C oxidase) and outer (rotenone-insensitive NADH:cytochrome C reductase) membrane markers, respectively. For the myelin and glia membrane marker, 4% of the 2′, 3′-cyclic nucleotide 3′-phosphohydrolase activity was detected in the synaptosomal fraction. 2′, 3′-Cyclic nucleotide 3′-phosphohydrolase activity has also been found to be associated with neural plasma membranes [42], and thus the actual myelin and glial contamination of the

synaptosomal preparation was even lower than the estimate (Table 3.1).

It is generally accepted that ouabain-sensitive Na^+-K^+-ATPase is a marker for the neural plasma membrane, and that the active site of this enzyme is facing intrasynaptosomally. Therefore the low specific enzyme activity detected in synaptosomes (26% of homogenate) indicates the presence of only small number of broken synaptosomes and/or inside-out synaptosomes. Indeed, the disruption of synaptosomes using 0.1% deoxycholic acid resulted in a substantial (40-fold) enhancement in the activity of Na^+-K^+-ATPase over that measured with intact synaptosomes (untreated), and 8-fold enhancement over that measured with deoxycholic acid treated homogenates.

These results suggest that the technique of Gray and Whittaker [16] as modified by White [36] offers a method of preparing highly purified intact synaptosomes with mostly outside-out orientation.

Stop solution

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 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. 3.1). These diluted synaptosomes were then filtered immediately or after a 5-90 s delay. Figure 3.1 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 Section 3.2). By contrast, stop solution at either 4 or 22^{o}C in the absence of dipyridamole resulted in a rapid loss of radioactivity from the synaptosomes. These results suggest that no significant loss of of ^{14}C-label is apparent with the ice-cold stop solution containing 50 μM dipyridamole, and thus this solution was routinely used in this study.

Metabolism

The data of Fig. 3.1 also indicate that approximately 40% of the radiolabel was retained by the synaptosomes after dilution at 22^{o}C. Calculation of the intrasynaptosomal concentration of [^{14}C]uridine yielded a value of 4 μM, which was close to that of the new extrasynaptosomal uridine concentration after dilution (3 μM), suggesting that both equilibration of uridine

across the synaptosomal membrane had occurred and that uridine was also not metabolized. The results of Table 3.2 confirm that no significant metabolism of uridine occurred within the first one minute of uptake, and >95% of the radioactivity co-chromatographed with uridine. The starting [^{14}C]uridine 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 extrasynaptosomal medium.

Time course of uridine influx

The time course of [^{14}C]uridine uptake (100 μM) for rat cerebral cortical synaptosomes in the presence of 20 μM NBMPR or 50 μM dipyridamole is shown in Fig. 3.2. The transport of [^{14}C]uridine by synaptosomes was linear for at least 10 s at 22^{o}C. Therefore, in subsequent kinetic experiments, initial rates of uridine uptake were determined by measuring the synaptosomal content of uridine after incubation intervals of 10 s at 22^{o}C. Under these conditions, the maximum intrasynaptosomal uridine levels did not exceed 10% of the extrasynaptosomal 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 μM) produced no further inhibition of uridine uptake. In

contrast, 20 μM NBMPR, a concentration 10^5-10^6 fold greater than the apparent dissociation constant for high affinity NBMPR binding to rat brain membranes [15, 17, 35, 38], only partially inhibited uridine influx.

Inhibition of uridine transport by transport inhibitors

To further investigate the effects of NBMPR and dipyridamole on uridine influx by rat cerebral cortical synaptosomes, dose response curves for inhibition of uridine transport by these compounds were compared (Fig. 3.3). 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 IC_{50} 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 uridi transport by rat cerebral cortical synaptosomes that can be distinguished on the basis of their sensitivity to inhibition by NBMPR. Fig. 3.3 also demonstrates that the dose response curve for inhibition of uridine transport by dilazep was also biphasic (approximate IC_{50} 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 with or without 500 nM NBMPR. Transport remaining in the presence of 500 nM NBMPR was defined as 'NBMPR-insensitive', whereas the difference between transport rates determined in the presence of 500 nM NBMPR and the total transport rates represents 'NBMPR-sensitive' transport.

Concentration dependence of uridine transport

The concentration dependence of uridine influx in the presence or absence of 500 nM NBMPR is shown in Fig. 3.4. Uptake of [^{14}C]uridine in the presence of 50 μM dipyridamole increased linearly with concentration (15 pmol/mg protein/s at 0.5 mM). By contrast, uridine transport by synaptosomes in the presence or absence of 500 nM NBMPR alone 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 transport component obeyed simple Michaelis Menten kinetics consistent with the existence of a single NBMPR-insensitive uridine transporter, the apparent K_m value was 180 $\pm$ 81 μM with V_{max} value of 17 $\pm$ 2.6 pmol/mg protein/s (estimates $\pm$ S.E.). The saturable NBMPR-sensitive transport component

of Fig. 3.4 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 with apparent K_m value of 135 ± 36 μM and V_{max} value of 12 ± 1 pmol/mg protein/s (estimates ± S.E.). The mean apparent K_m values for uridine influx for the NBMPR-sensitive and NBMPR-insensitive transport components from four separate experiments were 300 ± 51 and 214 ± 23 μM respectively, with V_{max} values of 12 ± 3 and 16 ± 3 pmol/mg protein/s respectively (means ± S.E.).

Inhibition of uridine transport by other nucleosides

The ability of other nucleosides to interact with the uridine transport system in rat cerebral 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 3.3 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 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. These results,

together with the determination of the K_m values for uridine transport, suggest that the NBMPR-sensitive and NBMPR-insensitive transport components of nucleoside transport in rat cerebral cortical synaptosomes exhibit similar affinities.

Uridine uptake versus binding

In the preceding sections, the [^{14}C]uridine associated with the synaptosomes 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 synaptosomes. Figure 3.5 shows the uptake of [^{14}C]uridine at equilibrium (5 minutes incubation) as a function of medium osmolarity. 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 intrasynaptosomal space without appreciable binding to the surface of the synaptosomes.

Concentration dependence of NBMPR binding

Using buffers identical to those of the transport assays, Fig. 3.6 shows the concentration dependence of [^{3}H]NBMPR binding to rat synaptosomes where membrane

associated binding is plotted against the equilibrium free concentration of the inhibitor. [^{3}H]NBMPR binding was resolved into two components, one of which was saturable whereas the other was proportional to the free [^{3}H]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 [^{3}H]NBMPR in the presence and absence of 20 μM NBTGR. Scatchard analysis (Fig. 3.7) of the specific binding data from Fig. 3.6 revealed a linear relationship, indicating a single population of high-affinity [^{3}H]NBMPR binding sites with an apparent K_d of 74 $\pm$ 16 pM and B_{max} of 421 $\pm$ 18 fmol/mg protein (estimates $\pm$ S.E.). The mean apparent K_d and B_{max} values for NBMPR binding from three different synaptosomal preparations were 58 $\pm$ 15 pM and 470 $\pm$ 26 fmol/mg protein, respectively (means $\pm$ S.E.).

Inhibition of NBMPR binding

Various compounds, including nucleosides and nucleoside transport inhibitors, were tested for their ability to inhibit the specific binding of [^{3}H]NBMPR to rat synaptosomes. Figure 3.8 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. 3.3). Adenosine was a more effective inhibitor than uridine. IC_{50} values were 60 and 700 μM respectively, a result consistent with the relative affinities of these two nucleosides for NBMPR-sensitive nucleoside transport (see Fig. 3.4 and Table 3.3). Addition of deoxycoformycin (0.1 μM), an inhibitor of adenosine deaminase, did not affect the IC_{50} value for adenosine inhibition of [^{3}H]NBMPR binding (Fig. 3.9). In other experiments, the nature of the inhibition by adenosine and uridine was explored using varying concentrations of both nucleoside and [^{3}H]NBMPR (Fig. 3.10). In both cases the inhibition profiles were consistent with a competitive type of inhibition with apparent K_i values of 15 $\pm$ 3 and 207 $\pm$ 20 μM for adenosine and uridine, respectively (estimates $\pm$ S.E.).

3.4. Discussion

This study shows 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 [4-7, 40], it was 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. Figure 3.1 clearly demonstrates that such a stop solution is unsatisfactory for quantitating uridine transport by rat cerebral cortical synaptosomes. In addition, these earlier studies also used incubation times that allowed substantial metabolism of adenosine to occur thereby complicating the interpretation of the data. The present results were obtained under conditions where initial rates of transport could be measured and no significant metabolism of radioactively labelled uridine could occur. 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. 3.3). 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. 3.4). These data demonstrate that 50 μM dipyridamole blocks

carrier mediated uridine transport in rat cerebral cortical synaptosomes, and therefore it is valid to use this concentration of dipyridamole to correct for non-mediated uridine uptake.

Transport of uridine by rat cerebral cortical synaptosome preparations was 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 can be defined as NBMPR-sensitive, was inhibited by nanomolar concentrations of NBMPR with an estimated apparent K_i value of approximately 0.35 nM and represented approximately 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 = 14 nM). Inhibition of uridine transport by NBMPR was associated with high affinity binding of [^{3}H]NBMPR to the synaptosomes. The dissociation constant for [^{3}H]NBMPR binding, 58 $\pm$ 15 pM (mean $\pm$ S.E.), was similar to values reported earlier using either crude P_2 fractions or synaptosomal membranes (range 0.05-0.18 nM) [15, 17, 22, 38]. Moreover, the similarity of the K_d value for [^{3}H]NBMPR binding with 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 transport 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 close to the inhibition constants for inhibiting high-affinity [^{3}H]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 [^{3}H]NBMPR binding in a competitive manner. These data contrast with earlier reports by Phillis and colleagues who reported major discrepancies between (1) the dissociation constant for [^{3}H]NBMPR binding in rat brain (0.05 nM) [38] and its K_i value for inhibition of adenosine uptake (30 μM) [6], and (2) the IC_{50} values for inhibition of [^{3}H]NBMPR and adenosine uptake into synaptosomes by nucleosides [6, 38]. As stated previously, 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. [15] that [^{3}H]NBMPR binds to adenosine-preferring transporter sites in rat brain that do not have a broad specificity for nucleosides. However, it does appear from this study 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 μM. Similarly, dilazep was also a much less potent inhibitor of this flux component (apparent K_i 1.5 μM) compared to inhibition of NBMPR-sensitive uridine transport by rat cerebral cortical synaptosomes. In other aspects, the two transport components are quite similar. Both mechanisms are facilitated diffusion systems with similar apparent K_m values for uridine. 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 [41]. Also, both transporters in rat cerebral cortical synaptosomes appear to be equally sensitive to inhibition by dipyridamole, as judged from the monophasic curve presented in Fig. 3.3.

In conclusion, the present results suggest that there are two components of facilitated diffusion nucleoside transport present in the 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.

3.5. References

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Table 3.1. Contamination of synaptosomal preparations by other subcellular constituents.

Marker Enzymes	Organelle	Specific marker enzyme activities in: Homo.	Synap.	% of Homogenate
NADPH:Cytochrome C Oxidoreductase	Microsome	8 ± 1	0.6 ± 0.2	7.5
Rotenone-insensitive NADH:Cytochrome C Oxidoreductase	Mitochon. outer Membrane	77 ± 14	6 ± 1	8
Cytochrome C Oxidase	Mitochon. inner Membrane	100 ± 21	3 ± 0.5	3
2′,3′-Cyclic Nucleotide 3′-Phosphohydrolase	Myelin and Glia Membrane	104 ± 18	4 ± 0.5	4
Na^+-K^+-ATPase	Plasma Membrane (intact)	15 ± 2	4 ± 1	26
Na^+-K^+-ATPase (0.1% deoxycholate)	Plasma Membrane (disrupted)	20 ± 6	160 ± 25	800

The specific enzyme activities expressed in nmol/mg protein/min are the means ± S.E. of two to four separate experiments. The percentage of homogenate is calculated as the ratio of specific enzyme activity of synaptosomes to that of the initial homogenates.

Table 3.2. Metabolism of [^{14}C]uridine by rat cerebral cortical synaptosomes.

[^{14}C]Uridine Concentration (μM)	Incubation Period	Distribution of radioactivity (% of total ^{14}C 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

Two concentrations of [^{14}C]uridine (5 and 100 μM) were incubated with synaptosomes for 10, 60 s and 30 min at 22^{o}C. Extraction and identification of the intrasynaptosomal and extrasynaptosomal products were performed as described in Section 3.2. Values are shown as a percentage distribution of total radioactivity analyzed from intrasynaptosomal extracts. Results for extrasynaptosomal extracts were not included as no product other than uridine was detected.
ND. Not detected.

Table 3.3. Effect of nucleosides on uridine transport by NBMPR-sensitive and NBMPR-insensitive transport components in rat cerebral cortical synaptosomes.

	NBMPR-sensitive		NBMPR-insensitive	
Nucleosides	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
2-Chloroadenosine	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

The uptake was initiated by addition of [^{14}C]uridine (final concentration 100 μM) and in the presence or absence of various nucleosides added simultaneously to the synaptosomes treated with or without NBMPR (500 nM). After 10 s at 22°C, uridine uptake was terminated as described in Section 3.2. IC_{50} values were obtained by extrapolation from the dose-response curves. K_i values were calculated from the equation $K_i = IC_{50}/(1 + L/K_m)$, where K_m values for NBMPR-sensitive and NBMPR-insensitive uridine transport were taken as 360 and 225 μM, respectively, and L = 100 μM uridine. Values are means ± S.E. of at least three separate experiments.

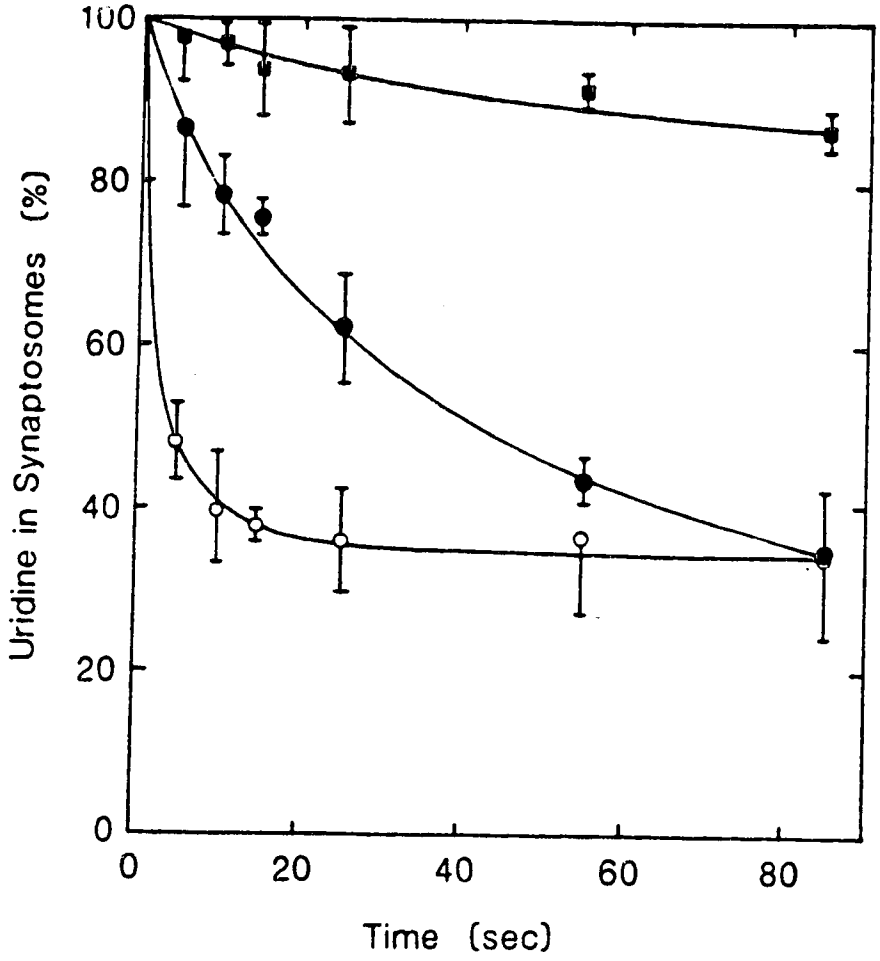

Fig. 3.1. Effect of various stop solutions on uridine influx by rat cerebral cortical synaptosomes. Synaptosomes were incubated with [^{14}C]uridine (100 μM) at 22°C for 30 s, and transport was terminated by the addition of 1 ml of stop solution composed of either incubation medium at 22°C (o), or ice-cold 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 synaptosomes is expressed as a percentage of the radioactivity retained at 0 s when ice-cold stop solution plus 50 μM dipyridamole was used. Values are means ± S.E. of triplicate determinations.

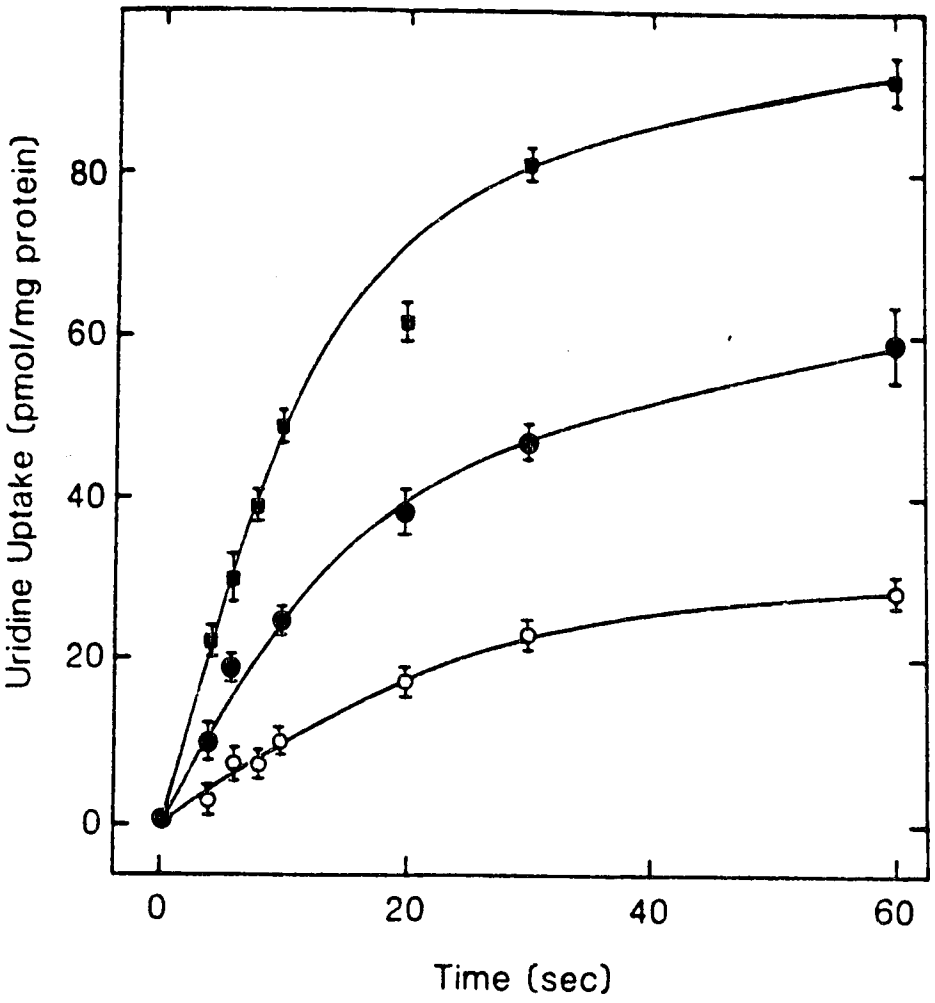

Fig. 3.2. Time course of uridine influx by rat cerebral cortical synaptosomes. Synaptosomes were preincubated with either buffer (■), 20 μM NBMPR (●) or 50 μM dipyridamole (o) for 20 min before the addition of [^{14}C]uridine (final concentration 100 μM). Values are means ± S.E. of triplicate determinations.

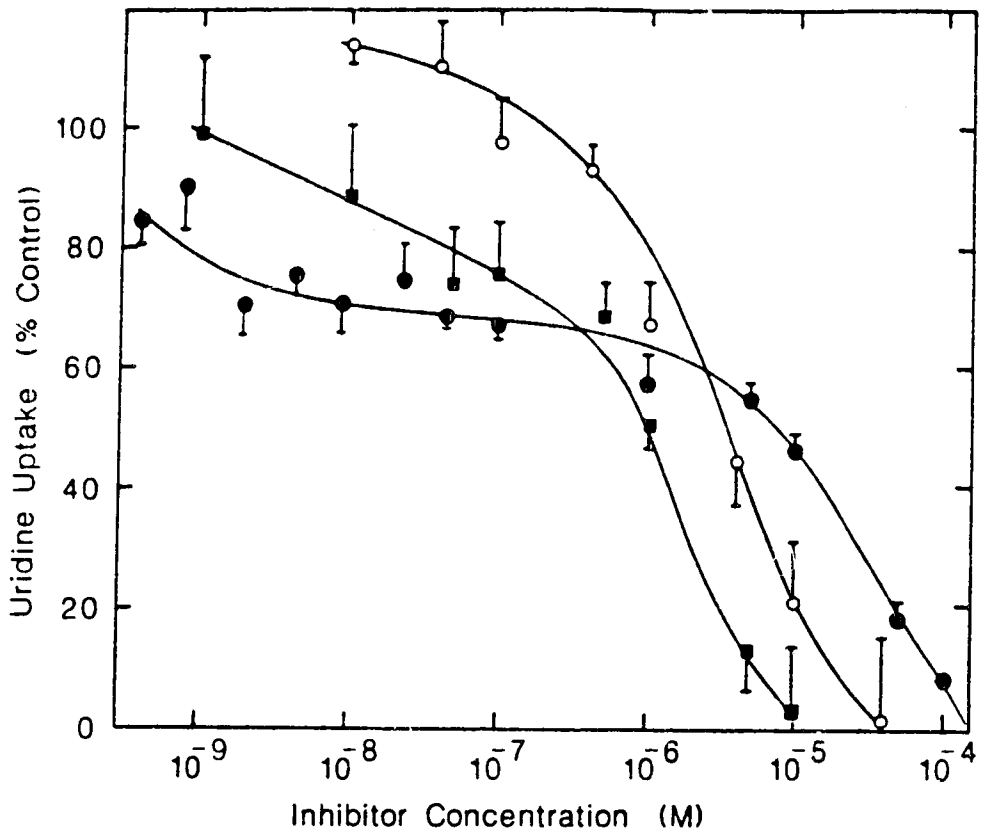

Fig. 3.3. Effect of NBMPR, dilazep and dipyridamole on uridine transport by rat cerebral cortical synaptosomes. Synaptosomes (0.1-0.2 mg) were preincubated with various concentrations of either NBMPR (●), dilazep (■) or dipyridamole (○). Transport of 100 μM [^{14}C]uridine was determined as described in Section 3.2, and the results are expressed as a percentage of the control transport rate versus the inhibitor concentration. The transport rates were corrected for non-carrier mediated transport determined in the presence of 50 μM dipyridamole. In the case of NBMPR, [^{3}H]NBMPR in the final concentration range 0-30 nM was used in parallel experiments to determine the final free concentration of the inhibitor. Values are means ± S.E. of at least three separate experiments conducted in triplicate. For clarity, data are presented with either positive or negative S.E..

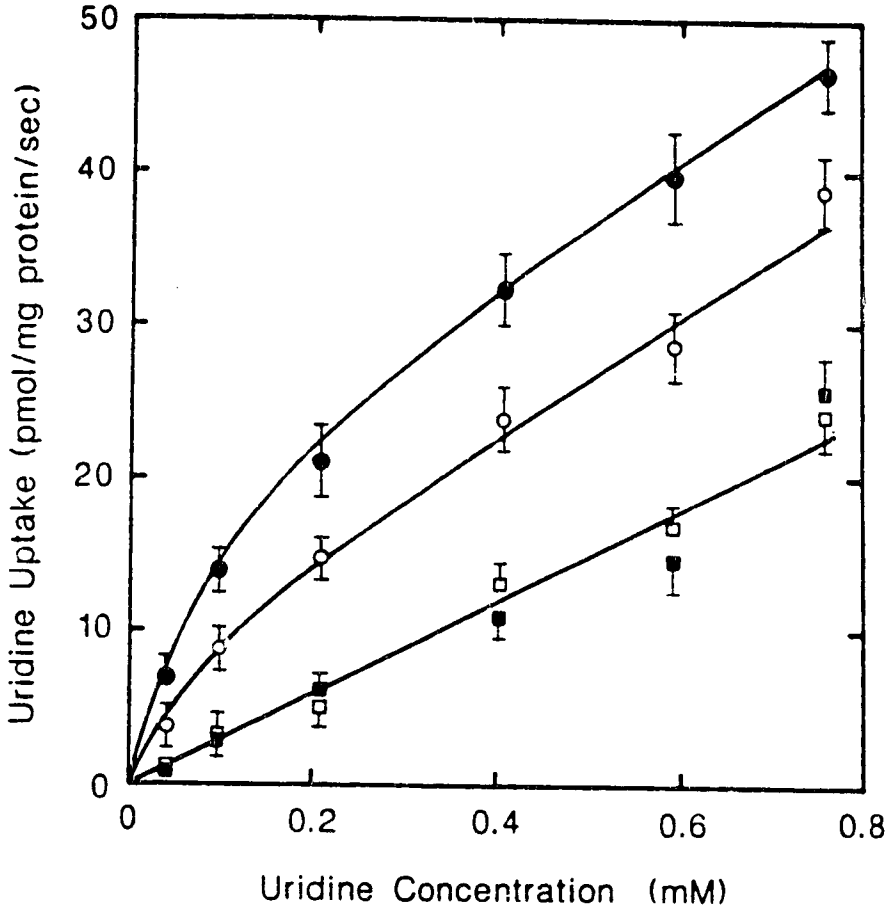

Fig. 3.4. Concentration dependence of uridine transport by control and NBMPR-treated rat cerebral cortical synaptosomes. [^{14}C]Uridine transport by synaptosomes in the presence (o) or absence (●) of 500 nM NBMPR or in the presence of 50 μM dipyridamole (□) or 50 μM dipyridamole plus 20 μM NBMPR (■) was determined as described in Section 3.2. The synaptosomes were preincubated for 20 min with inhibitors before addition of [^{14}C]uridine. The data are reported as means $\pm$ S.E. of triplicate determinations, for clarity, some data are presented with either positive or negative S.E.. 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 $\pm$ 36 and 180 $\pm$ 81 μM with V_{max} estimates of 12 $\pm$ 1 and 17 $\pm$ 2.6 pmol/mg protein/s for NBMPR-sensitive and -insensitive transport, respectively (estimates $\pm$ S.E.).

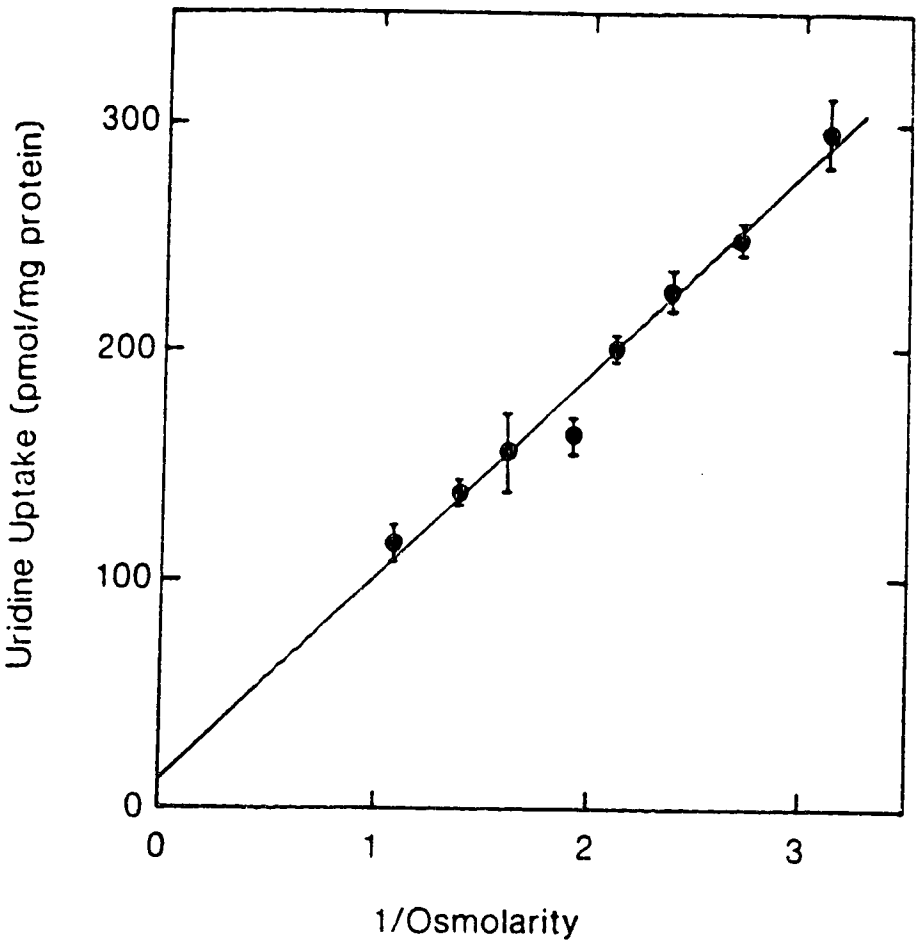

Fig. 3.5. Effect of extrasynaptosomal osmolarity on equilibrium uridine uptake by rat cerebral cortical synaptosomes. Uridine uptake by synaptosomes (5 min incubation, 100 μM [^{14}C]uridine) is plotted as a function of the reciprocal of the extrasynaptosomal osmolarity. Extrasynaptosomal osmolarity was adjusted with cellobiose and the osmolarity was calculated as the sum of contributions from all compounds, assuming ideal behaviour. The data are reported as means ± S.E. of triplicate determinations. The regression line was calculated by least-squares method ($r = 0.990$) with y-intercept value of 18.39 ± 11.26 pmol/mg protein (estimate ± S.E.).

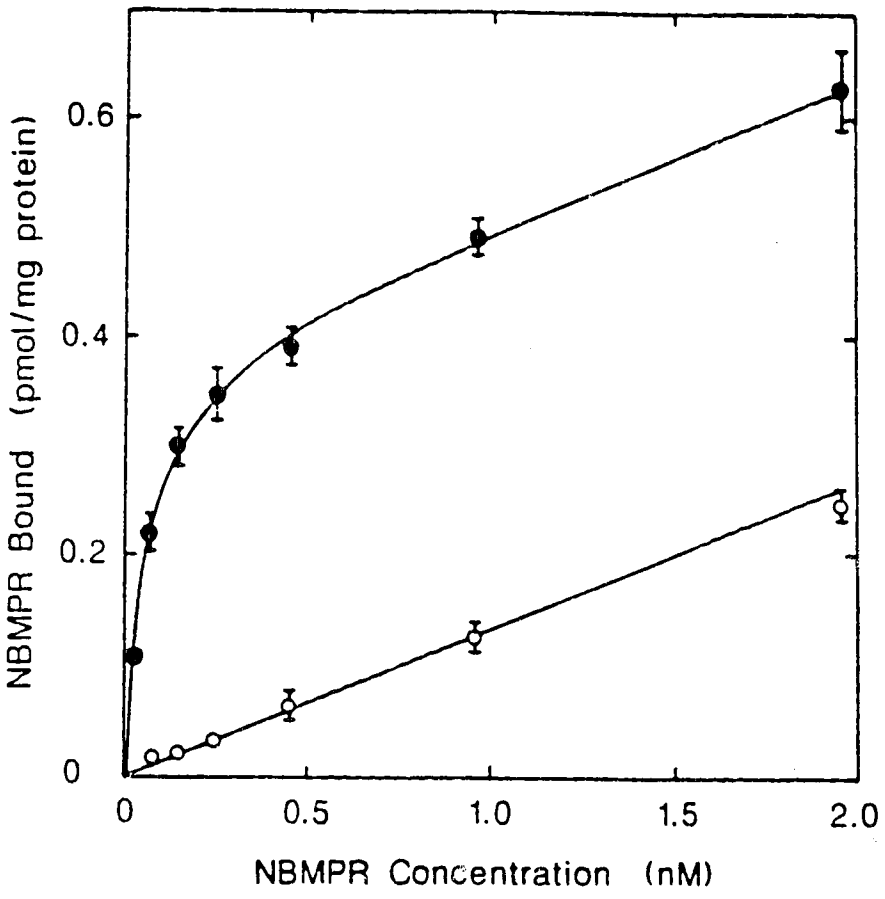

Fig. 3.6. Concentration dependence of [^{3}H]NBMPR binding to rat cerebral cortical synaptosomes. Synaptosomes were incubated with graded concentrations of [^{3}H]NBMPR for 30 min at 22°C in the absence (●) or presence (○) of 20 μM NBTGR. Specific binding of NBMPR represents the difference between membrane associated NBMPR in the presence and absence of NBTGR. Values are means ± S.E. of triplicate determinations, where absent, S.E. are smaller than symbol.

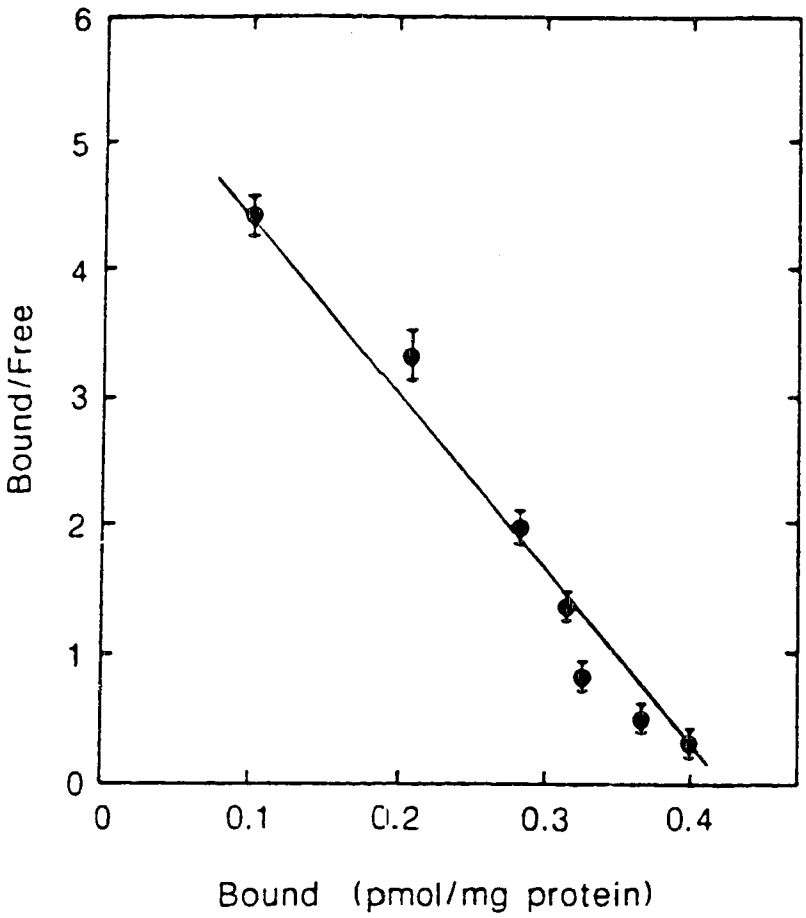

Fig. 3.7. Scatchard analysis of NBMPR binding data from Fig. 3.6. The regression line was calculated by least-squares method (r = 0.967). The data are reported as means ± S.E. of triplicate determinations. The binding constants for the data shown are K_d 74 ± 19 pM and B_{max} 421 ± 18 fmol/mg protein (estimates ± S.E.).

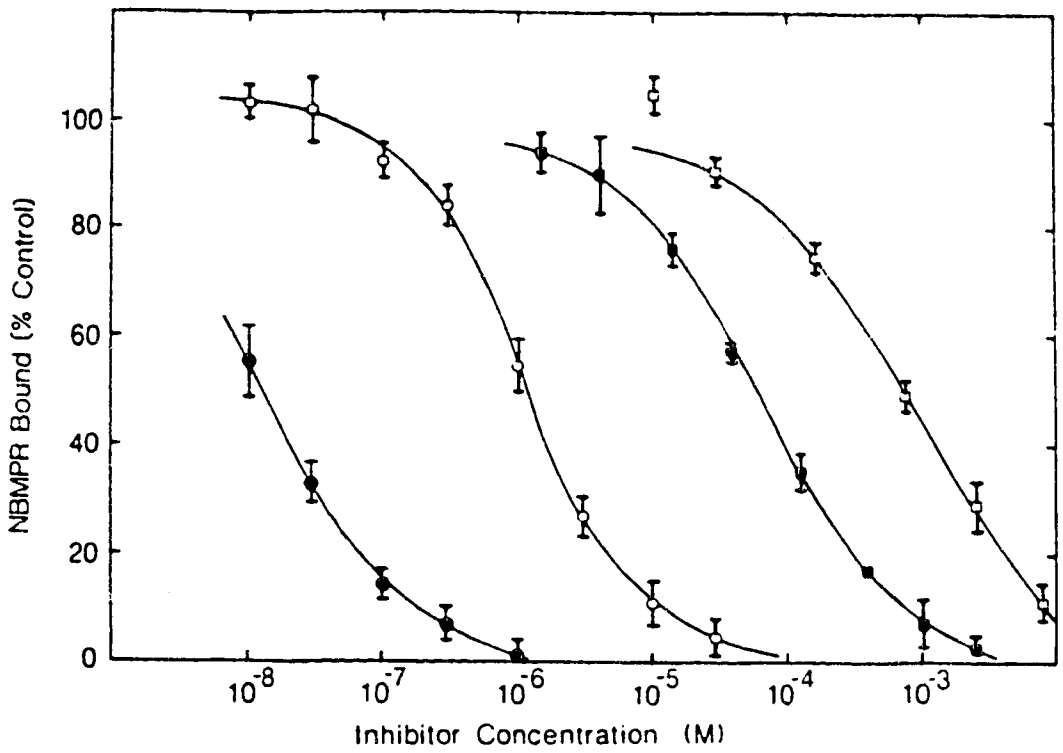

Fig. 3.8. Inhibition of site-specific binding of NBMPR to rat cerebral cortical synaptosomes. Synaptosomes were incubated with [^{3}H]NBMPR (0.1 nM) for 30 min at 22°C in the presence of either dipyridamole (o), dilazep (●), adenosine (■) or uridine (□). Results are plotted as a percentage of site-specific binding of NBMPR in the absence of inhibitor (480 fmol/mg protein). Values are means ± S.E. of triplicate determinations.

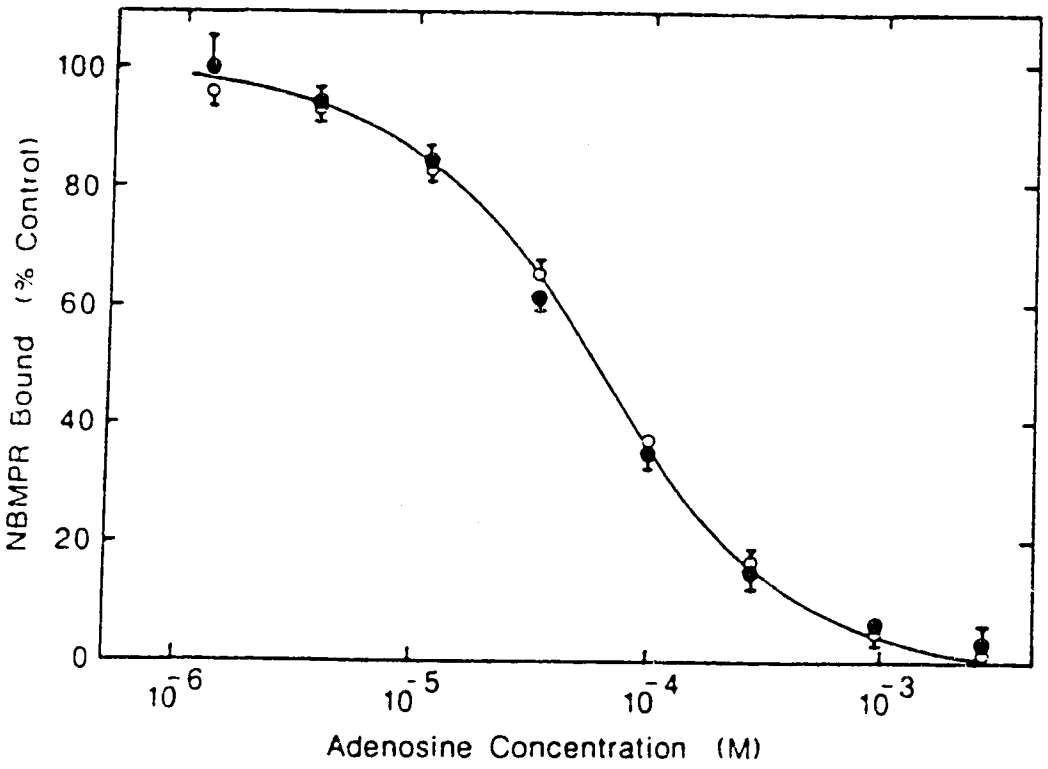

Fig. 3.9. Effect of deoxycoformycin on adenosine inhibition of site-specific NBMPR binding to rat cerebral cortical synaptosomes. Synaptosomes were incubated with [^{3}H]NBMPR (0.1 nM) and various concentrations of adenosine for 30 min at 22°C in the presence (●) or absence (o) of 0.1 μM deoxycoformycin. Results are plotted as a percentage of site-specific binding of NBMPR in the absence of adenosine versus the adenosine concentration. Values are means ± S.E. of triplicate determinations, for clarity, data are presented with either positive or negative S.E..

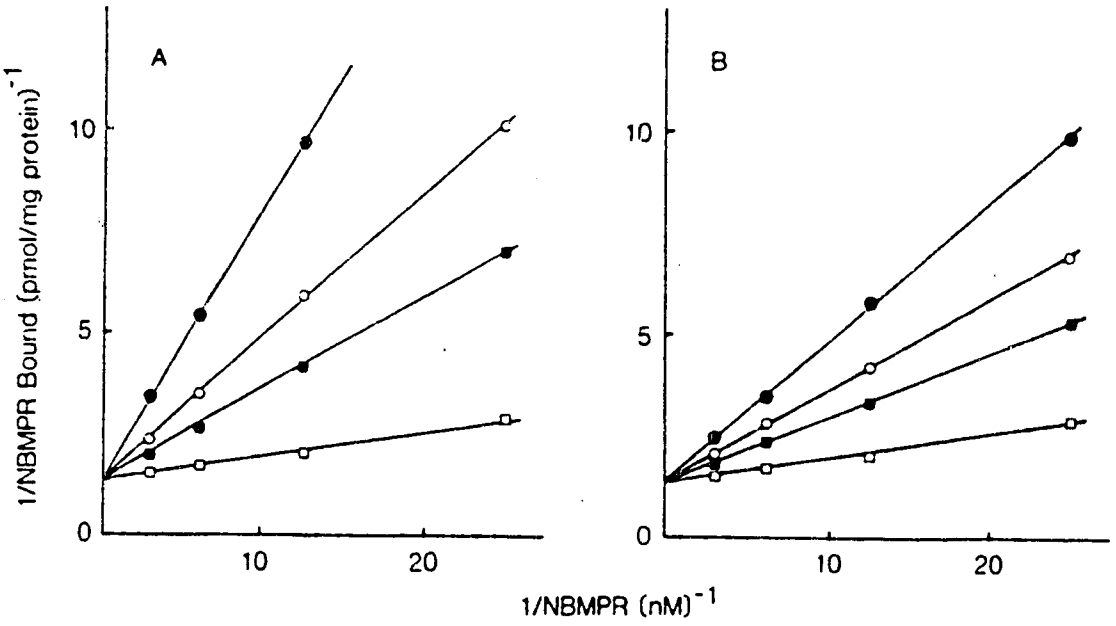

Fig. 3.10. Effect of adenosine and uridine on [^{3}H]NBMPR binding to rat cerebral cortical synaptosomes. The reciprocals of [^{3}H]NBMPR bound to specific sites in the presence of graded concentrations of adenosine (A) and uridine (B) at 22°C are plotted against the respective reciprocals of the free equilibrium concentrations of [^{3}H]NBMPR. (A) Initial concentrations (μM) of adenosine were 0 (□), 50 (■), 100 (○), and 200 (●). (B) Initial concentrations (μM) of uridine were 0 (□), 250 (■), 500 (○), and 1000 (●). Apparent K_i values for adenosine and uridine were 15 ± 3 and 207 ± 20 μM, respectively (estimates ± S.E.).

4. Study 2 : Adenosine transport in guinea pig cerebral cortical synaptosomes[1]

4.1. Introduction

Adenosine influences many body processes and there is substantial evidence supporting a neuromodulatory role for adenosine in the central nervous system. Adenosine and related analogues depress the spontaneous firing of neurons [8, 37], inhibit the release of several neurotransmitters [12, 13, 19], regulate cAMP formation [43] and display sedative properties and analgesic actions when administered [9, 35]. These effects are mediated by specific receptors on the extracellular surface of membranes [6, 7, 10, 43]. The level of adenosine in nervous tissue and the termination of the actions of adenosine are regulated primarily by the activity of facilitated diffusion transporter systems [46]. Inhibitors of these transporter systems potentiate the effects of adenosine [46]. It has also been suggested that benzodiazepines, drugs with anxiolytic, anticonvulsant and muscular relaxant properties, exert their effects by inhibiting adenosine uptake in brain [34, 36, 38, 40].

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[1] A version of this section has been published. Lee, C.W., and Jarvis, S.M. (1988). Neurochem. Int. 12:483-492.

As described earlier (Section 3.1), one approach to investigate the properties of adenosine transporters in brain has been to use [^{3}H]NBMPR, a potent specific inhibitor of nucleoside transport by facilitated diffusion in a wide variety of cells, as a ligand probe for the transporter [22, 48]. High-affinity, saturable [^{3}H]NBMPR binding sites (apparent K_d 0.1-1.0 nM) have been demonstrated in a variety of brain preparations [14, 17, 18, 23, 29]. The ability of various compounds to inhibit reversible [^{3}H]NBMPR binding to nervous tissue has been used as a mean to predict the potency of the compound to inhibit adenosine transport [14, 17, 33, 44]. However, the results from Section 3 have indicated that there are two components of facilitated diffusion nucleoside transport present in the rat cerebral cortical synaptosomes that differ in their sensitivity to inhibition by NBMPR. This leads to the suggestion that there are NBMPR-sensitive and -insensitive transport sites in the rat central nervous system. It is possible that such a multiplicity of nucleoside transporters could also be present in other mammalian species. Thus, the objectives of this study were to measure adenosine transport into guinea pig cerebral cortical synaptosomes to examine the effect of nucleoside transport inhibitors and several benzodiazepines on transport. Many of the earlier studies [1, 3-5, 15, 39, 40] used incubation times (30 s up to 15 min) such that there was metabolism

of adenosine by adenosine deaminase and adenosine kinase and the possible loss of adenosine by efflux during the incubation of adenosine with the cerebral synaptosomes. As a result, the measured accumulation of cellular radioactivity probably did not accurately reflect adenosine transport. In this study, an experimental protocol developed from an earlier study (Section 3) was used to measure the kinetic properties of adenosine transport without the complications arising from metabolism or efflux.

4.2. Methods

Synaptosome preparation

Synaptosomes were prepared according to the method of Gray and Whittaker [16] as modified by White [45] and was described in Section 3.2. The synaptosomal membranes were suspended in ice-cold incubation medium containing: 120 mM NaCl, 4.75 mM KCl, 1.18 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.77 mM $CaCl_2$, 5.5 mM D-glucose, 5.8 mM sucrose and 26 mM $NaHCO_3$ at pH 7.5 [45]. Synaptosomes were used on the same day of preparation. Protein was determined according to Lowry et al. [27] with bovine serum albumin as standard.

Adenosine transport by synaptosomes

The transport of [^{3}H]adenosine (50 μCi/ml) was measured using an inhibitor-stop filtration method similar to that described previously (Section 3.2). Briefly, a 10-μl aliquot of synaptosomes (100-200 μg protein) and 20 μl of [^{3}H]adenosine (final concentration 15 μM) were added separately on opposite sides of a polyethylene culture tube (16x100 mm) and preincubated at 22^{o}C for 5 min prior to initiation of the assay. In inhibition studies, test compounds and [^{3}H]adenosine were added simultaneously with the exception of NBMPR, dilazep and dipyridamole which were preincubated with the synaptosomes for 20 min. The transport of [^{3}H]adenosine

was initiated by continuous mixing with a vortexer such that the synaptosomes and the incubation medium came in contact. 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 fiber filter (GF/B) under suction. The filter was subsequently washed twice with 5 ml of ice-cold stop solution and dissolved in 4 ml of Scinti Verse Bio-HP (Fisher Scientific) after being dried. The radioactivity was determined using a liquid scintillation counter (LKB/Wallac 1217) with automatic quench correction and disintegration per minute conversion. 'Blank' values for transport, attributable to non-specific binding of [^{3}H]adenosine to the glass fiber filter and non-mediated permeation, were determined using synaptosomes pretreated with 50 μM dipyridamole. These blanks were subtracted from measurements of adenosine transport by synaptosomes to determine carrier-mediated transport rates. Radioactivity that became associated with synaptosomes during a transport interval of 0 s was determined using synaptosomes that were filtered immediately after exposure to medium containing both [^{3}H]adenosine and dipyridamole at 4^{o}C. This value was not significantly different from that obtained in the absence of synaptosomes ($P<.01$). The loss of protein during filtration and washing was measured and amounted

to approximately 5-10%. A correction was made for this loss of protein in calculating transport rates. Kinetic constants for mediated transport (apparent K_m and V_{max}) were determined by non-linear least squares fit of the equation:

$$v = V_{max} \cdot [S]/K_m + [S]$$

where v is the initial transport rate and [S] is the permeant concentration, using the computer programme "HYPMIC" [2]. Student's t-test was used to determine statistical significance.

[^{3}H]NBMPR binding assay

Equilibrium [^{3}H]NBMPR binding assays were initiated by adding 10-μl aliquots of synaptosomes (100-200 μg protein) to graded concentrations of [^{3}H]NBMPR (0.05-2 nM) in a total volume of 2 ml at 22^{o}C in the presence or absence of 20 μM NBTGR, a competitive nonradioactive ligand. When inhibitors of binding activity were evaluated, incubation mixtures contained both test compounds and 0.1 nM [^{3}H]NBMPR. After 30-min incubation the samples were rapidly filtered (Whatman GF/B) and washed twice with 5-ml aliquots of ice-cold incubation medium. The filters were dried and their radioactive content was determined as described above. Specific binding was defined as the difference in membrane content of [^{3}H]NBMPR in the presence and absence of 20 μM NBTGR.

Adenosine metabolism

Synaptosomes were incubated for 10 s, 1 and 30 min with either 5 or 50 μM [^{3}H]adenosine at 22^{o}C. To identify the intrasynaptosomal products, the incubation was stopped with 1 ml of ice-cold stop solution and the synaptosomes were rapidly centrifuged (12000 x g, 15 s). The pellet was retained and immediately solubilized with 50 μl of ice-cold perchloric acid (7%, w/v). The precipitate was removed by centrifugation (12000 x g, 60 s). To analyze for possible extrasynaptosomal products of adenosine metabolism, the synaptosomes were centrifuged at the end of the incubation period without dilution. Fifty microlitres of the synaptosome-free incubation medium were diluted with 50 μl of perchloric acid. Samples of both types of acid extracts were neutralized with 1 M $KHCO_3$ and centrifuged (12000 x g, 60 s) to remove precipitate. Supernatant (25 μl) was then chromatographed on both cellulose and silica-gel coated plates impregnated with fluorescent indicator, using (a) 1-butanol/ethylacetate/methanol/ammonium hydroxide (7:4:3:4) and (b) 1-butanol/acetone/acetic acid/ammonium hydroxide/water (7:5:3:3:2) as solvents, respectively. The standards (adenosine, adenine, inosine, AMP, ADP and ATP) were co-chromatographed with the samples. After drying, the individual standards were localized under ultraviolet light. R_f values for solvent (a) were 0.48, 0.35, 0.21, 0, 0 and 0, and solvent (b) were 0.76, 0.63,

0.67, 0.44, 0.26 and 0.11 for adenosine, adenine, inosine, AMP, ADP and ATP, respectively. Zones were scraped off the plate into scintillation vials and the remainder of the chromatogram was divided into 0.5-cm horizontal strips. The radioactivity associated with each sample was then extracted with 1 ml of water by shaking for at least 15 min before the addition of 10 ml of scintillation fluid.

4.3. Results

Stop solution

The previous study (Section 3) had demonstrated that ice-cold stop solution containing 50 μM dipyridamole, an inhibitor of nucleoside transport by facilitated diffusion, was able to prevent the rapid loss of radiolabelled uridine from rat cerebral cortical synaptosomes during filtration and washing. Figure 4.1 showed that this stop solution was also effective at preventing the loss of [^{3}H]adenosine from the guinea pig cerebral cortical synaptosomes. By contrast, stop solution without dipyridamole, at either 4°C or 22°C, resulted in a rapid loss of radioactivity from the synaptosomes. Thus, dipyridamole containing stop solution was employed in this study.

Metabolism

The results of Table 4.1 show that under the present experimental conditions no significant metabolism of adenosine occurred within the first 10 s of uptake, and >90% of the radioactivity co-chromatographed with adenosine. However, after a 30-min incubation with synaptosomes, a substantial amount of [^{3}H]adenosine was metabolized. Adenine nucleotides were found exclusively in the intrasynaptosomal extract (Table 4.1) while

inosine was found mainly in the extrasynaptosomal medium (Table 4.2).

Time course of adenosine influx

The time course of [^{3}H]adenosine influx (15 μM) for guinea pig cerebral cortical synaptosomes in the presence of 20 μM NBMPR, or 50 μM dipyridamole is shown in Fig. 4.2. The influx of [^{3}H]adenosine by synaptosomes was almost linear for about 5 s at 22^{o}C. Therefore, in subsequent kinetic experiments, initial rates of adenosine transport were determined by measuring the synaptosomal content of adenosine after incubation intervals of 5 s at 22^{o}C. Under these conditions, there was no significant metabolism of adenosine (see Table 4.1) and the maximum intrasynaptosomal adenosine levels did not exceed 20% of the extrasynaptosomal concentrations under most circumstances. Dipyridamole was a potent inhibitor of adenosine transport and reduced the rate of influx to one-fifth. In contrast, 20 μM NBMPR, a concentration 10^5-10^6 fold greater than the apparent dissociation constant for high affinity binding to guinea pig brain membranes [17, 18, 23, 44], only partially inhibited adenosine influx. To test for the possibility that adenosine binding may be contributing to the adenosine transport values, the time course of [^{3}H]adenosine binding to lysed synaptosomes in the presence and absence of 50 μM dipyridamole was determined

(Fig. 4.3). [^{3}H]Adenosine binding reached a steady state value by 60 s of approximately 10 pmol/mg protein at an extrasynaptosomal adenosine concentration of 16 μM. Dipyridamole (50 μM) had no effect on the time course of adenosine binding and the final equilibrium value for binding. This result demonstrates that the present protocol only determines adenosine transport and that there is no contribution from direct [^{3}H]adenosine binding to the synaptosome preparation.

Inhibition of adenosine transport by transport inhibitors

To further investigate the effect of nucleoside transport inhibitors on adenosine influx by guinea pig cerebral cortical synaptosomes, the dose response curves for inhibition of adenosine transport by NBMPR, dilazep and dipyridamole were compared. Figure 4.4 shows that the inhibition curves for both NBMPR and dilazep were biphasic; about 60% of the transport activity was inhibited by NBMPR and dilazep with IC_{50} values of 0.7 and 1 nM, repectively. No further inhibition was observed until the NBMPR and dilazep concentration exceeded 1 μM. The wide plateau in the inhibition curves suggested that there may be two mechanisms for adenosine transport by guinea pig cerebral cortical synaptosomes that can be distinguished on the basis of their senitivity to inhibition by either NBMPR or dilazep. Figure 4.5 demonstrates that the dose response curve for

the inhibition of adenosine transport by dipyridamole was shallow and consistent with two different components (approximate IC_{50} values for the two phases were 9 nM and 6 μM). The dipyridamole-sensitive adenosine transport component was abolished by pretreating the synaptosomes with 500 nM dilazep or NBMPR (Fig. 4.5). Thus, further characterization of these two transport components was performed using synaptosomes pretreated with or without 500 nM NBMPR. Transport remaining in the presence of 500 nM NBMPR was defined as NBMPR-insensitive while the difference between transport rates determined in the presence and absence of 500 nM NBMPR represents NBMPR-sensitive transport.

Na^+-independent adenosine influx

Recent studies have demonstrated that in some mammalian cells nucleoside transport proceeds via a Na^+-dependent system that is insensitive to inhibition by NBMPR [20, 25, 26]. Thus, the effect of replacing Na^+ with K^+ on the rate of adenosine transport was investigated. The time course of adenosine influx (15 μM) in the presence and absence of 20 μM NBMPR with an initial 100 mM NaCl gradient (out>in) was identical to that observed with a 100 mM KCl gradient (out>in) (Fig. 4.6).

Concentration dependence of adenosine transport

The concentration dependence of adenosine transport in the presence and absence of 500 nM NBMPR was resolved into two components: (i) a linear component, and (ii) a saturable component which obeyed simple Michaelis Menten kinetics. Figure 4.7 shows the carrier mediated transport component after subtraction of the non-carrier mediated transport component estimated in the presence of 50 μM dipyridamole. The apparent K_m values of adenosine transport for the NBMPR-sensitive and -insensitive transport components from 8 separate experiments were 17 $\pm$ 3 and 68 $\pm$ 8 μM, respectively, with V_{max} values of 2.8 $\pm$ 0.3 and 6.1 $\pm$ 0.4 pmol/mg protein/s, respectively (means $\pm$ S.E.). The difference in transport affinity for these two transport components was also observed with uridine as a permeant (Fig. 4.8), with K_m values of 117 $\pm$ 30 and 238 $\pm$ 55 μM; V_{max} values of 7.5 $\pm$ 1.4 and 13.7 $\pm$ 3.9 pmol/mg protein/s for NBMPR-sensitive and -insensitive transport components, respectively (estimates $\pm$ S.E.).

Inhibition of adenosine transport by other nucleosides

The ability of other nucleosides to interact with the adenosine transport mechanisms in guinea pig cerebral cortical synaptosomes was studied by investigating the effects of inosine, thymidine and uridine on adenosine

transport by NBMPR-treated and untreated synaptosomes. Figure 4.9 shows that adenosine transport by untreated synaptosomes was inhibited by these nucleosides and thymidine was the most effective inhibitor. The apparent K_i values estimated from IC_{50} values for inhibition of adenosine transport by the NBMPR-sensitive and -insensitive transport mechanisms were, respectively, 107 ± 12 and 164 ± 15 μM for thymidine, 109 ± 24 and 235 ± 14 μM for uridine, and 265 ± 10 and 403 ± 16 μM for inosine (means ± S.E. of three separate experiments). The apparent K_i values for uridine were similar to the apparent K_m values measured directly.

Inhibition of adenosine transport by benzodiazepines

Various benzodiazepines were also tested for their effect on adenosine transport by guinea pig cerebral cortical synaptosomes. Table 4.3 shows that adenosine transport by the NBMPR-sensitive and -insensitive transport components was effectively inhibited by diazepam and midazolam, with apparent K_i values of 36 and 80-160 μM, respectively. The peripheral binding site ligand, Ro 5-4864, also inhibited adenosine transport by both transport components with a potency slightly less than that of the benzodiazepine agonists. Interestingly, the benzodiazepine Ro 11-3128 was 20-fold more potent as an inhibitor of NBMPR-sensitive adenosine transport compared to NBMPR-insensitive transport.

Concentration dependence of NBMPR binding

Figure 4.10 shows the concentration dependence of [^{3}H]NBMPR binding to guinea pig cerebral cortical synaptosomes where membrane associated binding is plotted against the equilibrium free concentration of the inhibitor. [^{3}H]NBMPR binding was resolved into two components, one of which was saturable, whereas the other was proportional to the free [^{3}H]NBMPR concentration. NBTGR (20 μM) eliminated saturable binding but had no effect on the linear component. Hence, specific binding is defined as the difference in membrane content of [^{3}H]NBMPR in the presence and absence of 20 μM NBTGR. A Scatchard plot (Fig. 4.11) of the specific binding data from Fig. 4.10 revealed a linear relationship, indicating a single population of high-affinity [^{3}H]NBMPR binding sites with an apparent K_d of 78 $\pm$ 14 pM and B_{max} of 619 $\pm$ 25 fmol/mg protein (estimates $\pm$ S.E.). The mean apparent K_d and B_{max} values for NBMPR binding from three different synaptosomal preparations were 88 $\pm$ 6 pM and 632 $\pm$ 12 fmol/mg protein, respectively (means $\pm$ S.E.).

Inhibition of NBMPR binding

Nucleoside transport inhibitors such as dipyridamole and dilazep were tested for their ability to inhibit specific binding of [^{3}H]NBMPR to guinea pig cerebral cortical synaptosomes. Figure 4.12 shows dilazep was

more potent than dipyridamole with calculated K_i values of 0.3 and 1.5 nM for dilazep and dipyridamole respectively. These values were similar to those calculated for inhibiting NBMPR-sensitive adenosine transport (Figs. 4.4 and 4.5). The dipyridamole inhibition curves were not parallel to that observed with dilazep suggesting the presence of a small population of NBMPR binding sites (10-20% of the total) that have a relatively low affinity for dipyridamole. A previous study also noted NBMPR binding site heterogeneity in guinea pig brain cortical membranes [17].

4.4. Discussion

During the past decade much attention has been focused on the neuromodulatory properties of adenosine in the nervous system and the role that adenosine uptake plays in these processes [46]. However, most of the studies have investigated adenosine uptake (a process that represent both transport and metabolism) rather than adenosine transport alone. In this study the detailed kinetics, substrate specificity and inhibitor susceptibility of adenosine transport into guinea pig cerebral cortical synaptosomes at 22°C were investigated.

The data presented in this study demonstrate that there are two distinct components of adenosine transport in guinea pig cerebral cortical synaptosomes that are most readily distinguished on the basis of their sensitivity to inhibition by the transport inhibitors, NBMPR, dilazep and to a lesser extent, dipyridamole. The first is inhibited by nanomolar concentrations of NBMPR with an estimated K_i value of approximately 0.4 nM and represents approximately 50-60% of the total adenosine flux at 15 μM extrasynaptosomal adenosine. Dilazep and dipyridamole were also potent inhibitors of this system (apparent K_i values, 0.5 and 4.8 nM, respectively), and this transport system is defined as NBMPR-sensitive. It is of interest to note that the proportion of adenosine transport that was sensitive to either NBMPR, dilazep or

dipyridamole inhibition was about the same (about 60%) suggesting the possibility that these compounds bind to the same site on the synaptosome membranes. Consistent with this view is the previous finding that dilazep and dipyridamole are competitive inhibitors of [^{3}H]NBMPR binding to guinea pig cortical membranes [17]. The second transport route, defined as NBMPR-insensitive, was not inhibited by concentrations of NBMPR, dilazep and dipyridamole as high as 1 μM. Both transport components are saturable facilitated-diffusion systems of broad specificity. Replacement of Na^+ gradient (out>in) with K^+ had no effect on adenosine influx. The systems do not discriminate between purine and pyrimidine nucleosides, although adenosine had the highest affinity of the nucleosides tested, having an apparent K_m for influx of 17 $\pm$ 3 and 68 $\pm$ 8 μM for the NBMPR-sensitive and -insensitive transport components, respectively (means $\pm$ S.E.). Similarly, the affinity of the NBMPR-insensitive transport component is approximately one-half less for uridine, thymidine and inosine than the NBMPR-sensitive system. Previous results have suggested that a similar difference in substrate affinities for NBMPR-sensitive and -insensitive nucleoside transport is also observed in rat erythrocytess [24] and Chinese hamster ovary cells [41]. However, results from earlier study (Section 3) demonstrated no difference in the affinity of these two transport components in rat cerebral cortical

synaptosomes. A further difference between nucleoside transport in rat and guinea pig cerebral cortical synaptosomes is that dipyridamole inhibits the NBMPR-sensitive transport component with an approximate 600-fold increased affinity than the NBMPR-insensitive transport component in guinea pig (IC_{50} values of 9 nM and 6 μM, respectively), while both transport components in rat cerebral cortical synaptosoms are equally sensitive to dipyridamole inhibition with an IC_{50} value of 3 μM (Fig. 3.3). The low sensitivity of rat nucleoside transporters to dipyridamole has been observed for many different tissues and is a unique characteristic of rat [18, 24, 42, 44, 47, Section 3].

NBMPR-sensitive and -insensitive adenosine transport has been detected using guinea pig brain slices [11]. It is not known if the two transporters are evenly dispersed throughout the cerebral cortex or whether the heterogeneity detected represents the anatomical complexity of the cortex. Further experiments will be needed to examine the detailed distribution of adenosine transporters within the cortex.

The usefulness of NBMPR as a binding probe for nucleoside transport sites in brain has been questioned since there is a wide discrepancy between the concentration of NBMPR required to inhibit adenosine uptake (approximately 1 μM) and its binding potency (0.1-

1 nM) [39, 46]. The present findings do not support those arguments. The results show that the K_i value of NBMPR inhibition of NBMPR-sensitive adenosine transport is similar to that of the apparent K_d value of NBMPR binding to guinea pig cerebral cortical synaptosomes. Moreover, the order of potency of nucleosides as inhibitors of NBMPR binding to guinea pig cortical membranes [17], is similar to the affinity of nucleosides for the NBMPR-sensitive transporter. In both cases adenosine had the highest affinity. Therefore, the present results demonstrate that there is no major discrepency between the concentrations required to inhibit NBMPR-sensitive adenosine transport and the K_d value of NBMPR binding to brain membranes. The reason for the difference between these findings and those of Phillis and Wu [39, 46] is that these latter workers determined adenosine uptake rates rather than adenosine transport rates. Uptake rates reflect metabolism, nucleoside entry and efflux and passive diffusion, and therefore inhibition of total adenosine uptake cannot be interpreted as inhibition of membrane transport. For example, the Hill coefficient for NBMPR inhibition of adenosine uptake by guinea pig synaptosomes is calculated to be only 0.19 [39], indicating multiple processes.

Benzodiazepines have been proposed to produce some of their central effects through the inhibition of cellular accumulation of adenosine [34, 36, 38, 40]. In

this study, benzodiazepines apparently have a low affinity for both the NBMPR-sensitive and -insensitive routes in guinea pig cerebral cortical synaptosomes (K_i for diazepam of 36-81 μM) compared to their affinities for the "central neuronal" benzodiazepine binding site (K_i for diazepam of 7.4 nM) [31]. Furthermore, the peripheral site ligand Ro 5-4864 also inhibited adenosine transport with a similar affinity to diazepam although its affinity for the "neuronal" benzodiazepine binding site is at least 10^4 less than that of diazepam. These data extend the earlier observations that benzodiazepines were both weak inhibitors of NBMPR binding to brain membranes [17, 33] and total adenosine transport by rat synaptosomes [32]. The present results, together with earlier observations, demonstrate that the sedative and anxiolytic actions of benzodiazepines are not due to inhibition of adenosine transport. It is interesting to note that the R conformation of the benzodiazepine, [R]-5-[o-chlorophenyl] 1,3-dihydro-3-methyl-7-nitro-2H-1, 4 benzo-diazepin-2-one (Ro 11-3624) is 50-fold more potent as an inhibitor of NBMPR-insensitivie adenosine influx than the corresponding S stereoisomer Ro 11-3128 (K_i values of 11 $\pm$ 2 and 500 $\pm$ 82 μM, respectively). No difference in affinity for these two benzodiazepine isomers was observed for NBMPR-sensitive adenosine transport.

In conclusion, the present results suggest that there are separate NBMPR-sensitive and -insensitive nucleoside transporter sites in the central nervous system. These separate sites appear to be present in a wide range of species, guinea pig (this study), rat (Section 3) and rabbit (Appendix 4.1), and can be detected by direct transport studies and possibly by the use of ligand probes [28, 30]. The relationship between these two types of transporter are unknown but they may represent either different functional states of the same protein or products of closely related genes.

4.5. References

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Table 4.1. Metabolism of [^{3}H]adenosine by guinea pig cerebral cortical synaptosomes: Intrasynaptosomal products.

[^{3}H]Adenosine Concentration (μM)	Incubation Period	Distribution of radioactivity (% of total ^{3}H recovered)			
		Ado	Ino	Ade	Nucleotides
5	10 s	95	ND	ND	5
	60 s	86	ND	ND	14
	30 min	8	9	4	79
50	10 s	100	ND	ND	ND
	60 s	93	2	ND	5
	30 min	60	8	5	27

Two concentrations of [^{3}H]adenosine (5 and 50 μM) were incubated with synaptosomes for 10, 60 s and 30 min at 22^{o}C. Extraction and identification of the intrasynaptosomal products were performed as described in Section 4.2. Values are shown as a percentage distribution of total radioactivity analyzed from intrasynaptosomal extracts.
ND - not detected.

Table 4.2. Metabolism of [^{3}H]adenosine by guinea pig cerebral cortical synaptosomes: Extrasynaptosomal products.

[^{3}H]Adenosine Concentration (μM)	Incubation Period	Distribution of radioactivity (% of total ^{3}H recovered)			
		Ado	Ino	Ade	Nucleotides
5	10 s	100	ND	ND	ND
	60 s	100	ND	ND	ND
	30 min	88	12	ND	ND
50	10 s	100	ND	ND	ND
	60 s	100	ND	ND	ND
	30 min	98	2	ND	ND

Two concentrations of [^{3}H]adenosine (5 and 50 μM) were incubated with synaptosomes for 10, 60 s and 30 min at 22^{o}C. Extraction and identification of the extrasynaptosomal products were performed as described in Section 4.2. Values are shown as a percentage distribution of total radioactivity analyzed from extrasynaptosomal extracts.
ND - not detected.

Table 4.3. Effect of benzodiazepines on NBMPR-sensitive and NBMPR-insensitive transport of [^{3}H]adenosine by guinea pig cerebral cortical synaptosomes.

	K_i (μM)	
Benzodiazepines	NBMPR-sensitive	NBMPR-insensitive
Diazepam	36 ± 7	81 ± 12
Midazolam	36 ± 3	162 ± 48
Ro 11-3624	36 ± 6	11 ± 2
Ro 11-3128	27 ± 6	500 ± 82
Ro 5-4864	105 ± 11	163 ± 20

The transport was initiated by addition of [^{3}H]adenosine (final concentration 15 μM) and in the presence or absence of various benzodiazepines simultaneously to the synaptosomes treated with or without NBMPR (500 nM). After 5 s at 22°C, adenosine transport was terminated as described in Section 4.2. 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 adenosine transport were taken as 17 and 68 μM, respectively, and L = 15 μM adenosine. The values are the means ± S.E. of 3 separate experiments.

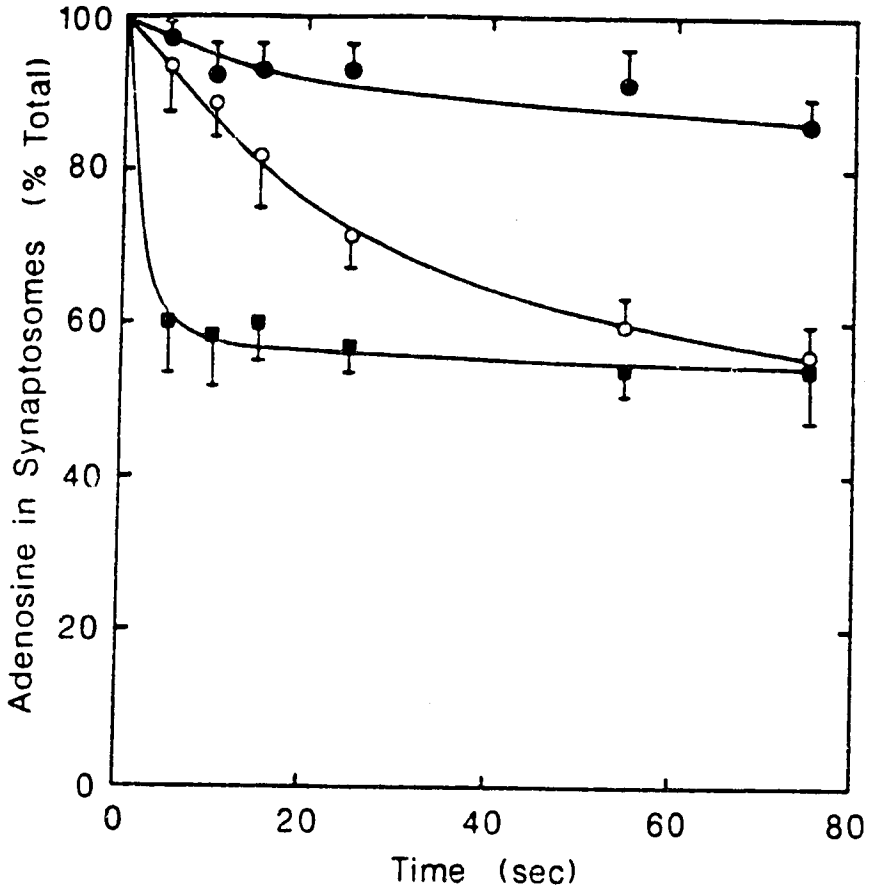

Fig. 4.1. Effect of various stop solutions on adenosine influx by guinea pig cerebral cortical synaptosomes. Synaptosomes were incubated with [^{3}H]adenosine (15 μM) at 22^{o}C for 30 s, and the transport was terminated by the addition of 1 ml of stop solution composed of either incubation medium at 22^{o}C (■), ice-cold incubation medium (o), 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 synaptosomes is expressed as a percentage of the radioactivity retained at 0 s when ice-cold stop solution plus 50 μM dipyridamole was used. Values are means $\pm$ S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E..

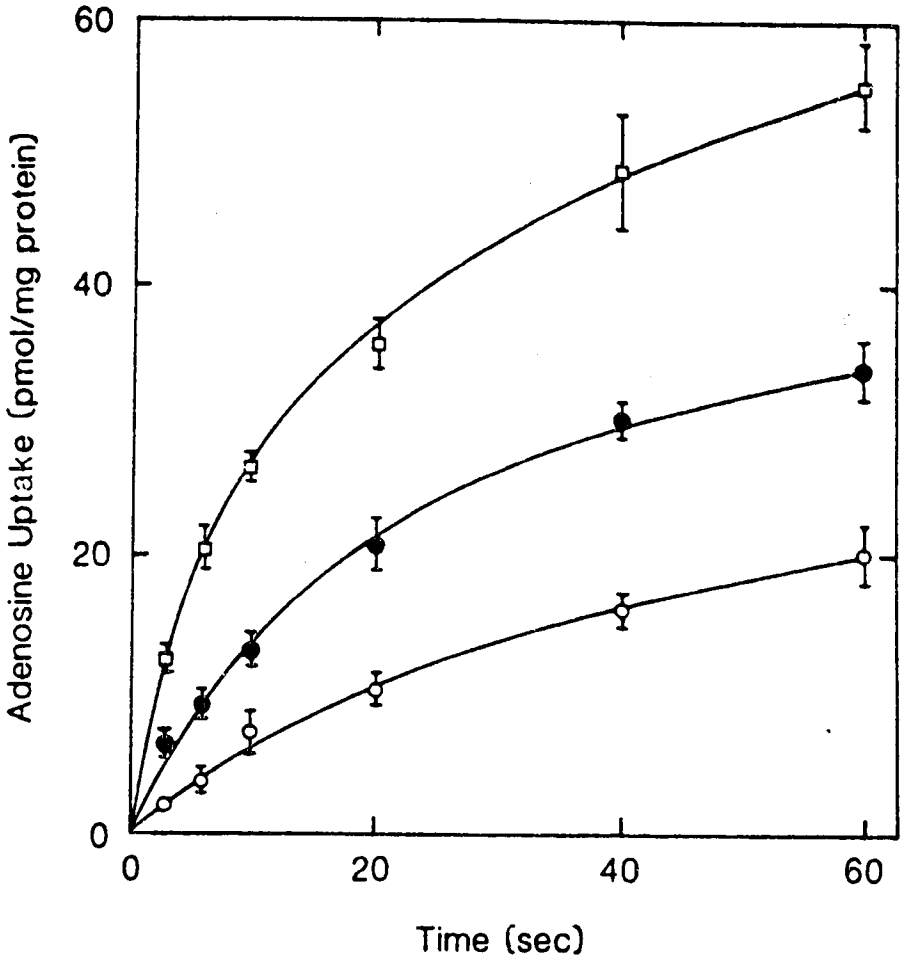

Fig. 4.2. Time course of adenosine influx by guinea pig cerebral cortical synaptosomes. Synaptosomes were preincubated with either buffer (□), 20 μM NBMPR (●) or 50 μM dipyridamole (o) for 20 min before the addition of [^{3}H]adenosine (final concentration 15 μM). Values are means ± S.E. of triplicate determinations.

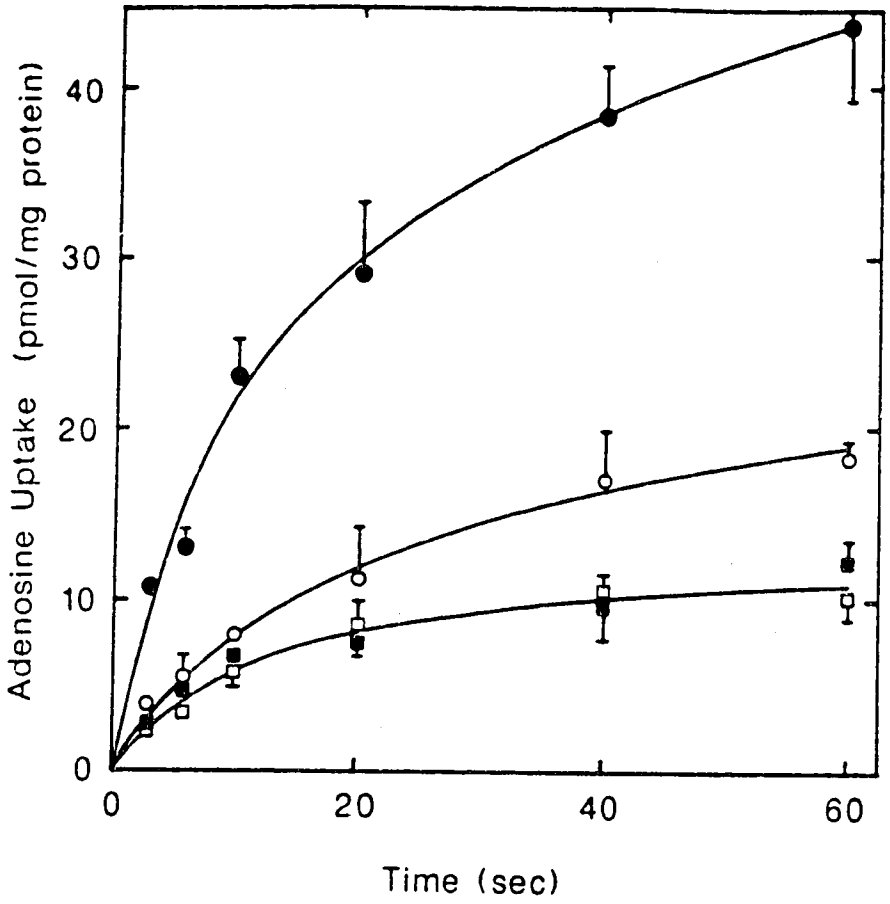

Fig. 4.3. Time course of adenosine influx by lysed guinea pig cerebral cortical synaptosomes. Synaptosomes were treated with (■,□) or without (●,○) 1 mg/ml saponin. [^{3}H]Adenosine (15 μM) influx were measured in the presence (○,□) or absence (●,■) of 50 μM dipyridamole. Values are means ± S.E. of triplicate determinations, for clarity, data are presented with either positive or negative S.E..

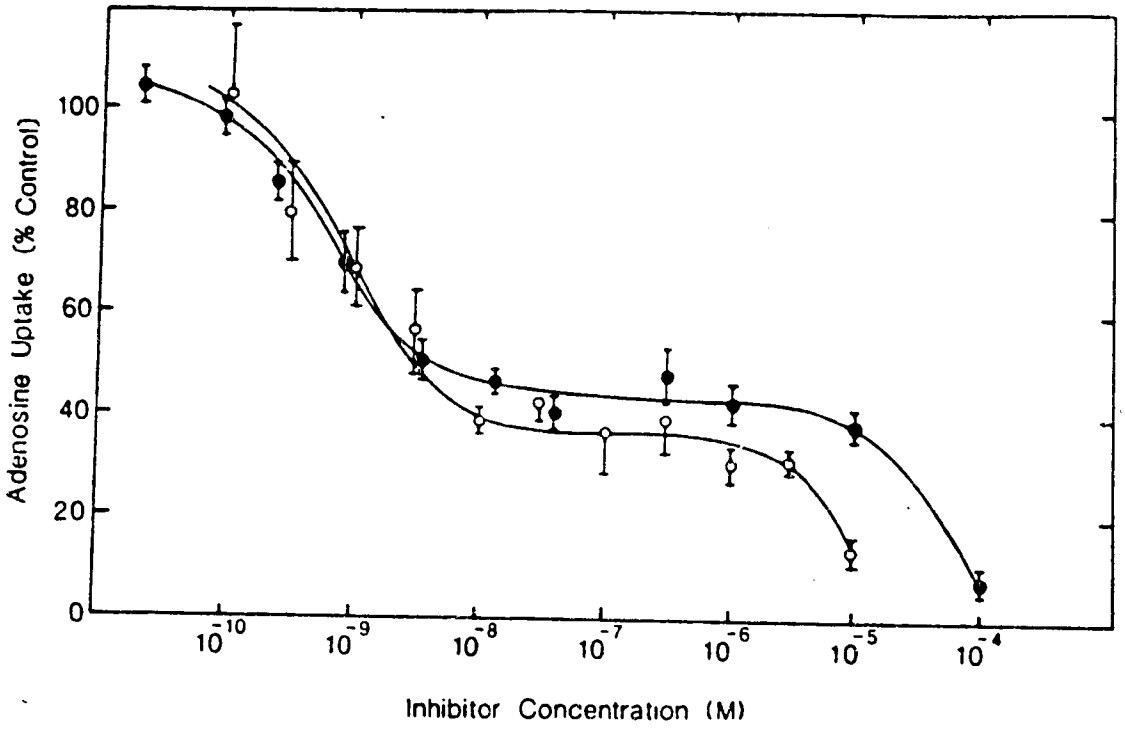

Fig. 4.4. Effect of NBMPR and dilazep on adenosine transport by guinea pig cerebral cortical synaptosomes. Synaptosomes were preincubated with various concentrations of either NBMPR (●) or dilazep (o) for 20 min before the addition of 15 μM [^{3}H]adenosine. The results are expressed as a percentage of the control transport rate versus the final inhibitor concentration. In the case of NBMPR, [^{3}H]NBMPR in the concentration range of 0 to 30 nM was used in parallel experiments to determine the final free concentration of the inhibitor. Values are means ± S.E. of at least three separate experiments conducted in triplicate. For clarity, some data are presented with either positive or negative S.E..

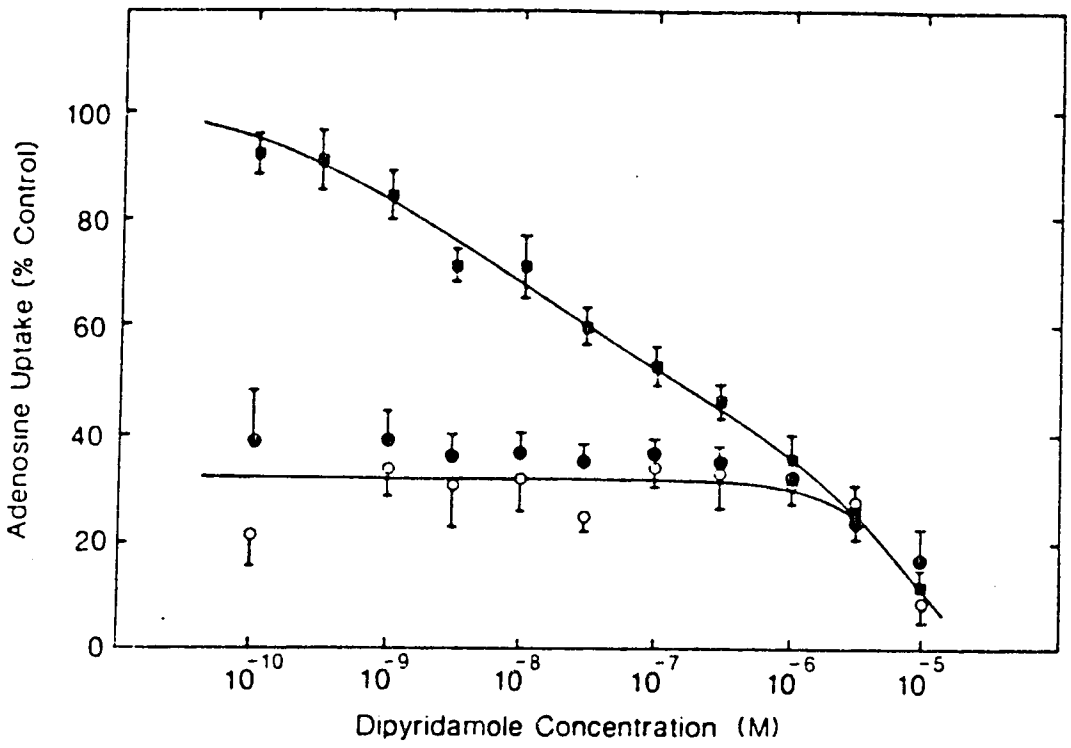

Fig. 4.5. Effect of dipyridamole on adenosine transport by guinea pig cerebral cortical synaptosomes. Synaptosomes treated with either buffer (■), or 500 nM NBMPR (●) or dilazep (o) were preincubated with various concentrations of dipyridamole for 20 min before the addition of 15 μM [^{3}H]adenosine. The results are expressed as a percentage of the control transport rate versus the final dipyridamole concentration. Values are means ± S.E. of three separate experiments conducted in triplicate. For clarity, some data are presented with either positive or negative S.E..

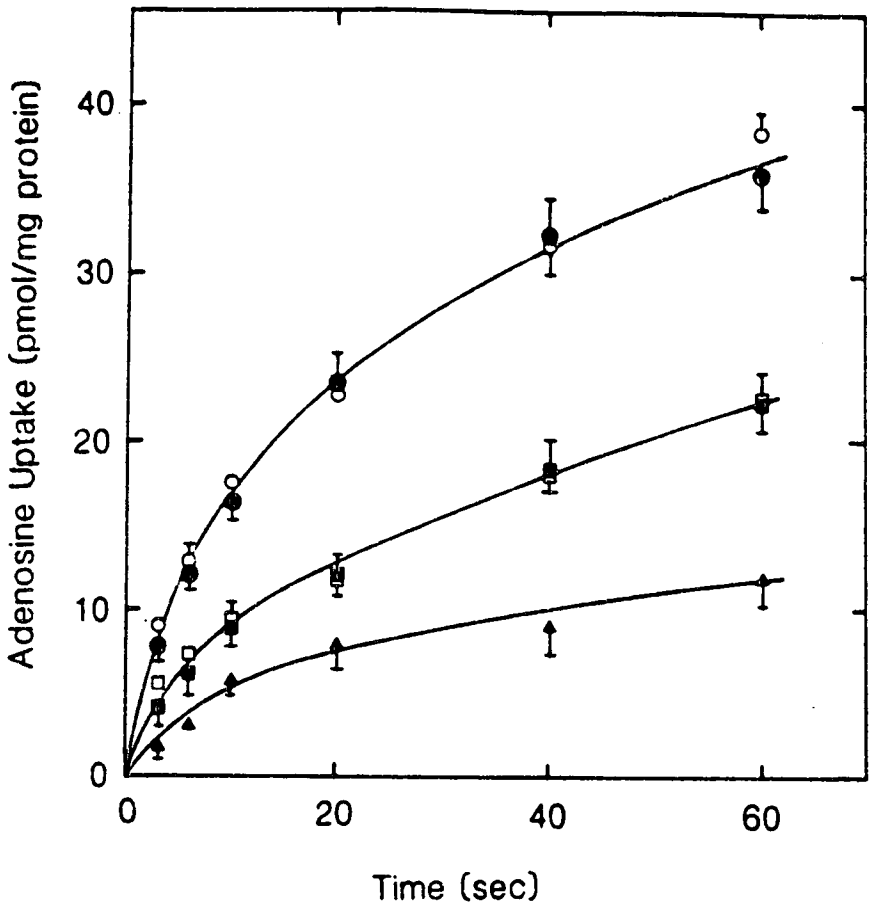

Fig. 4.6. Effect of a Na^+-gradient on adenosine influx by guinea pig cerebral cortical synaptosomes. Synaptosomes were preincubated with either buffer (●,○), 20 μM NBMPR (■,□) or 50 μM dipyridamole (▲) for 20 min prior to measurement of uptake. [^{3}H]Adenosine (15 μM) influx was measured in the presence of 100 mM extrasynaptosomal NaCl (○,□) or KCl (●,■,▲). Values are means ± S.E. of triplicate determinations, where absent, S.E. are smaller than symbol. For clarity, data are presented with either positive or negative S.E..

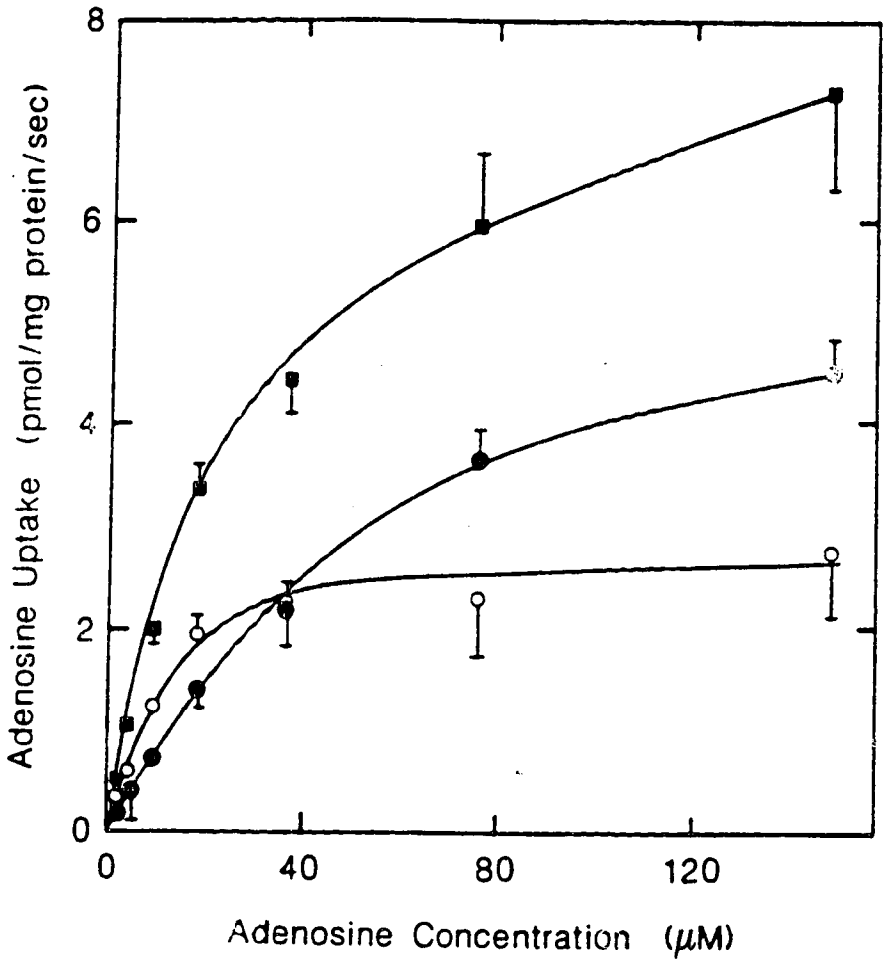

Fig. 4.7. Concentration dependence of adenosine transport by control and NBMPR-treated guinea pig cerebral cortical synaptosomes. [^{3}H]Adenosine transport by synaptosomes in the presence (●) or absence (■) of 500 nM NBMPR was determined as described in Section 4.2. The synaptosomes were preincubated with NBMPR for 20 min before addition of [^{3}H]adenosine and the data (means ± S.E. of triplicate determinations) shown are corrected for the non-carrier mediated transport component determined in the presence of 50 μM dipyridamole. NBMPR-sensitive transport (o) represents the difference between the total transport rates (■) and the transport rates determined in the presence of NBMPR (●). For clarity, data are presented with either positive or negative S.E. The kinetic constants for the data shown are K_m values of 13 ± 3 and 73 ± 8 μM and V_{max} values of 2.9 ± 0.3, and 6.9 ± 0.5 pmol/mg protein/s for NBMPR-sensitive and -insensitive transport, respectively (estimates ± S.E.).

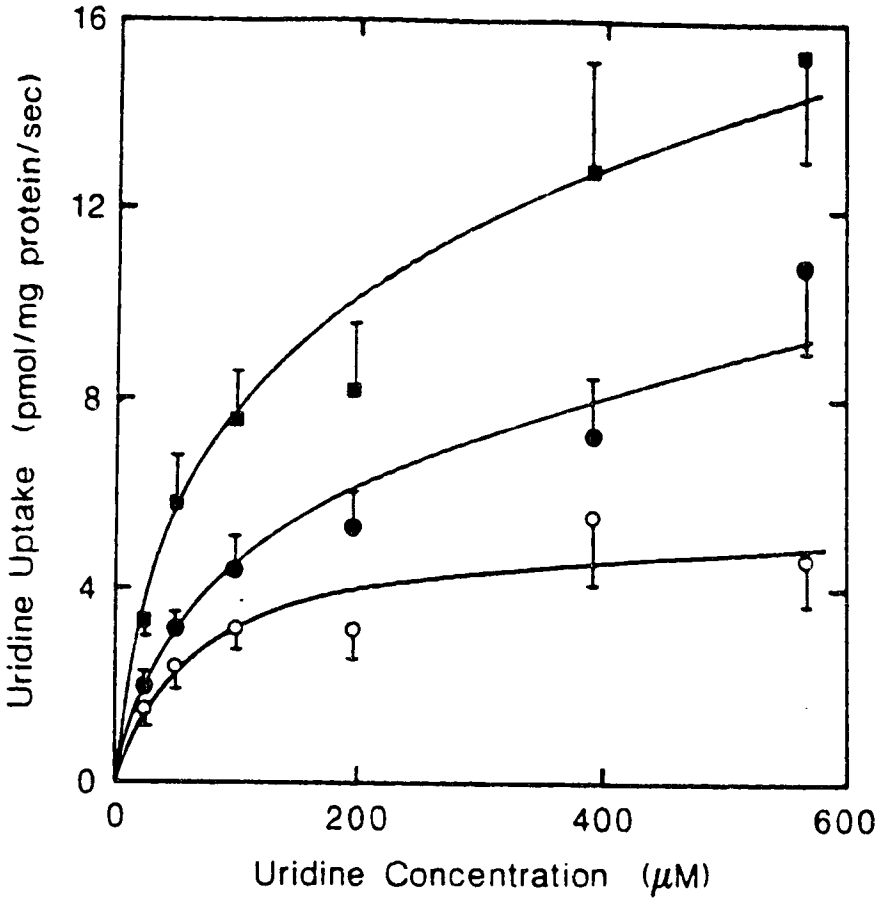

Fig. 4.8. Concentration dependence of uridine transport by control and NBMPR-treated guinea pig cerebral cortical synaptosomes. [^{3}H]Uridine transport by synapatosomes in the presence (●) or absence (■) of 500 nM NBMPR was determined as described in Section 4.2. The synaptosomes were preincubated for 20 min with NBMPR before addition of [^{3}H]uridine and the data (means ± S.E. of triplicate determinations) shown are corrected for the non-carrier mediated transport component determined in the presence of 50 μM dipyridamole. NBMPR-sensitive transport (o) represents the difference between the total transport rates (■) and the transport rates determined in the presence of NBMPR (●). For clarity, data are presented with either positive or negative S.E.. The kinetic constants for the data shown are K_m values of 74 ± 45 and 238 ± 155 μM, and V_{max} values of 5.5 ± 1.0 and 13.7 ± 3.9 pmol/mg protein/s for NBMPR-sensitive and -insensitive transport, respectively (estimates ± S.E.).

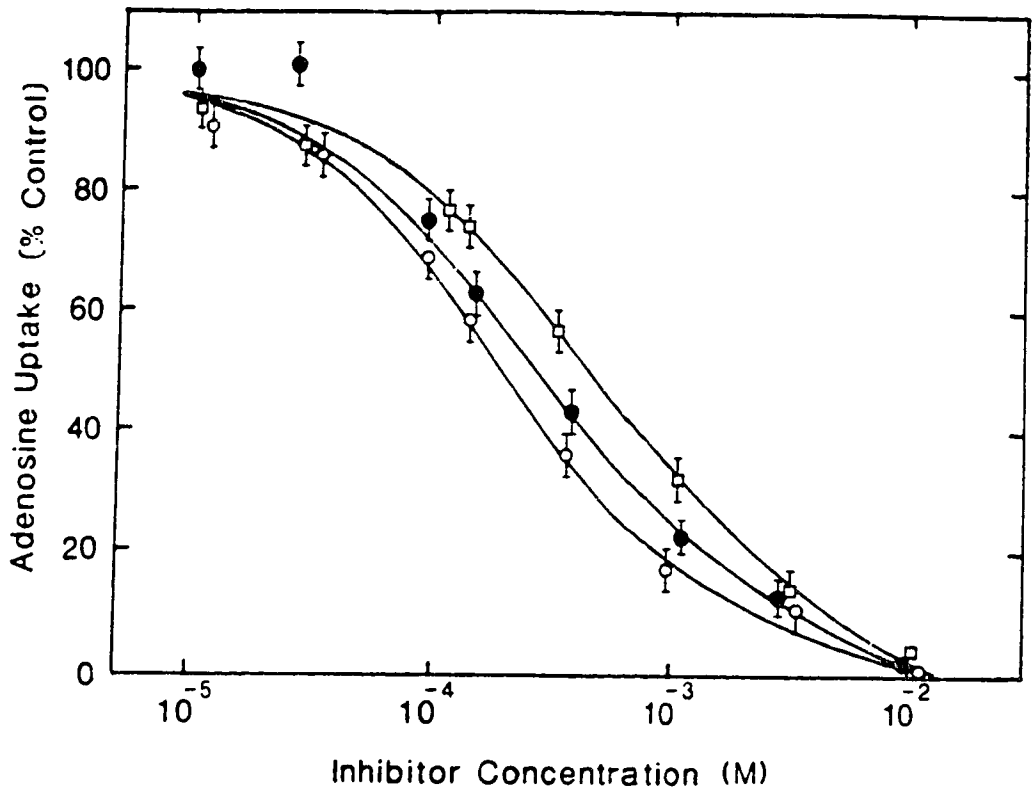

Fig. 4.9. Effect of nucleosides on adenosine transport by guinea pig cerebral cortical synaptosomes. [^{3}H]Adenosine (15 μM) and unlabelled nucleosides (□, inosine; ●, uridine; and ○, thymidine) were added simultaneously to guinea pig synaptosomes in the presence or absence of NBMPR (500 nM). After 5 s at 22°C, adenosine transport was terminated as described in Section 4.2. For clarity, only NBMPR-free fluxes of adenosine are plotted. The results are expressed as a percentage of the control transport rate versus the inhibiting nucleoside concentration. Values are means ± S.E. of three separate experiments conducted in triplicate. For clarity, some data are presented with either positive or negative S.E..

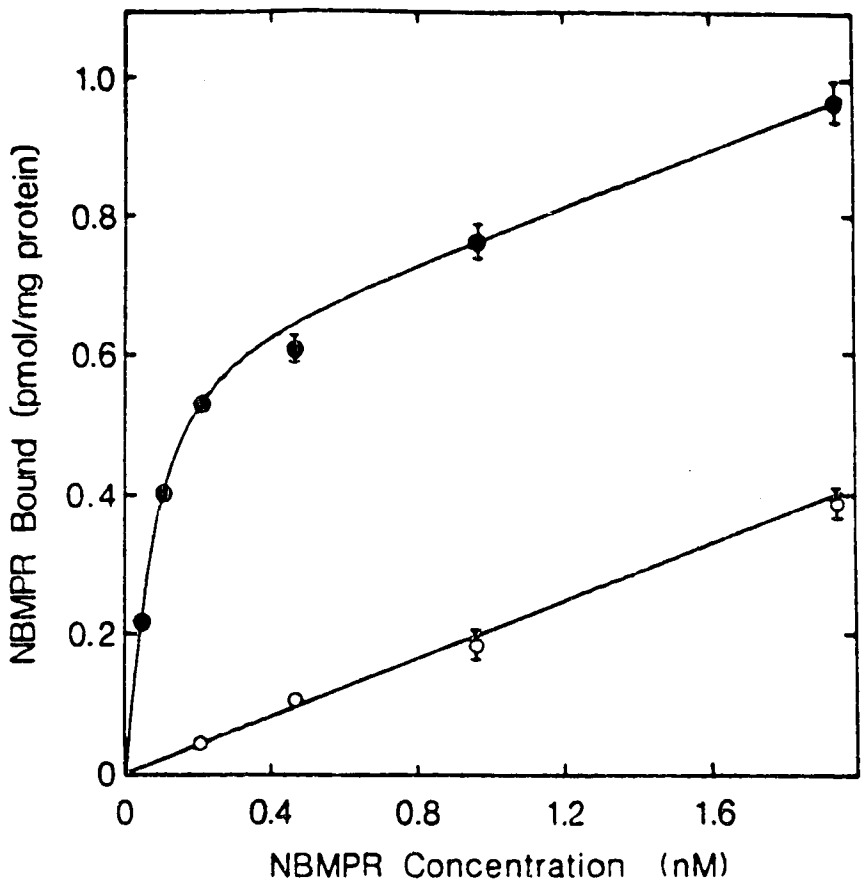

Fig. 4.10. Concentration dependence of [^{3}H]NBMPR binding to guinea pig cerebral cortical synaptosomes. Synaptosomes were incubated with graded concentrations of [^{3}H]NBMPR for 30 min at 22°C in the presence (o) or absence (•) of 20 μM NBTGR. Specific binding of NBMPR represents the difference between membrane-associated NBMPR in the presence and absence of NBTGR. Values are means ± S.E. of triplicate determinations, where absent, S.E. are smaller than symbol.

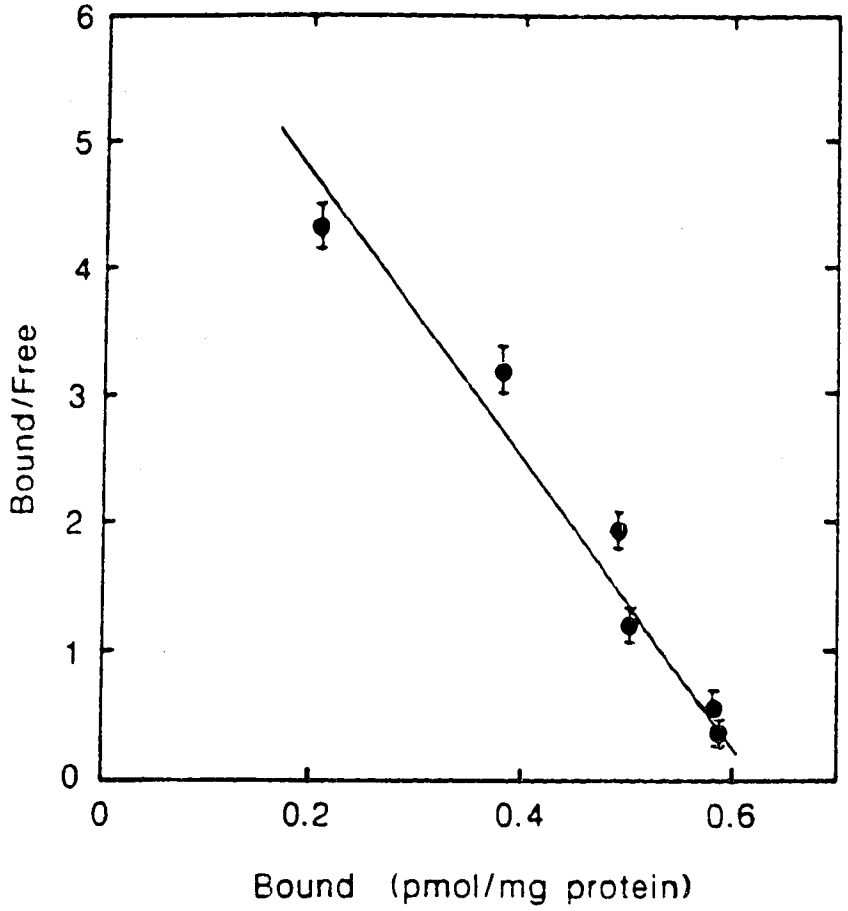

Fig. 4.11. Scatchard analysis of NBMPR binding data from Fig. 4.10. The regression line was calculated by the least-squares method ($r = 0.910$). The data are reported as means ± S.E. of triplicate determinations. The binding constants for the data shown are K_d 78 ± 14 pM and B_{max} 619 ± 25 fmol/mg protein (estimates ± S.E.).

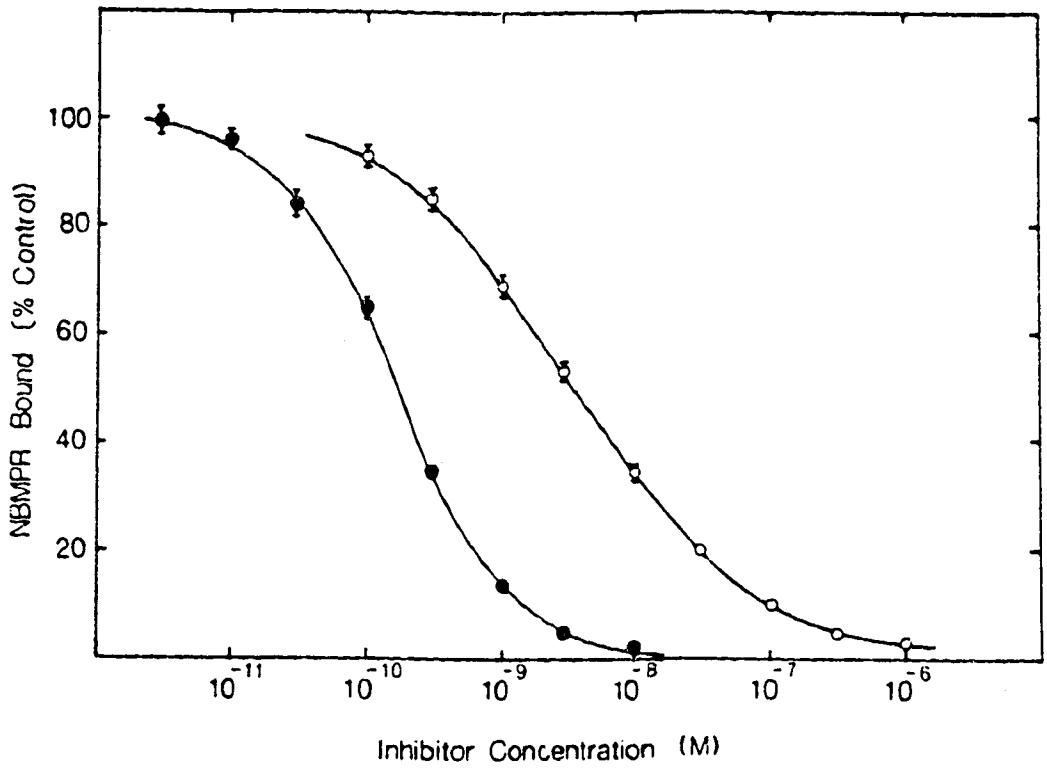

Fig. 4.12. Inhibition of site-specific binding of [^{3}H]NBMPR to guinea pig cerebral cortical synaptosomes. Synaptosomes were incubated with [^{3}H]NBMPR (0.1 nM) for 30 min at 22°C in the presence of either dilazep (●) or dipyridamole (o). Results are plotted as percentages of site-specific NBMPR binding activity in the absence of inhibitor. Values are means ± S.E. of three separate experiments conducted in triplicate, where absent, S.E. are smaller than symbol.

5. Study 3 : Uridine transport in rat renal cortex brush border membrane vesicles[1]

5.1. Introduction

A large number of nucleoside analogues have been synthesized and tested for antitumor, antiviral and antiparasitic activity [8, 10, 24, 30]. A major obstacle to the clinical use of these substances is toxicity to the host tissues. The kidney is particularly sensitive to therapeutic treatment with inhibitors of adenosine deaminase which results in increased concentrations of adenosine and even more of 2'-deoxyadenosine in serum [12, 25]. Both nucleosides are potentially cytotoxic [11]. Nephrotoxicity has also been observed when nucleoside analogues, such as tubercidin and 2'-deoxycoformycin, have been administered to experimental animals [4, 12, 13, 17]. In addition, the physiolcgical nucleoside adenosine may play a role in the intrinsic control of glomerular filtration rate and renin release [33, 34]. The mechanism(s) by which the kidney handles high concentrations of nucleosides in the filtrate will therefore be an important factor in the therapeutic administration of nucleoside drugs.

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[1] A version of this section has been published. Lee, C.W., Cheeseman, C.I., and Jarvis, S.M. (1988). Biochim. Biophys. Acta 942:139-149.

Measurements of the renal clearance of adenosine in humans and mice led to the hypothesis in 1982 that an active transport system exists in the kidney with the function of reabsorping the filtered adenosine [18]. Subsequent studies with rat renal brush border membrane vesicles demonstrated that adenosine uptake and the uptake of a variety of purine and pyrimidine nucleosides did not proceed by classical facilitated diffusion, as seen in most cell types [2, 28, 29, 36], but rather by an active concentrative process which required the presence of a Na^+-gradient (out>in) as a driving force [20-22]. Such ion gradients are maintained in the living cell by Na^+-K^+-ATPase. However, Na^+ gradients cannot be maintained across isolated brush border membrane vesicles because the Na^+-K^+-ATPase is present only in the basolateral membrane of the tubular cell, not in the brush border membrane [9]. Therefore, the "overshoot" uptake phenomenon observed in brush border membrane vesicles can be abolished by dissipating the driving force. Stimulation of nucleoside uptake by a Na^+ gradient is not a consequence of Na^+/H^+ exchange across the membrane as a H^+ gradient (in>out or out>in) did not affect the uptake of adenosine in the presence of a Na^+ gradient [22]. This suggests that Na^+ interacts directly with the nucleoside carriers, rather than indirectly through the formation of a H^+ gradient. Since Na^+-nucleoside cotransport is accompanied by a net transport

of positive charges from the medium into the vesicles, nucleoside influx can be stimulated by an inside-negative membrane potential [22]. It is not known whether there is a single Na^+-dependent nucleoside carrier with a broad specificity as observed for the facilitated diffusion nucleoside transport systems [15, 28, 29, 36] or whether there are a number of different active nucleoside carriers. In addition to the capacity for concentrative transport, the nucleoside carrier(s) in the renal brush border membranes also has a 20-100 fold greater affinity for nucleosides [20, 22] when compared to most of the NBMPR-sensitive and -insensitive facilitated diffusion nucleoside transport systems [15, 28, 29, 36].

Clearly the proximal tubule of the kidney is equipped with a system(s) for the reabsorption of nucleosides which differ from the facilitated diffusion nucleoside carrier of most cell types. To have a more complete view on this system(s), transport characteristics such as membrane potential dependency, monovalent cation specificity, Na^+:nucleoside stoichiometry and substrate structural specificity were investigated.

5.2. Methods

Preparation of brush border membrane vesicles

Brush border membranes from rat kidney cortex were prepared by a two-step Mg^{2+}-precipitation method [3]. All homogenization, isolation and centrifugation steps were undertaken at 4°C. Two male Sprague-Dawley rats (200-250 g) were anesthetized with sodium pentobarbital by intraperitoneal injection. The kidneys were removed, decapsulated and placed into ice-cold buffer 'A' (300 mM mannitol, 5 mM EGTA and 12 mM Tris-HCl, pH 7.4). Kidney cortex tissues, after being dissected from the kidney slices (approximately 1 mm thick), were homogenized in 65 ml of buffer 'A' with a Polytron on setting '5' for 2 min. The homogenate was then added to 65 ml of cold distilled water plus 18 ml of 100 mM $MgCl_2$ and stirred on ice for 15 min before being centrifuged at 2420 x g for 15 min. The supernatant (S_1) was centrifuged at 30600 x g for 30 min. The resultant pellet (P_2) was homogenized in 30 ml of ice-cold buffer 'B' (150 mM mannitol, 2.5 mM EGTA and 6 mM Tris-HCl, pH 7.4). Four millilitres of 100 mM $MgCl_2$ was again added to the suspension and stirred for 15 min on ice before centrifugation (2420 x g, 15 min). The supernatant (S_3) was recentrifuged for 30 min at 30600 x g. The pellet (P_4) was washed with an ice-cold vesicle suspension medium containing 300 mM mannitol and 5 mM Tris-HCl, pH

7.4 (30600 x g, 30 min). Finally, the pellet (P_5, brush border membranes) was suspended in the ice-cold vesicle suspension medium and stored on ice. Vesicles were used on the same day of preparation. Protein was assayed according to Lowry et al. [23] with bovine serum albumin as a standard. The purity of the preparation was assessed by the specific activity of alkaline phosphatase [19] and ouabain-sensitive Na^+-K^+-ATPase [30] in the vesicles as compared to the initial homogenate. Alkaline phosphatase (a brush border membrane marker) was enriched by 10 fold, while ouabain-sensitive Na^+-K^+-ATPase (a basolateral membrane marker) was reduced by 15 fold (Table 5.1).

Uptake studies

The uptake of [^{3}H]uridine (50 μCi/ml) was measured using an inhibitor-stop filtration method. A 10-μl aliquot of brush border membrane vesicle suspension (30-80 μg protein) and a 20-μl aliquot of incubation medium, containing [^{3}H]uridine and appropriate salt concentrations (with choline chloride substituted to maintain osmolarity) were placed separately on opposite sides of a polyethylene culture tube (16x100 mm) and preincubated at 22^{o}C for 5 min. The uptake of [^{3}H]uridine was initiated by continuous mixing with a vortexer such that the brush border membrane vesicles and the incubation medium came into contact. The detailed

final compositions of the incubation media are given in the figure legends. At the initiation of the incubation, the vesicles did not contain any substrate and hence the uptake was measured under "zero trans" conditions. All experiments were performed at room temperature (22-24^{o}C). After an appropriate time interval, the uptake was terminated by addition of 1 ml of ice-cold stop solution (100 mM mannitol, 100 mM NaCl, 1 mM phloridzin and 5 mM Tris-HCl at pH 7.4). The suspension was immediately filtered through an MSI nitrocellulose filter (pore size 0.45 μm, Fisher Scientific) under suction. The filter was subsequently washed once with 5 ml of ice-cold stop solution and, after being dried, was dissolved in 4 ml of Scinti Verse E (Fisher Scientific). The radioactivity was determined using an LKB/Wallac 1217 liquid scintillation counter with automatic quench correction and disintegration per minute conversion. The entire stopping and washing process took less than 15 s and control experiments (Fig. 5.1) established that no significant loss of [^{3}H]uridine from the vesicles occurs during this procedure. The radioactivity retained on the filter in the absence of membrane vesicles was used as the blank value for the uptake assays. These blanks were subtracted from measurements of uridine associated with the brush border membrane vesicles to determine uridine uptake rates.

Efflux studies

Brush border membrane vesicles were preequilibrated with incubation medium containing (final concentrations) 50 μM [^{3}H]uridine, 100 mM mannitol, 5 mM Tris-HCl (pH 7.4) and 100 mM of either $NaNO_3$, KNO_3 or choline chloride for 20 min at room temperature. The efflux was then initiated by mixing a 20-μl aliquot of the above vesicle suspension with 1 ml of incubation medium but lacking [^{3}H]uridine. This resulted in a fifty-fold dilution of the vesicles and their preincubation medium to produce conditions approaching those required for "zero trans" efflux. After an appropriate time interval, the reaction was stopped and washed once with 5 ml of ice-cold stop solution. The radioactivity retained on the filter was determined as described in the preceding section. For measuring the total initial intravesicular [^{3}H]uridine concentration, the reaction was initiated by mixing the vesicle suspension with 1 ml of ice-cold stop solution and filtering immediately. Other procedures remained unchanged.

The intravesicular volume was determined by measuring the intravesicular concentration of [^{3}H]D-glucose (100 μM) at equilibrium (90 min incubation). The mean $\pm$ S.E. of the intravesicular volume obtained was 1.81 $\pm$ 0.10 μl/mg protein from 21 determinations.

Uridine metabolism

Brush border membrane vesicles were incubated for 10 s, 1 and 30 min with 5 μM [^{3}H]uridine at 22^{o}C in the presence of either a Na^+- or K^+-gradient (out>in). To identify the intravesicular products, the reaction was terminated as described in uptake studies and the filter stirred for 30 min at room temperature in 250 μl of 2 N NH_4OH. To analyze for possible extravesicular products of uridine metabolism, the membrane vesicles were rapidly centrifuged at 90000 rpm for one minute (Beckman Airfuge) at the end of the incubation period without adding stop solution. Samples of the vesicle-free supernatant were diluted with an equal volume of 2 N NH_4OH. The NH_4OH extract was then chromatographed on silica gel coated plates impregnated with fluorescent indicator (Whatman K6F silica gel, 20 cm x 20 cm x 250 μm). The standards (uridine, uracil, UMP, UDP and UTP) were co-chromatographed with the samples. The solvent system used for the chromatographic analysis was 1-butanol (saturated with water). After drying, the zones bearing the standards were localized under UV light (Rf values of 0.4, 0.5, 0.03, 0 and 0 for uridine, uracil, UMP, UDP and UTP, respectively). The rest of the lane was equally divided into individual zones (1 cm). Each zone was scraped from the plate into a scintillation vial. Radioactivity in the powder was extracted with 1 ml of

water with shaking for at least 15 min before the addition of scintillation cocktail.

5.3. Results

Stop solution

The rapid filtration technique used here to measure solute transport by renal brush border membrane vesicles is only valid if all transport ceases after the addition of the stop solution and no loss of radioactivity from the vesicles occurs during filtration. Therefore, in preliminary experiments, the effect of different stop solutions on the retention of [^{3}H]uridine by the rat renal brush border membrane vesicles was tested. Renal brush border membrane vesicles were incubated with [^{3}H]uridine in the presence of a Na^+-gradient (out>in) for 10 s then subsequently diluted 1:33 into various stop solutions (see Fig. 5.1). These diluted vesicles were then filtered immediately or after a 5- to 60-s delay. Fig. 5.1 shows that using an ice-cold stop-solution containing 1 mM phloridzin, a Na^+-dependent D-glucose transport inhibitor [1, 35], total retention of radioactivity was observed for at least 1 min after addition of the phloridzin stop solution. In contrast, ice-cold stop solution containing 1 mM $HgCl_2$ or buffer alone resulted in rapid loss of radioactivity from the vesicles. The rate of uridine loss was further enhanced if stop solution at room temperature was used. Thus, the ice-cold stop solution containing 1 mM phloridzin was routinely used in the present study. These results,

however, were not in agreement with those reported by Le Hir and Dubach [20-22] that ice-cold buffer solution alone was able to prevent the rapid efflux of nucleosides from rat renal brush border membrane vesicles. The reason for these differences are unknown but it is clear that a stop solution containing NaCl and phloridzin is necessary for uridine transport studies using the present renal brush border membrane vesicle preparation.

Metabolism

Control experiments confirmed that no significant metabolism of uridine occurred within the first minute of incubating renal brush border membrane vesicles with [^{3}H]uridine in the presence of either Na^+- or K^+-gradient (out>in) and only 2% of the intravesicular radioactivity was recovered in the uracil fraction after 30 minutes (Table 5.2). No metabolites of uridine were detected in the extravesicular medium. Therefore, uridine metabolism does not complicate the present study of uridine transport by rat renal brush border membrane vesicles.

Time course of uridine influx

The time course of [^{3}H]uridine (5 μM) uptake by rat renal brush border membrane vesicles is illustrated in Fig. 5.2. In the presence of an initial 100 mM NaCl gradient (out>in), a transient overshoot of the

intravesicular uridine concentration above its equilibrium value was observed. The initial rate of uridine uptake (2 s) was about 300 times the initial rate in the absence of the gradient. Accumulation of uridine reached a maximum value at about 15 s and then decreased afterwards, indicating uridine efflux. In the absence of NaCl or in the presence of a 100 mM choline chloride gradient no transient overshoot was observed (Fig. 5.2). However, a 100 mM KCl gradient (out>in) produced a significant increase in uridine influx relative to choline chloride and moreover a transient overshoot was also observed (Fig. 5.2). The maximal accumulation of uridine with an initial 100 mM KCl gradient was 35 pmol/mg protein compared to 120 pmol/mg protein with an initial 100 mM NaCl gradient. In other experiments in which the equilibrium value for uridine uptake was measured as a function of extravesicular osmolarity, it was shown that the uptake of uridine occurred into an osmotically reactive intravesicular space without appreciable binding to the surface of the vesicles (Fig. 5.3). The superimposition of the choline chloride and mannitol curves (Fig. 5.2) rules out the possibility that the effect of a Cl^- generated membrane potential alone is an explanation for the overshoot seen in the presence of K^+. Thus, the above overshoot phenomena indicate that rat renal brush border membrane vesicles are capable of catalyzing the concentrative uptake of uridine in the

presence of inwardly directed transient electrochemical gradients formed by NaCl or KCl. Due to the absence of a stimulatory effect of choline chloride on uridine uptake and high solubility of this compound, choline chloride was used in this study to measure cation-gradient-independent uridine transport and to maintain isoosmolarity of the incubation medium.

Effect of anions

The effect of various anions on Na^+- and K^+-dependent uptake of [^{3}H]uridine is shown in Figs. 5.4 and 5.5. In the presence of a Na^+-gradient (out>in) (Fig. 5.4), the anions increased the amplitude of the overshoot in the following order: $NO_3^->SCN^->Cl^->SO_4^{2-}$. A similar order was also found in the presence of a K^+-gradient (out>in) (Fig. 5.5). This anion effect is very similar to what would be predicted from the order of diffusibility of these ions and the resultant magnitude of the membrane potential ($SCN^->NO_3^->Cl^->SO_4^{2-}$) [26], with the exception that the amplitude of the overshoot was higher with NO_3^- than SCN^- in this system. Therefore in subsequent experiments, NO_3^- salts were used to generate the chemical gradient.

Effect of salt concentrations

Increasing the concentration of $NaNO_3$ or KNO_3 in the extravesicular medium (isoosmolarity maintained with choline chloride) resulted in the amplitude of the overshoot for uridine uptake being increased for both Na^+ (Fig. 5.6) and K^+ (Fig. 5.7). Moreover, the initial rate of uridine uptake was increased and the Na^+ and K^+ dependence of approximate initial rates of uridine uptake (2 s for Na^+ and 5 s for K^+) was explored further by measuring the Na^+ and K^+ activation curves at a fixed uridine concentration (Fig. 5.8). As the extracellular Na^+ concentration increased from 0 to 200 mM, there was a hyperbolic activation of uridine uptake that conformed to simple saturation kinetics with apparent K_{Na} value of 18 $\pm$ 4 mM (estimate $\pm$ S.E.) consistent with a single Na^+ binding site on the uridine transporter. In contrast, the K^+ activation curve for uridine uptake was sigmoidal suggesting a K^+:uridine coupling stoichiometry of >1:1. An approximate estimate for the K_K was 280 $\pm$ 70 mM (estimate $\pm$ S.E.).

Interactions between Na^+ and K^+

The above results show that both a Na^+- and a K^+-gradient (out>in) may elicit active transport of uridine into renal brush border membrane vesicles. Thus, in further experiments the possible interactions between Na^+

and K^+ were investigated when these ions were both present in the incubation medium on the same or opposite sides of the membrane. Fig. 5.8 shows that the approximate initial rates of Na^+-dependent uridine uptake can be enhanced by the addition of a K^+-gradient (out>in). Greatest stimulation by K^+ was observed at extravesicular Na^+ concentrations ranging from 10 to 40 mM. The stimulation, however, was not profound at extravesicular Na^+ concentrations above 100 mM. The K_{Na} value in the presence of a 100 mM K^+ gradient was decreased 3-fold compared to the absence of K^+ (6 $\pm$ 1 and 18 $\pm$ 4 mM, respectively, means $\pm$ S.E.). Likewise, increasing the extravesicular K^+ concentration from 100 to 200 mM did not further stimulate the initial rate of uridine uptake. Therefore, a Na^+ and K^+ concentration of 20 and 100 mM, respectively, were used in subsequent experiments.

The stimulatory effect of K^+ on the Na^+-dependent uridine uptake is further illustrated in Fig. 5.9 (Na^+ : out>in, K^+ : out>in; curve a). Both the initial rate and the amplitude of uptake were significantly higher than the uptake in the presence of Na^+-gradient alone (Na^+ : out>in; curve b). This suggests that Na^+ and K^+ are not interacting at the same carrier site. However, when K^+ is equilibrated on both sides of the membrane (Na^+ : out>in, K^+ : out=in; curve c), uridine uptake is greatly reduced. The inhibitory effect was

further increased when an outwardly directed K^+-gradient was present (Na^+ : out>in, K^+ : out<in; curve d).

In Na^+-equilibrated conditions (Fig. 5.10), no net transport was observed in an outwardly directed K^+-gradient (Na^+ : out=in, K^+ : out<in). Also, an inwardly directed K^+-gradient with an outwardly directed Na^+-gradient (Na^+ : out<in, K^+ : out>in) was unable to increase uptake above the simple diffusion level (choline chloride : out=in). These two situations were therefore, not plotted in Fig. 5.10. However, uridine uptake was elicited with either Na^+ or K^+ equilibrated on both sides of the membrane (Na^+ : out=in; curve c, or K^+ : out=in; curve d), and reached the equilibrium concentration rapidly at 10 and 120 s for Na^+- and K^+-equilibrated membranes, respectively, with no overshoot observed (compared with >5 min for simple diffusion). These results suggest that both Na^+ and K^+ can stimulate uridine transport and the overshoot phenomenon is only observed if an inwardly directed gradient of either Na^+ or K^+ is imposed. No difference in uridine uptake was observed in vesicles equilibrated with only Na^+ (Na^+ : out=in; curve c) and those equilibrated with both Na^+ and K^+ (Na^+ : out=in, K^+ : out=in, data not plotted). As already described in Fig. 5.9, an overshoot induced by the presence of an inwardly directed Na^+-gradient can be greatly reduced when K^+ was present on the inside. It was also observed that when vesicles were equilibrated

with Na^+, the amplitude of overshoot induced by K^+-gradient was reduced (Na^+ : out=in, K^+ : out>in; curve b; Fig. 5.10). These observations can best be explained by postulating the involvement of more than one transport system for uridine in renal brush border membranes. The inhibition of K^+-dependent uridine influx by intravesicular Na^+ could be due to the rapid removal of intravesicular uridine via the Na^+-dependent transport system. Similarly, inhibition of Na^+-dependent uridine influx by intravesicular K^+ could be due to K^+-dependent efflux of intravesicular uridine.

Time course of uridine efflux

Efflux experiments were performed to provide further evidence that uridine transport is bidirectional and that it is the rapid removal of the intravesicular uridine by the presence of the intravesicular Na^+ or K^+ that caused the decreased in the magnitude of uridine overshoot observed in Figs. 5.9 and 5.10. A higher concentration of uridine (50 μM) was used in these studies to improve the signal to noise ratio. Fig. 5.11 shows that both Na^+ and K^+ (out=in) stimulated the efflux of intravesicular uridine although Na^+ had a greater effect. When the cation gradient was oriented from in to out, the rate of uridine efflux was further increased although again Na^+ had a larger effect than K^+.

Effect of lithium and rubidium on uridine uptake

To further investigate the possible involvement of other monovalent cations in eliciting uridine transport, both $LiNO_3$ and $RbNO_3$ were used to generate an electrochemical gradient for uridine uptake. Fig. 5.12 shows that both Li^+ and Rb^+ were able to induce active transport of uridine but were less potent than Na^+ and K^+ in producing an overshoot. It is possible that the active transport of uridine elicited by Li^+ and Rb^+ could be via either the Na^+- or the K^+-uridine cotransport systems. As shown in Fig. 5.13 the approximate initial rates of Na^+-dependent uridine uptake were reduced by the addition of a Li^+-gradient (out>in). A Lineweaver-Burk plot (Fig. 5.14) of these data showed that Li^+-gradient (out>in) increased the slope of the regression line with no effect on the y-intercept suggesting that Li^+ is acting as an apparent competitive inhibitor of Na^+-dependent uridine transport by competing with Na^+ for the same binding site. In contrast, Li^+ had little effect on the K^+-dependent system (Fig. 5.13). It is likely that the molecular size of Li^+ is too small to emulate K^+ in the K^+-dependent system, and hence it was ineffective in competing with K^+ for the ligand binding site. Similarly, Rb^+ only inhibited K^+-dependent uridine transport and not the Na^+-dependent system (Fig. 5.15).

5.4. Discussion

Intracellular metabolism of nucleosides often makes the characterization of the mechanisms of their transport across cell membranes difficult [28, 29]. It has been demonstrated that adenosine is rapidly metabolized during uptake into renal brush border membrane vesicles [20]. However, metabolism of uridine was not observed under the present experimental conditions. Thus uridine was used as the permeant to investigate nucleoside transport in rat renal brush border membrane vesicles.

The present results demonstrate that in the presence of a Na^+- or K^+-gradient (out>in), uridine uptake showed a transient overshoot phenomenon indicating active Na^+- and K^+-dependent transport of uridine (Fig. 5.2). Shunting of the driving force by equilibration of Na^+ and K^+ on both sides of the membrane abolished the overshoot (Fig. 5.10). In addition, uridine was taken up into an osmotically active intravesicular space, efflux from which was blocked by phloridzin and by reducing the temperature. Therefore an alternative explanation that the uridine overshoot phenomenon reflects cation dependent uridine binding to the vesicle membrane is not likely. Although evidence for a Na^+-dependent uptake of nucleosides by renal brush border membrane vesicles [20-22], isolated intestinal epithelial cells [31], murine splenocytes [6] and cultured intestinal IEC-6 cells [14]

has been documented, this is the first time that evidence for a K^+-dependent uptake of uridine has been presented. The results also show that the K^+-dependent uptake of uridine cannot be explained due to the action of a membrane potential induced by Cl^- movement as choline chloride failed to elicit increased uptake when compared to mannitol (Fig. 5.2). Thus, the above overshoot phenomena indicate that the rat renal brush border membrane vesicle preparation is capable of catalysing the concentrative uptake of uridine in the presence of inwardly directed transient electrochemical gradients generated by Na^+ and K^+.

One can predict that if Na^+- and K^+-uridine cotransport systems are electrogenic there will be a net transfer of positive charge across the membrane. Therefore a change in membrane potential should affect the transport process. The membrane potential dependency of uridine transport in Na^+- and K^+-gradient (out>in) conditions was analysed with different anions. Fig. 5.4 and 5.5 show the amplitude of the overshoot was increased by the use of more lipophilic anions. The reactive order of NO_3^->SCN^->Cl^->SO_4^{2-} observed is similar to what would be predicted from the order of membrane diffusibility of these ions and the resultant reduction of the membrane potential [24] with the exception of the NO_3^-. These findings suggest that the transport of uridine in rat

renal brush border membrane vesicles is an electrogenic process.

The relationship between approximate initial rates of uridine uptake and extravesicular Na^+ or K^+ concentrations showed that there is a higher affinity for Na^+ than K^+ for uridine transport (K_{Na} 18 mM versus K_K 280 mM, Fig. 5.8) . It is possible, therefore, that either the ligand binding site on the uridine carrier has a different affinity for the two cations or alternatively there are two uridine transport systems, one requiring Na^+ and the other K^+. The latter suggestion appears to be the more likely and the evidence supporting the presence of two uridine transporters in rat renal brush border membrane vesicles is discussed below.

Studies have shown that high external K^+ concentration can lead to an inhibition of Na^+-dependent transport processes [5, 7, 16, 27]. The inhibitory effect of high external K^+ concentration appears to be due to the fact that K^+ is competing with Na^+ for the Na^+-dependent transport system. However, such inhibition was not observed in this study. Instead, when both Na^+ and K^+ were present outside the membrane uridine uptake was stimulated. This finding rules out the possible competition between these ions for a common binding site on a single carrier. Also with vesicles suspended in media adjusted to isoosmolarity with choline chloride, it

appears that K^+ at the same concentration on both sides of the membrane could effectively reduce the Na^+-dependent accumulation of uridine. This could be due to the rapid removal of intravesicular uridine via the K^+-dependent system. Control experiments showed that in the presence of salt but absence of a driving force (out=in), uridine equilibrated rapidly across the membrane (Fig. 5.10). This rapid equilibration of uridine across the membrane was further illustrated in efflux experiments (Fig. 5.11), and suggests that uridine transport is bidirectional and Na^+ and K^+ are mandatory for the transport process. Both Li^+ and Rb^+ were also able to elicit active transport of uridine (Fig. 5.12), and it would appear that Li^+ can substitute for Na^+ (Fig. 5.13 and 5.14) and Rb^+ for K^+ (Fig. 5.15).

Further evidence suggesting that these two uridine transport systems are separate entities comes from the studies on the activation of uridine transport by Na^+ and K^+. The curve for Na^+ activation of uridine transport was hyperbolic and conformed to simple saturation kinetics suggesting that the Na^+:uridine coupling stoichiometry is 1:1 (Fig. 5.8). In contrast, the K^+ activation of uridine transport curve was sigmoidal, indicating that the K^+:uridine coupling stoichiometry is likely to be >1:1.

In conclusion, both Na^+- and K^+-gradients (out>in) can provide a driving force for the active transport of uridine in rat renal brush border membrane vesicles. The physiological significance of K^+-dependent transport of uridine into renal proximal tubule is not clear. However, it is possible that this system could be involved in a secretory process. Clearly, detailed kinetic analysis and the permeant specificity of these two active uridine transporters should be performed to gain a more comprehensive view of active nucleoside transport in the kidney.

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Table 5.1. Purity of rat renal brush border membrane vesicle preparations.

Marker Enzymes	Membrane	Specific marker enzyme activities in: Homogenate	Final pellet	E.F.
Alkaline phosphatase	Brush Border	158 ± 46	1635 ± 258	+10
Na^+-K^+-ATPase (0.1% deoxycholate)	Baso-lateral	3.91 ± 0.63	0.26 ± 0.01	-15

The specific enzyme activities expressed in pmol/mg protein/min are means ± S.E. of three separate experiments. The enrichment factor (E.F.) is calculated as the ratio of specific enzyme activity of the final pellet to that of the initial homogenates. The Na^+-K^+-ATPase was assayed in the presence of 0.1% deoxycholic acid to rupture the membrane vesicles.

Table 5.2. Metabolism of [^{3}H]uridine by rat renal brush border membrane vesicles.

Incubation period	Distribution of radioactivity (% of total ^{3}H recovered)		
	Uridine	Uracil	Nucleotides
10 s	100	ND	ND
60 s	100	ND	ND
30 min	98	2	ND

[^{3}H]Uridine (5 μM) was incubated with vesicles for 10, 60 s and 30 min at 22°C in the presence of Na^+-gradient (100 mM). Extraction and identification of the intravesicular and extravesicular products were performed as described in Section 5.2. Values are shown as a percentage distribution of total radioactivity analyzed from intravesicular extracts. Results for extravesicular extracts were not included as no product other than uridine was detected. Similar results were obtained with vesicles incubated with [^{3}H]uridine (5 μM) for 10, 60 s and 30 min at 22°C in the presence of K^+-gradient (100 mM).
ND - Not detected.

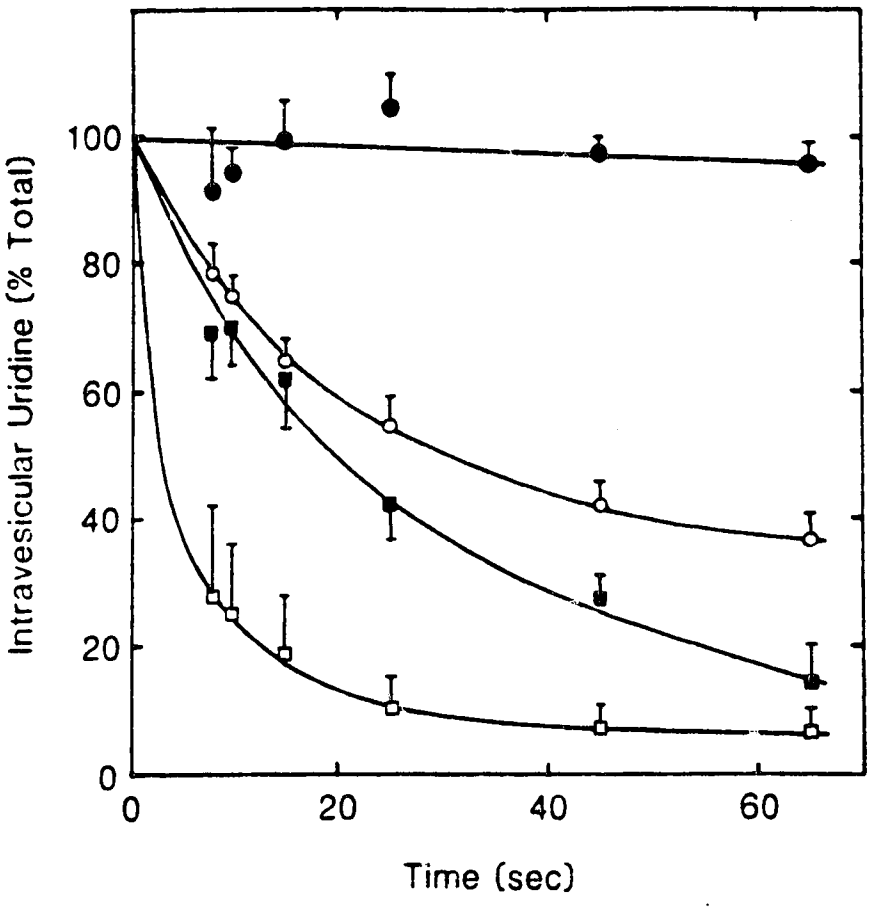

Fig. 5.1. Effect of various stop solutions on uridine uptake by rat renal brush border membrane vesicles. Ten µl of the vesicle suspension were incubated with 20 µl of incubation medium containing (final concentrations) 5 µM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and 100 mM NaCl for 10 s at 22°C. Transport was stopped by adding 1 ml of stop solution (100 mM mannitol, 100 mM NaCl and Tris-HCl, pH 7.4) at room temperature (□), ice-cold stop solution (■), ice-cold stop solution containing 1 mM $HgCl_2$ (o) or ice-cold stop solution containing 1 mM phloridzin (●). The suspension was poured onto the filter at different times after being stopped and subsequently washed once with 5 ml of respective solution. The data (means ± S.E. of triplicate determinations) expressed as percentage of the radioactivity retained by the vesicles when the incubation medium was filtered immediately with ice-cold stop solution containing 1 mM phloridzin (90 pmol/mg protein). For clarity, data are presented with either positive or negative S.E..

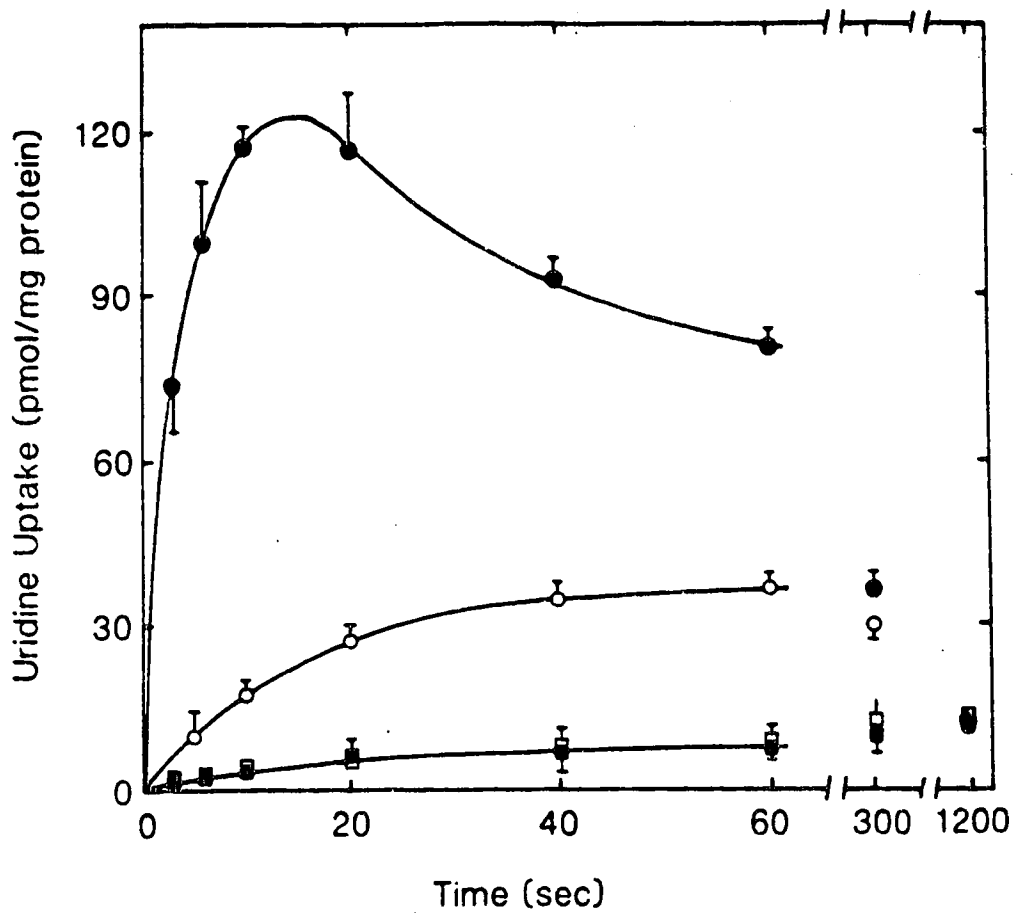

Fig. 5.2. Time course of uridine uptake by rat renal brush border membrane vesicles. Ten μl of the vesicle suspension were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and either 100 mM NaCl (●), 100 mM KCl (○), 100 mM choline chloride (■), or 200 mM mannitol (□). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E..

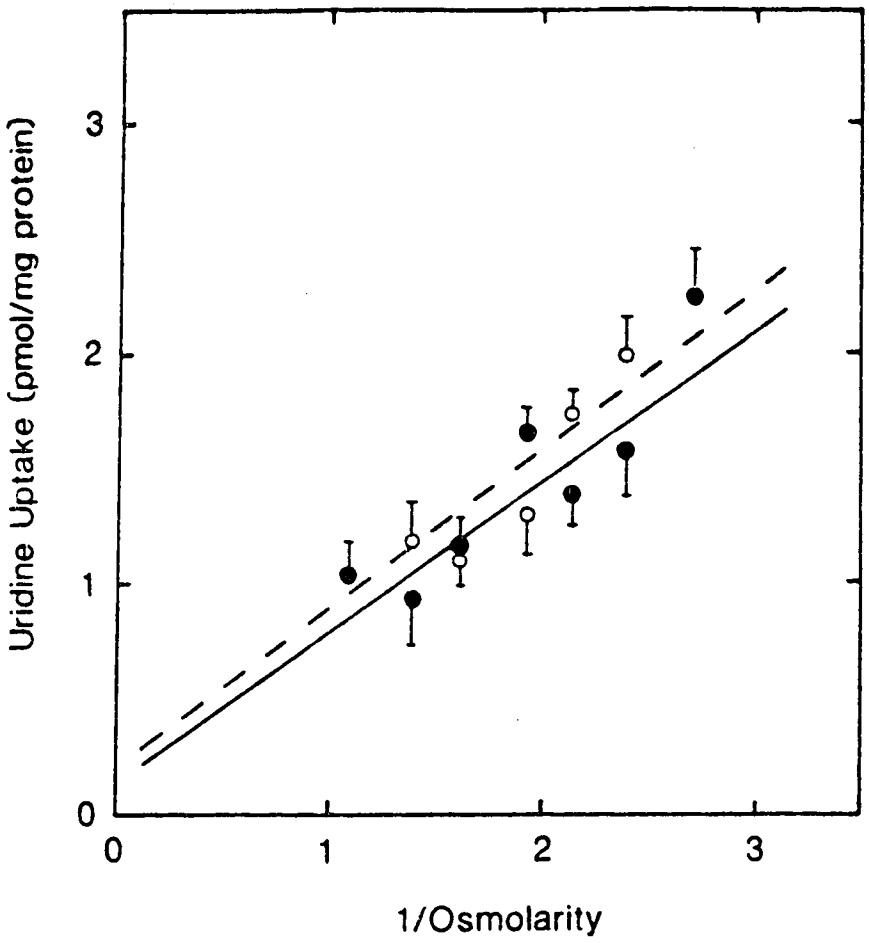

Fig. 5.3. Effect of extravesicular osmolarity on equilibrium uridine uptake by rat renal brush border membrane vesicles. [^{3}H]Uridine (5 μM) uptake by membrane vesicles (5-min incubation) in the presence of either 100 mM $NaNO_3$ (o--o) or KNO_3 (●—●) is plotted as a function of the reciprocal of the extravesicular osmolarity. Extravesicular osmolarity was adjusted with cellobiose and the osmolarity was calculated as the sum of contributions from all compounds, assuming ideal behaviour. The data are reported as means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E.. The regression lines were calculated by least-squares method (regression coefficient of 0.925 and 0.918 for Na^+- and K^+-dependent uridine uptake, respectively). The y-intercepts were 0.14 ± 0.50 and 0.21 ± 0.74 pmol/mg protein for Na^+- and K^+-dependent uridine uptake, respectively (estimates ± S.E.).

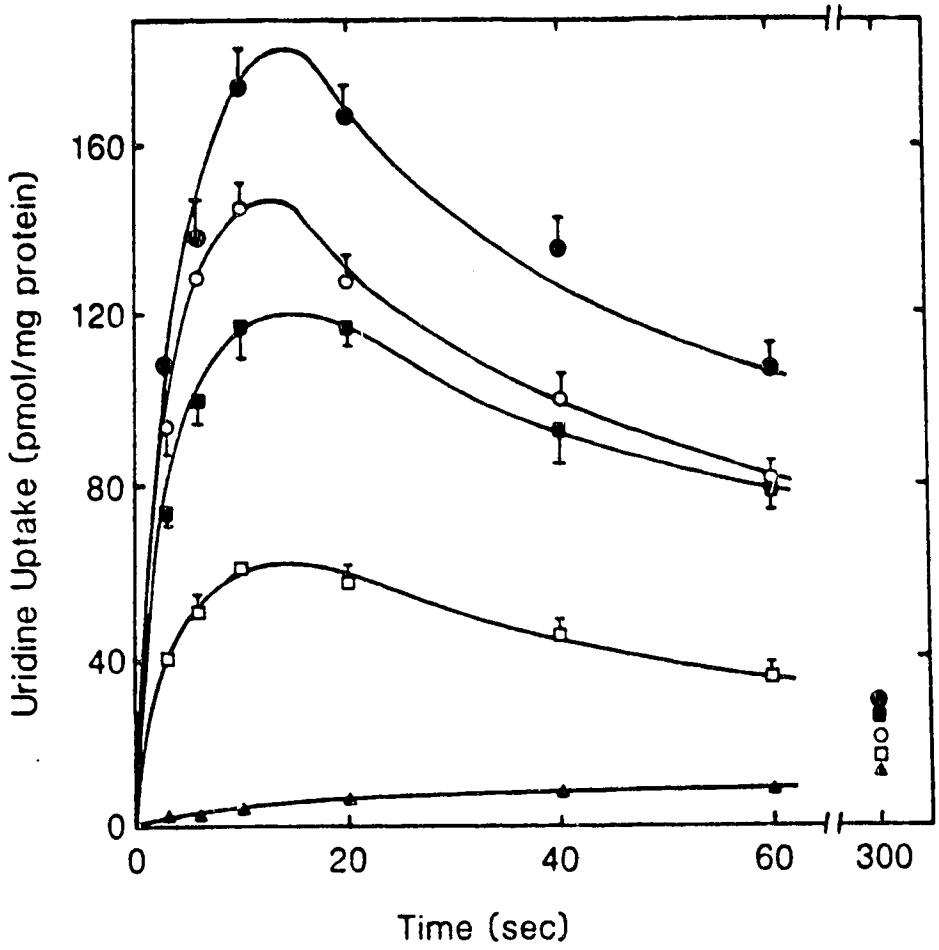

Fig. 5.4. Effect of anions on Na^+-dependent uridine uptake by rat renal brush border membrane vesicles. Ten μl of the vesicles suspension were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and either 100 mM $NaNO_3$ (●), 100 mM NaSCN (○), 100 mM NaCl (■), 50 mM Na_2SO_4 (□) or 100 mM choline chloride (▲). Values are means ± S.E. of triplicate determinations, where absent, S.E. are smaller than symbol. For clarity, data are presented with either positive or negative S.E..

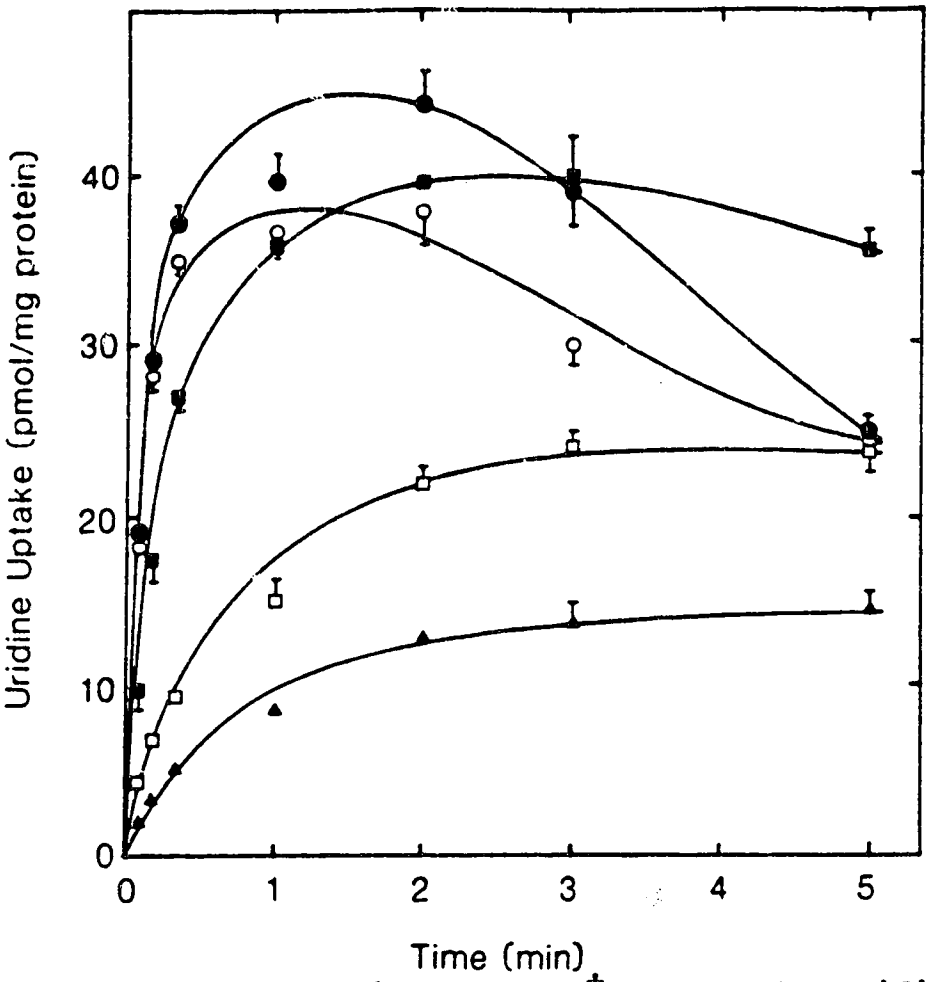

Fig. 5.5. Effect of anions on K^+-dependent uridine uptake by rat renal brush border membrane vesicles. Ten μl of the vesicle suspension were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and either 100 mM KNO_3 (●), 100 mM KSCN (○), 100 mM KCl (■), 50 mM K_2SO_4 (□) or 100 mM choline chloride (▲). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

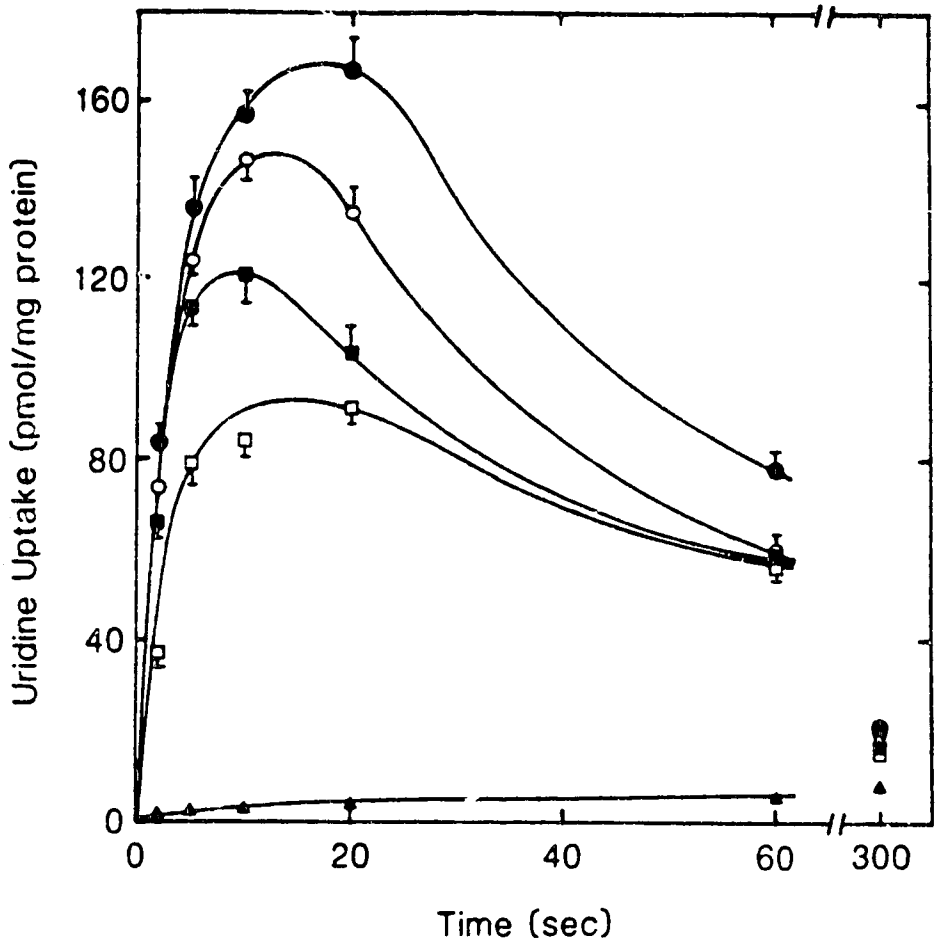

Fig. 5.6. Effect of external salt concentrations on Na^+-dependent uridine uptake by rat renal brush border membrane vesicles. Ten μl of the vesicle suspension were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and 200 mM total salt concentrations. Variable external Na^+ concentrations from 0 to 200 mM were obtained by substitution with choline chloride. Final external Na^+ concentrations were 200 mM (●), 100 mM (○), 50 mM (■), 10 mM (□) and 0 mM (▲). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

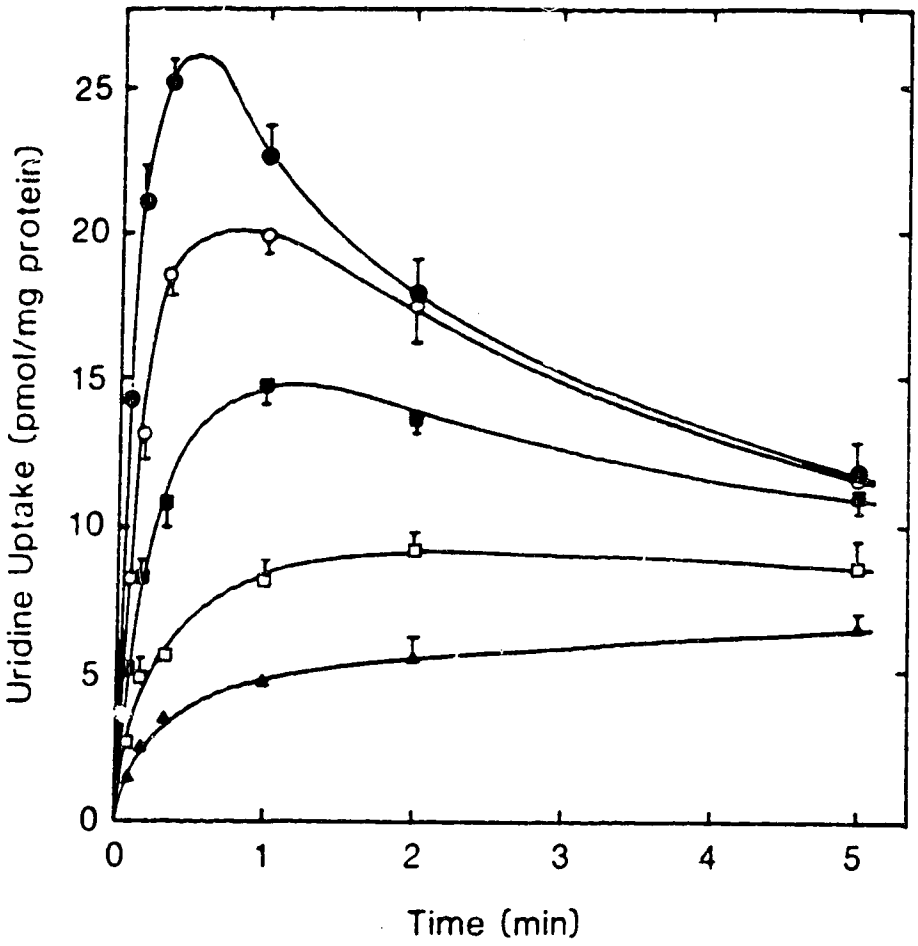

Fig. 5.7. Effect of external salt concentrations on K^+-dependent uridine uptake by rat renal brush border membrane vesicles. Ten μl of the vesicle suspension were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and 200 mM total salt concentrations. Variable external K^+ concentrations from 0 to 200 mM were obtained by substitution with choline chloride. Final external K^+ concentrations were 200 mM (●), 100 mM (○), 50 mM (■), 10 mM (□) and 0 mM (▲). Values are means ± S.E. of triplicate. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

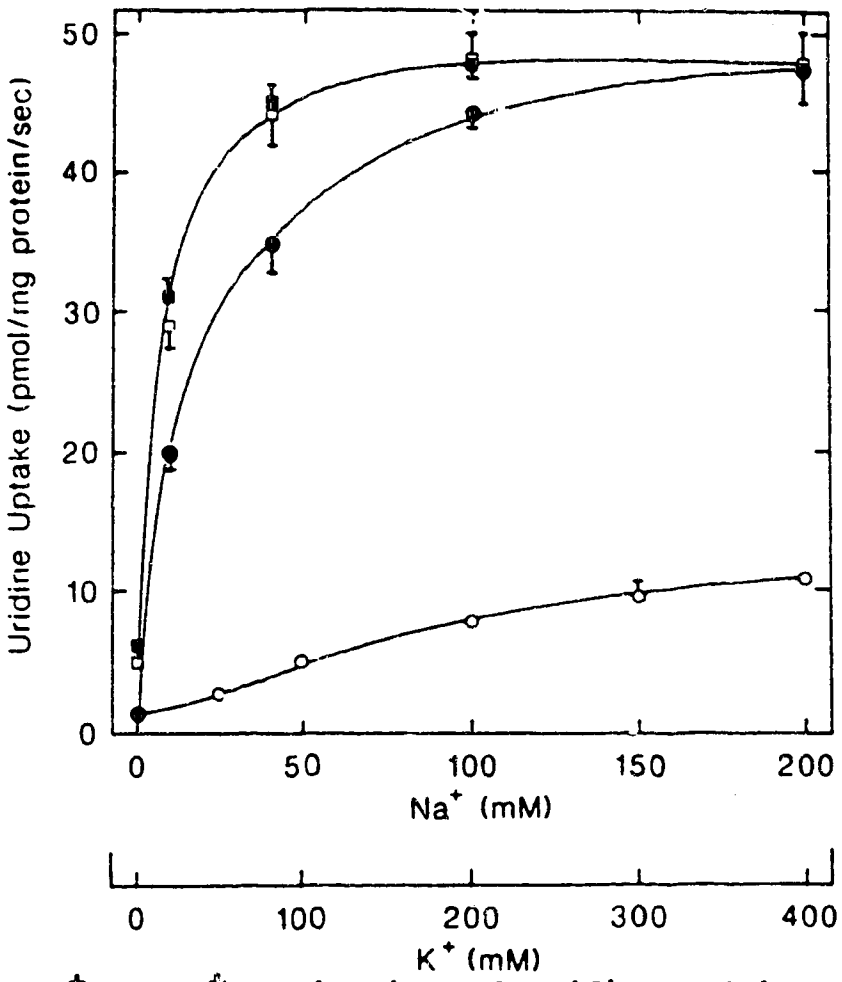

Fig. 5.8. Na^+ and K^+ activation of uridine uptake. Ten μl of the vesicle suspension were incubated with 20 μl of media containing either (final concentrations) 200 mM KNO_3 (■), 100 mM KNO_3 plus 100 mM choline chloride (□) or 200 mM choline chloride (●), 5 μM [^{3}H]uridine, 5 mM Tris-HCl buffer (pH 7.4) and variable Na^+ concentrations. Effect of external K^+ concentration alone (○) on uridine uptake was also determined. Variable external Na^+ and K^+ concentrations from 0 to 200 mM and to 400 mM respectively were obtained by substitution with choline chloride. Approximate initial rates of uridine uptake were estimated from 2 s (■,□,●) and 5 s (○) uptake intervals. Values are means ± S.E. of triplicate. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

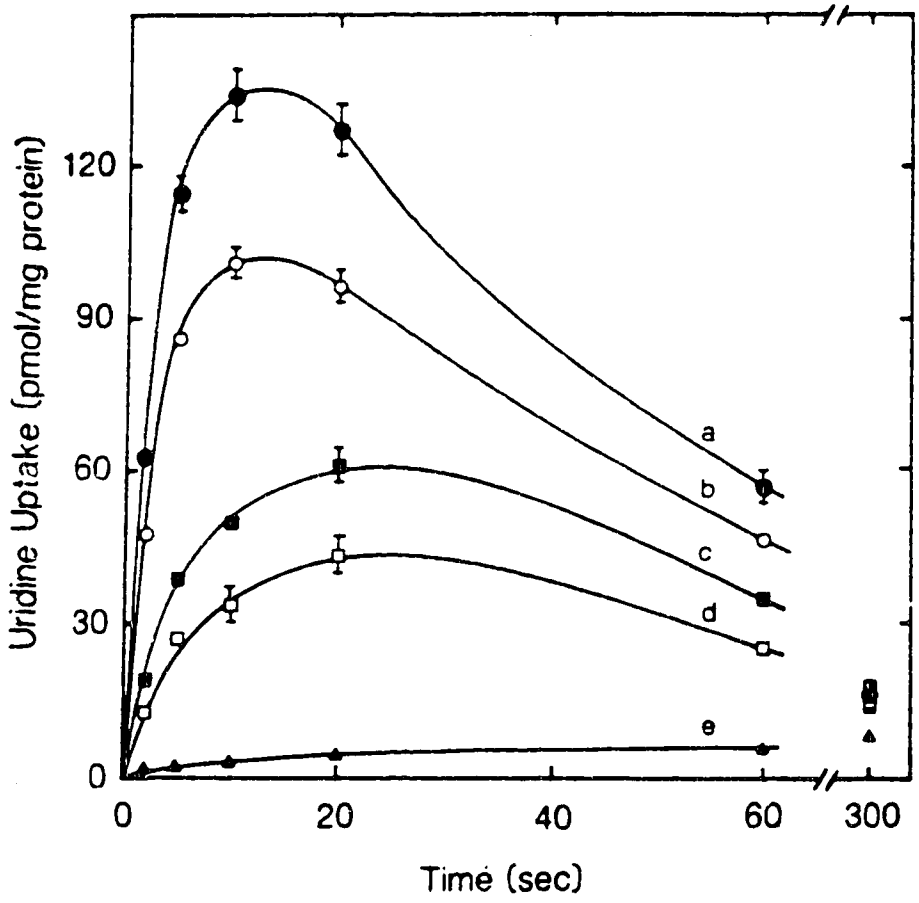

Fig. 5.9. **Interactions between Na^+ and K^+ for uridine uptake by rat renal brush border membrane vesicles.** Vesicles were resuspended in media containing 300 mM mannitol, 5 mM Tris-HCl (pH 7.4), and 100 mM of either KNO_3 or choline chloride. Ten μl of the vesicles were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and either 20 mM $NaNO_3$ plus 100 mM KNO_3 or 20 mM $NaNO_3$ plus 100 mM choline chloride or 120 mM choline chloride. Symbols have the following meanings: inward Na^+- and K^+-gradient (●, curve a), inward Na^+-gradient (○, curve b), inward Na^+-gradient with K^+-equilibrated (■, curve c), inward Na^+-gradient with outward K^+-gradient (□, curve d), choline chloride (▲, curve e). Values are means ± S.E. of triplicate determinations, where absent, S.E. are smaller than symbol.

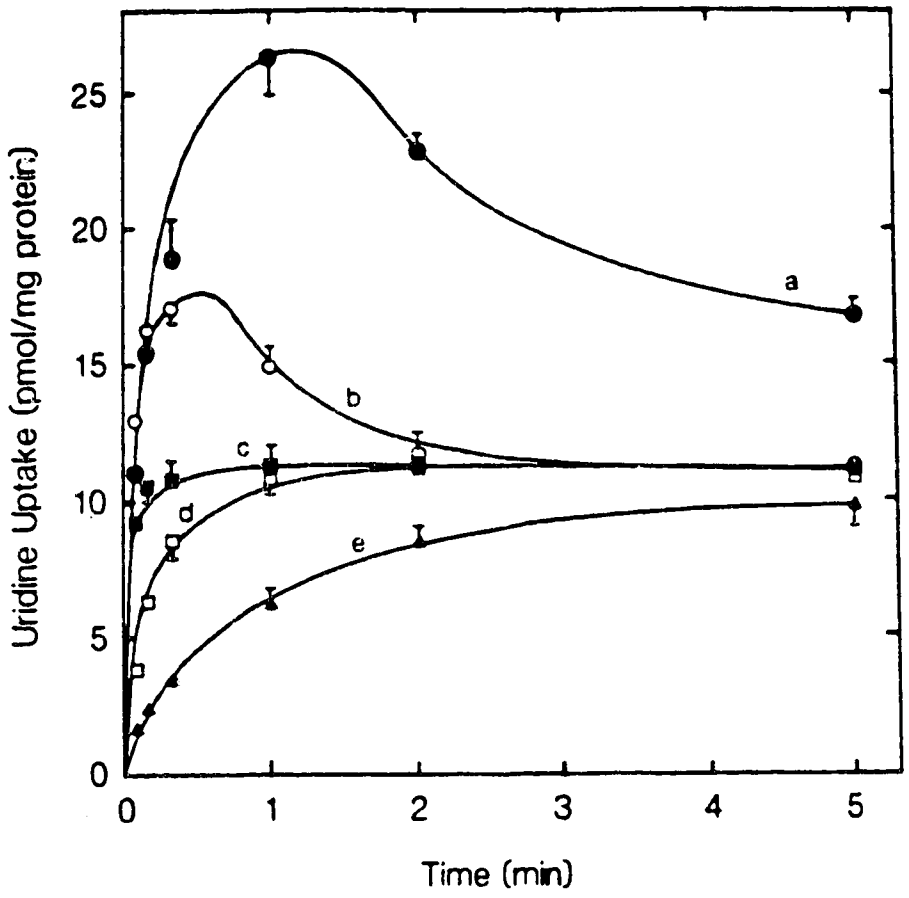

Fig. 5.10. Interactions between Na^+ and K^+ for uridine uptake by rat renal brush border membrane vesicles. Vesicles were resuspended in media containing 300 mM mannitol, 5 mM Tris-HCl (pH 7.4), and either 20 mM $NaNO_3$ plus 100 mM KNO_3 or 20 mM $NaNO_3$ plus 100 mM choline chloride or 120 mM choline chloride. Ten μl of the vesicles were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and either 20 mM $NaNO_3$ plus 100 mM KNO_3 or 20 mM $NaNO_3$ plus 100 mM choline chloride or 100 mM KNO_3 plus 20 mM choline chloride or 120 mM choline chloride. Eight combinations were generated and analysed for uridine uptake. Only the significant results are presented. The symbols represent: inward K^+-gradient (●, curve a), inward K^+-gradient with Na^+-equilibrated (○, curve b), Na^+-equilibrated (■, curve c), K^+-equilibrated (□, curve d), choline chloride (▲, curve e). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

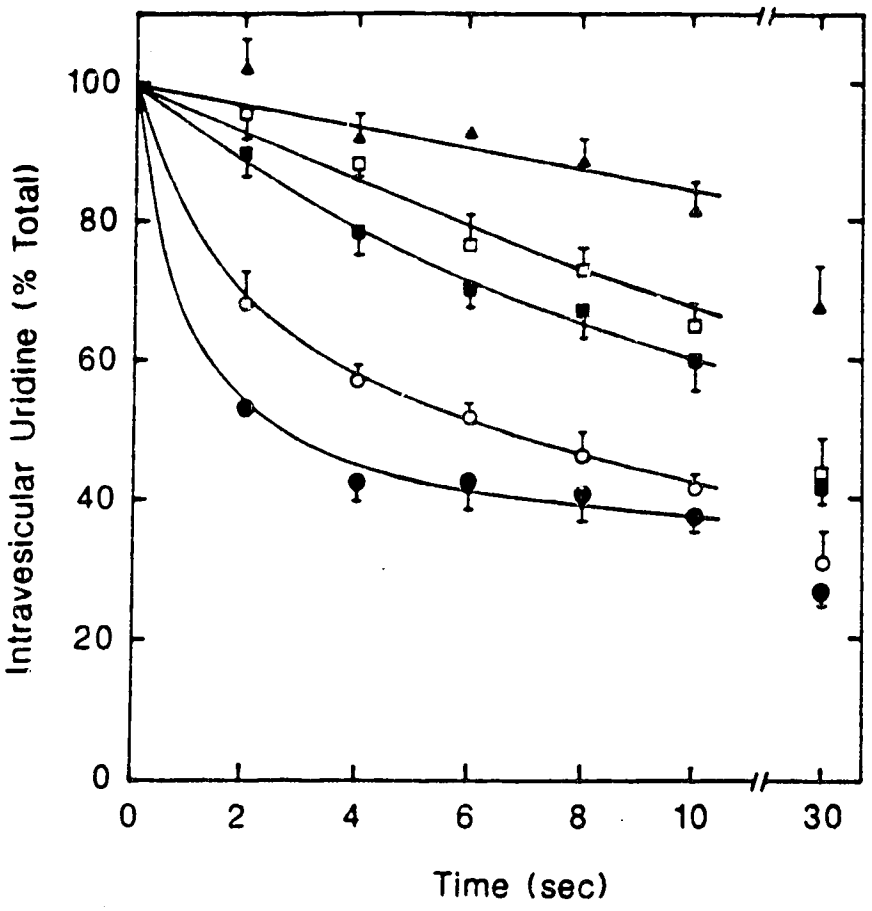

Fig. 5.11. Time course of uridine efflux by rat renal brush border membrane vesicles. Experimental conditions were as described in Section 5.2. Symbols have the following meaning: outward Na^+-gradient (●), Na^+-equilibrated (○), outward K^+-gradient (■), K^+-equilibrated (□), and choline chloride equilibrated (▲). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

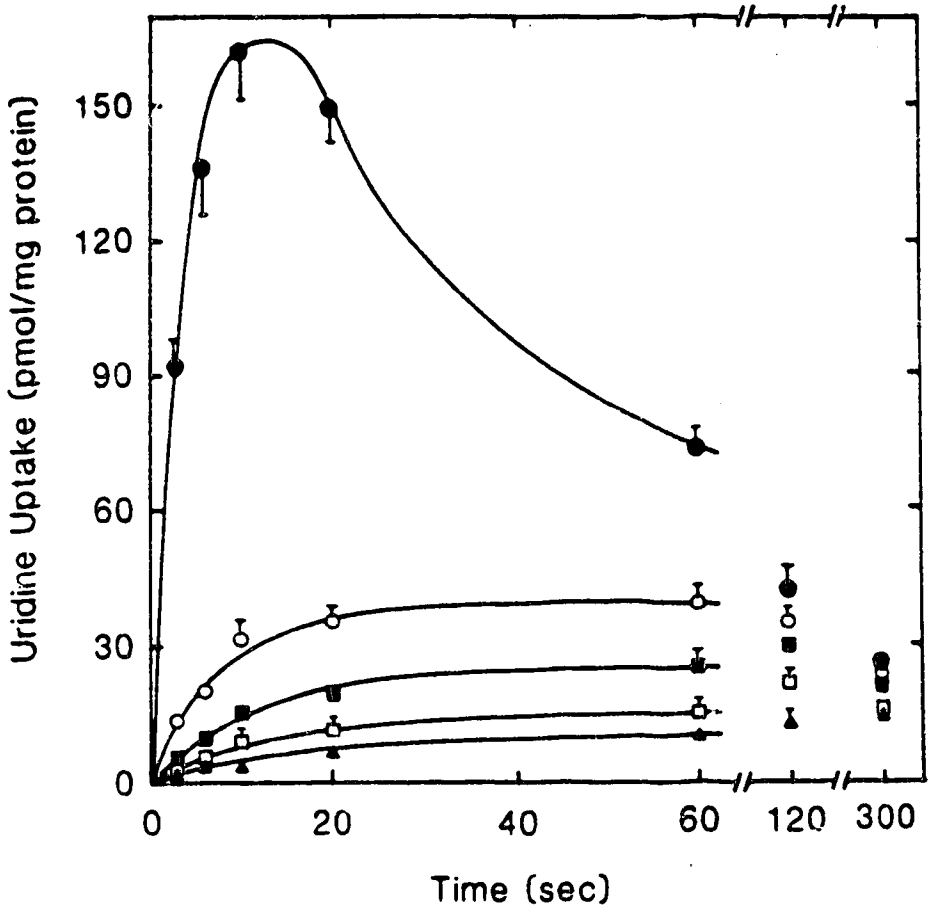

Fig. 5.12. Time course of cation-dependent uridine transport by rat renal brush border membrane vesicles. Ten μl of the vesicle suspension were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and 100 mM of either $NaNO_3$ (●), KNO_3 (○), $LiNO_3$ (■), $RbNO_3$(□), or choline chloride (▲). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

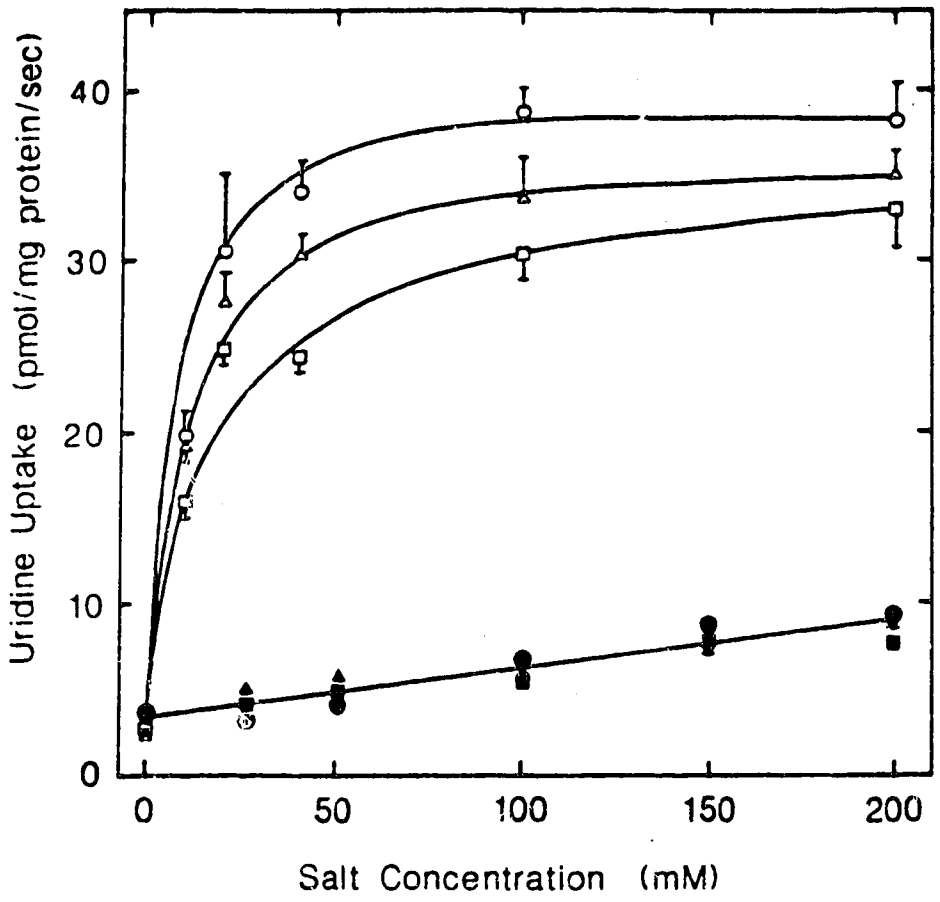

Fig. 5.13. Effect of external Li^+ concentration on Na^+- and K^+-dependent uridine uptake by rat renal brush border membrane vesicles. Ten µl of the vesicle suspension were incubated with 20 µl of media containing (final concentrations) 5 µM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4), either 200 mM $LiNO_3$ (■,□), 100 mM $LiNO_3$ plus 100 mM choline chloride (▲,△) or 200 mM choline chloride (●,○) and variable $NaNO_3$ (○,△,□) or KNO_3 (●,▲,■) concentrations. Variable external $NaNO_3$ and KNO_3 concentrations from 0 to 200 mM were obtained by substitution with choline chloride. Approximate initial rates of uridine uptake were estimated from 2 (○,△,□) and 5 s (●,▲,■) uptake intervals. Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

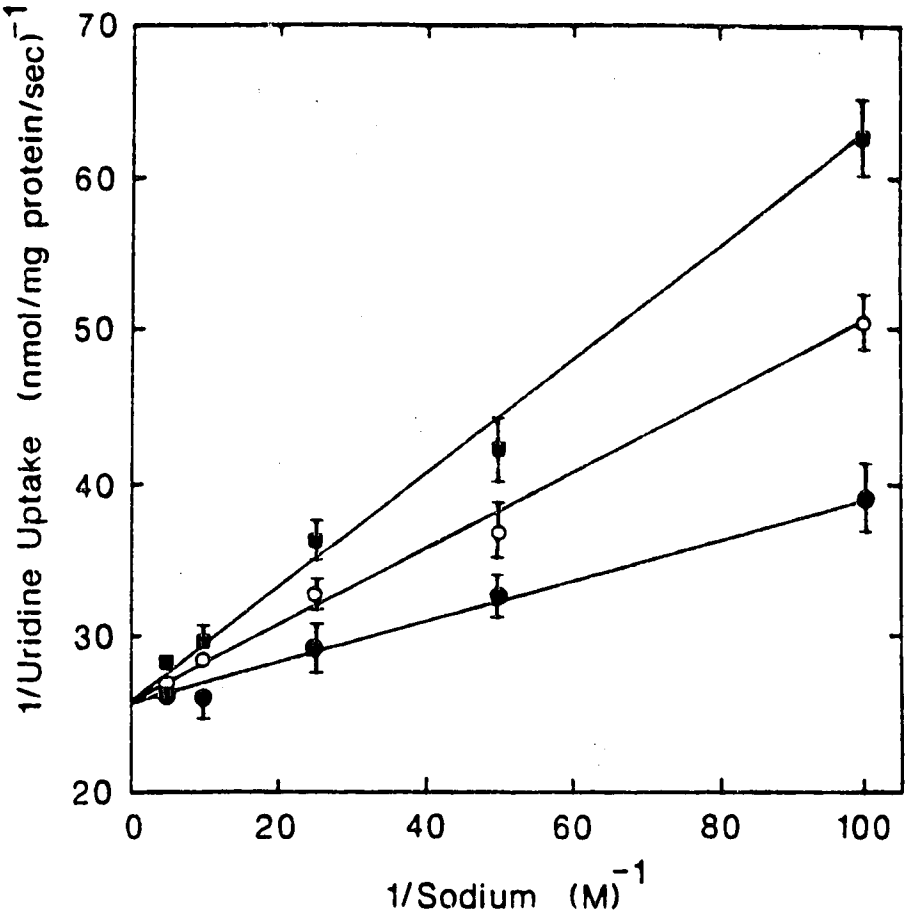

Fig. 5.14. Lineweaver-Burk plot of extravesicular Li^+ concentrations on Na^+-dependent uridine uptake by rat renal brush border membrane vesicles. Ten μl of the vesicle suspension were incubated with 20 μl of media containing either (final concentrations) 200 mM $LiNO_3$ (■), 100 mM $LiNO_3$ plus 100 mM choline chloride (○) or 200 mM choline chloride (●), 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and 200 mM salt concentrations. Variable external Na^+ concentrations from 0 to 200 mM were obtained by substitution with choline chloride. Approximate initial rates of uridine uptake were taken at 2 s. Values are means $\pm$ S.E. of triplicate determinations. For clarity, some data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

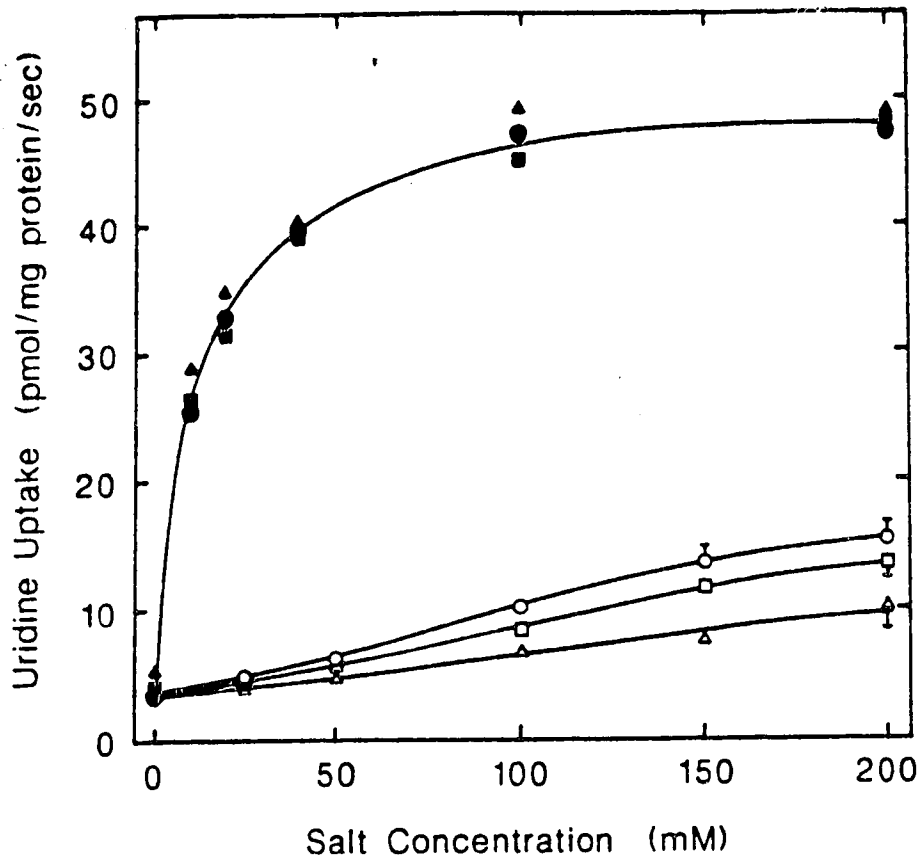

Fig. 5.15. Effect of external Rb^+ concentration on Na^+- and K^+-dependent uridine uptake by rat renal brush border membrane vesicles. Ten μl of the vesilces suspension were incubated with 20 μl of media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4), either 200 mM $RbNO_3$ (▲,△), 100 mM $RbNO_3$ plus 100 mM choline chloride (■,□) or 200 mM choline chloride (●,○) and variable $NaNO_3$ (●,■,▲) or KNO_3 (○,□,△) concentrations. Variable external $NaNO_3$ and KNO_3 concentrations from 0 to 200 mM were obtained by substitution with choline chloride. Approximate initial rates of uridine uptake were estimated from 2 (●,■,▲) and 5 (○,□,△) uptake intervals. Values are means ± S.E. triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

6. Study 4 : Further characterization of uridine transport in rat renal cortex brush border membrane vesicles[1]

6.1. Introduction

The transport of nucleosides across the cell membrane of most animal cells is mediated by specific carrier elements. The best studied of these transport routes is a facilitated diffusion process that has a broad substrate specificity and is inhibited by low concentrations (nanomolar) of NBMPR [8, 11, 24]. Using a variety of approaches, including NBMPR binding activity, reconstitution, photoaffinity labelling and antibodies, this nucleoside transporter protein has been identified and partially purified from mammalian erythrocytes as a band 4.5 glycoprotein with an apparent molecular weight of 65000-45000 [10, 12-14, 33, 39, 41]. In addition to this NBMPR-sensitive nucleoside transport system, there also exists a facilitated diffusion nucleoside carrier that is insensitive to inhibition by NBMPR [3, 25]. Further heterogeneity of nucleoside transport is apparent in recent studies which have shown that rat kidney brush border membrane vesicles take up nucleosides in a sodium-dependent manner [18-20]. The active reabsorption of electrolytes and non-electrolytes, e.g.

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[1] A version of this section has been submitted for publication.

glucose, amino acids, sulphate, phosphate and mono- and dicarboxylic acids, by mammalian kidney epithelial cells has been well documented [26-29, 34, 37]. In contrast, apart from measurements of the kinetic constants of sodium cotransport of nucleosides by rat renal brush border membrane vesicles, few studies have been conducted to investigate the substrate specificity and stoichiometry of sodium-dependent nucleoside transport in the kidney.

Section 5 demonstrated that in addition to a Na^+-dependent uridine transport system, uridine transport in rat renal brush border membrane vesicles can also be driven by a K^+-gradient. K^+-dependent uridine uptake was not due to the substitution of K^+ for Na^+ in the Na^+-dependent system, as stimulation rather than inhibition was observed when both Na^+ and K^+ were present extravesicularly. This result suggested that Na^+ and K^+ are not interacting at the same carrier site. The physiological significance of K^+-dependent uridine transport in renal proximal tubule is not clear. However it is possible for this system to be involved in the secretion of nucleosides. Secretion of 2'-deoxyadenosine by an active transport system in kidney epithelial cells has been reported [17]. Therefore, the aim of this study was to further characterize and compare the transport properties of Na^+- and K^+-dependent uridine transport in order to gain a more comprehensive view of the manner in

which nucleosides cross the apical membrane surface of proximal tubular cells.

6.2. Methods

Preparation of brush border membrane vesicles

Rat renal cortex brush border membrane vesicles were prepared by a two-step $MgCl_2$-precipitation method [6] as described in Section 5.2. The purified vesicles were suspended in an ice-cold medium containing 300 mM mannitol and 5 mM Tris-HCl at pH 7.4. They were used on the same day of preparation. Protein was assayed according to Lowry et al. [21] with bovine serum albumin as a standard.

Uptake studies

The uptake of [^{3}H]uridine (50 μCi/ml) was measured using a phloridzin-stop filtration method detailed previously (Section 5.2). The uptake assay was initiated at room temperature (22-24^{o}C) by mixing a 10-μl aliquot of brush border membrane vesicles (30-80 μg protein) treated with or without gramicidin D (5 μg/mg protein) or valinomycin (2 μg/mg protein), with a 20-μl aliquot of transport buffer, containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and appropriate salt concentrations (with choline chloride substituted to maintain the osmolarity). In the inhibition studies, test compounds and [^{3}H]uridine were added simultaneously with the exception of dilazep, dipyridamole and NBMPR which were preincubated with the membrane vesicles for 5

min prior to the uptake assay. The detailed composition of the final incubation media is given in the figure legends. After an appropriate time interval, the incubation was terminated by the rapid addition of 1 ml of ice-cold stop solution (100 mM mannitol, 100 mM NaCl, 1 mM phloridzin and 5 mM Tris-HCl, pH 7.4). Approximate initial rates of uridine transport were determined using a time interval of 2 and 5 s for the Na^+- and K^+-dependent systems, respectively. The suspension was immediately filtered through an MSI nitrocellulose filter (pore size 0.45 μm, Fisher Scientific) under suction. The filter was subsequently washed once with 5 ml of ice-cold stop solution and, after being dried, was dissolved in 4 ml of scintillation fluid (Scinti Verse E, Fisher Scientific). The radioactivity was determined using an LKB/Wallac 1217 liquid scintillation counter with automatic quench correction and disintegration per minute conversion. The termination and washing process was completed within 10 s. From the previous study (Fig. 5.1), no significant loss of [^{3}H]uridine from the vesicles occurred using this stop solution. The radioactivity retained on the filter in the absence of membrane vesicles was used as the blank value for the uptake assay. Na^+- and K^+-independent uridine uptake was determined by exposing vesicles to Na^+ and K^+ free medium for the same incubation period. These values were subtracted from measurements of uridine uptake by

vesicles in the presence of Na^+ or K^+ to determine Na^+- or K^+-dependent transport rates, respectively. All experiments were performed in triplicate. Unless otherwise indicated, only the results of representative experiments are illustrated. Kinetic constants for the Na^+- and K^+-dependent systems (apparent K_m and V_{max}) were determined by nonlinear least squares fit of the equation:

$$v = V_{max} \cdot [S]/K_m + [S]$$

where v is the initial transport rate and [S] is the permeant concentration using the computer program HYPMIC [2].

Fluorescence quenching measurements

Fluorescence changes of 3,3′-dipropylthiadicarbocyanine iodide (DiS-C3(5)) were measured at an excitation wavelength of 622 nm and emission wavelength of 669 nm with a Turner spectrofluorometer (model 430, G.K. Turner Associates, Palo Alto, CA). Ten μl of DiS-C3(5) ethanol stock was added to 3 ml of buffer solution (100 mM mannitol, 5 mM Tris-HCl, pH 7.4, and 100 mM appropriate salt) to give a final concentration of 3 μM. Twenty μl of membrane vesicles (150 μg protein) were then added to set a new base line for the fluorescence quenching curve. The substrate (100 μl) was injected immediately after the new base line was obtained as indicated in Figs. 6.6 and 6.7.

The data presented were performed with the same membrane preparation for the purpose of comparison. All experiments were repeated at least twice with two membrane preparations. Within one experiment, an experimental condition was always carried out in triplicate. The triplicate traces were identical and the experimental traces obtained under a given condition but with different membrane preparation were comparable (variation 10-20%).

6.3. Results

Concentration dependence of uridine uptake

The concentration dependence of uridine uptake in the presence or absence of an inwardly directed Na^+- or K^+-gradient measured as a function of the uridine concentration is shown in Fig. 6.1. Uptake of [^{3}H]uridine in the absence of Na^+ and K^+ was linear with increasing uridine concentration. In contrast, uridine transport in the presence of extravesicular Na^+ or K^+ could be resolved into two components: (i) a linear component, and (ii) a saturable component. The kinetic constants for the Na^+- or K^+-gradient dependent transport components were determined after subtraction of the linear component estimated in the absence of Na^+ and K^+. The saturable data of Fig. 6.1 conformed to simple Michaelis Menten kinetics. The apparent K_m values in the presence of 100 mM Na^+ and K^+ from at least five determinations were 6.7 $\pm$ 0.5 and 28 $\pm$ 3 μM, respectively, with V_{max} estimates of 70 $\pm$ 1 and 15 $\pm$ 4 pmol/mg protein/s, respectively (means $\pm$ S.E.). In a further study, the effect of varying the extravesicular Na^+ or K^+ concentration on the kinetic constants for uridine influx was determined (Figs. 6.2 and 6.3). Increasing extravesicular Na^+ from 5 to 200 mM caused a decrease in K_m (Fig. 6.2) but had little effect on the V_{max} (Fig. 6.3) for uridine influx. In contrast, the K_m

for uridine influx by the K^+-dependent system was not significantly affected by extravesicular K^+ varying from 50-300 mM (Fig. 6.2). However, the V_{max} of the K^+-dependent system was increased by approximately 3-fold in a linear manner as the concentration of extravesicular K^+ was increased from 50 to 300 mM (Fig. 6.3).

Inhibition of uridine uptake

The substrate specificities of the uridine uptake systems were examined by studying the interaction of various nucleoside analogues on the transport process in the presence of 100 mM extravesicular Na^+ or K^+. Table 6.1 shows that pyrimidine nucleosides such as thymidine and 2′-deoxyuridine were effective inhibitors of uridine uptake into renal brush border membrane vesicles, with K_i values of 5 and 8 μM for the Na^+-dependent system and 70 and 100 μM for the K^+-dependent system, respectively. Adenosine, 2′-deoxyadenosine and 2-chloroadenosine were also potent uridine uptake inhibitors (K_i values of approximately 3-7 μM and 40-85 μM for the Na^+- and K^+-dependent systems, respectively). In contrast, other purine nucleosides such as 8-azidoadenosine, inosine, 2′-deoxyinosine, guanosine and 2′-deoxyguanosine were less effective at blocking uridine influx (apparent K_i values of 80-550 μM and 400-2700 μM for the Na^+- and K^+-dependent systems, respectively). Neither uptake system was inhibited by uracil, D-glucose and D-ribose at

concentrations as high as 1 mM. Both uptake systems were also insensitive to uptake inhibition by 30 μM NBMPR and dipyridamole, inhibitors of facilitated diffusion nucleoside transport. In contrast, dilazep, another potent inhibitor of nucleoside transport by facilitated diffusion was able to inhibit uridine uptake in both systems with apparent K_i values of 34 and 90 μM for the Na^+- and K^+-dependent systems, respectively. The uridine uptake was also inhibited by phloridzin, a Na^+-dependent D-glucose transport inhibitor [1, 31] which was used in this study to terminate the transport assay, with apparent K_i values of 60 and 100 μM for Na^+- and K^+-dependent systems, respectively.

The kinetics of inhibition of uridine uptake by various nucleosides were studied in more detail. The mode of inhibition and the apparent K_i values were determined by Dixon plots and confirmed by Lineweaver-Burk plots. Table 6.2 shows that adenosine and thymidine were potent competitive inhibitors of uridine uptake with K_i values of 3 and 40-80 μM for Na^+- and K^+-dependent systems, respectively. Competitive inhibition was also observed with nucleosides such as inosine, guanosine and 8-azidoadenosine, with apparent K_i values of approximately 100-500 and 300-1300 μM for Na^+- and K^+-dependent systems, respectively. The apparent K_i values for adenosine and thymidine for the Na^+-dependent system are similar to the apparent K_m values reported previously

for Na^+-dependent adenosine and thymidine influx [18, 20] consistent with the suggestion that adenosine, thymidine and uridine are permeants for the same system. In contrast, the apparent K_i values for inosine and guanosine (300 ± 12 and 92 ± 4 μM, respectively, means ± S.E.) are significantly different from the apparent K_m's for Na^+-dependent inosine and guanosine influx by rat kidney brush border membrane vesicles (2 and 3.5 μM, respectively) [19].

Clamping the transmembrane electrical potential

In most stoichiometry studies, the electrical potentials across the brush border membrane have been effectively clamped by using the conventional K^+/valinomycin treatment [9, 16, 34]. However using the same technique, an inhibitory effect was observed (Fig. 6.4). An additional problem associated with this approach is that K^+, the ion used to clamp the electrical potential, is also a substrate for one of the transport systems. Thus, the Na^+:uridine and K^+:uridine stoichiometric ratios were evaluated by using the highly lipophilic anion iodide to clamp the membrane potential. This anion has been successfully used to quantitate the generation of membrane potential differences in vesicle studies [4, 5].

Preliminary control studies demonstrated that iodide had no effect on uridine transport. The time course for uridine uptake in Na^+-equilibrated conditions (100 mM) with the membrane potential clamped to zero with gramicidin D was identical with either nitrate or iodide as the only anion present in the incubation media (Fig. 6.5). As expected, no overshoot was observed and uridine transport reached a steady state by 10 s. Similar results were obtained in K^+-equilibrated conditions (100 mM) with the membrane potential clamped to zero using valinomycin although the time course of uptake was considerably slower than that observed with Na^+.

To show iodide was able to clamp the electrical potential in the present experimental conditions, the cyanine dye DiS-C3(5) was used to measure the membrane potential of renal brush border membrane vesicles related to Na^+- or K^+-dependent uridine uptake. Figs. 6.6 and 6.7 show fluorescence signals of DiS-C3(5) following the addition of uridine and D-glucose respectively to renal brush border membrane vesicles under various conditions. Uridine increased the fluorescence of the dye in the presence of extravesicular Na^+ or K^+ indicating the formation of an inside positive membrane potential. In the absence of Na^+ or K^+ (substituted with mannitol) no signal was observed. The anions increased the amplitude of fluorescence changes in both Na^+ and K^+ conditions in the following order: $SO_4^{2-}>Cl^->NO_3^->SCN^->I^-$. This anion

effect is similar to what would be predicted from the order of diffusibility of these anions and the resultant magnitude of the membrane potential [22]. In iodide media, the addition of uridine did not lead to a significant change in the fluorescence. This suggests that the high diffusibility of iodide had effectively clamped the electrical potential generated during the Na^+- or K^+-uridine cotransport process. For comparison, the fluorescence change related to the transport of D-glucose was also studied (Fig. 6.7). The addition of D-glucose in sodium media provoked a similar anion effect as observed with uridine. However in potassium media, no change in the fluorescence was observed. This is due to the fact that D-glucose transport in renal brush border membrane is sodium dependent and cannot be driven by potassium. Therefore, the changes in fluorescence observed were indeed related to the transport process.

Stoichiometry of Na^+- and K^+-dependent uridine transport

The "activation method" [9, 35, 36] was used to determine the stoichiometry of Na^+- and K^+-dependent uridine transport using vesicles in which the membrane potential was clamped with I^-. The initial rates of uridine influx were measured as a function of the extravesicular Na^+ or K^+ concentration over the range 0-200 mM. Fig. 6.8 shows a plot of sodium- and potassium-dependent uridine flux (5 μM) versus the respective

cation concentration. The curve for Na^+ activation of uridine uptake was hyperbolic consistent with the notion of a Na^+:uridine coupling stoichiometry of 1:1. In contrast, the uridine flux data with K^+ showed a marked sigmoidal dependence, which is indicative of multiple potassium binding sites [30]. To determine the Na^+:uridine and K^+:uridine stoichiometry from these data, the Hill-type equation as described by Turner and Moran [35, 36] was used:

$$Flux = V_{max} \cdot [Cation]^n / (Z + [Cation]^n)$$

where Z is a constant comprising the interaction factors between binding sites and the intrinsic association constants, and n is the Hill coefficient [30]. According to this equation, a plot of $Flux/[Cation]^n$ against Flux for the correct value of n will yield a straight line with slope 1/Z. Figs. 6.9 and 6.10 show a series of such plots for Na^+ and K^+ using the data of Fig. 6.8. Visual inspection of Figs. 6.9 and 6.10 indicate that Hill coefficients of 1 and 1.5 provide the best fit to these data for Na^+ and K^+, respectively.

An alternative approach for determining stoichiometric coupling ratios is by the "Static-head method" [9, 35, 36]. However due to the poor signal to noise ratio as a consequence of the low uridine transport rate, the precise stoichiometric determination using this method was not possible.

Effect of anions

The permeability of iodide exceeds the permeability of other anions in the present experimental conditions (permeability order $I^->SCN^->NO_3^->Cl^->SO_4^{2-}$), and thus one would predict that the magnitude of the overshoot should be greater in the presence of I^- than the other anions. However, Fig. 6.11 shows the chemical gradients generated by nitrate salts still give a higher overshoot in both uptake systems than those generated by iodide salts. These results were consistent with the earlier finding that NO_3^- has a higher reactive order for uridine uptake than SCN^-, Cl^- and SO_4^{2-} (Section 5.3).

One possible explanation for these observations is the involvement of anions in the transport processes. Such a possibility was tested by equilibrating 200 mM Na^+ (Fig. 6.12) or K^+ (Fig. 6.13) across the plasma membranes and varying the extravesicular nitrate or chloride concentration from 0 to 200 mM with gluconate replacement to maintain isoosmolarity. Gramicidin D or valinomycin was added to clamp the transmembrane electrical potential for Na^+- and K^+-dependent systems, respectively. The results show that increasing the extravesicular concentration of NO_3^- resulted in a hyperbolic activation of uridine uptake with a $K_{nitrate}$ values of approximately 42 ± 13 and 163 ± 54 mM for Na^+- and K^+-dependent systems, respectively (estimates

± S.E.). In contrast, no hyperbolic activation of uridine uptake was observed with vesicles equilibrated with nitrate (in=out). This result rules out the possibility that nitrate was acting solely as a modifier. When chloride instead of nitrate was used in the extravesicular medium, the rate of uridine transport activated by anion gradient was lower and no saturation of uptake was observed at chloride concentrations as high as 200 mM (Figs. 6.12 and 6.13). These results thus suggest the possible involvement of anions in uridine transport via the Na^+- and K^+-dependent routes in rat renal brush border membrane vesicles.

6.4. Discussion

In this section, the transport properties of the Na^+- and K^+-dependent uridine transporters in rat renal brush border membrane vesicles were further characterized. Kinetic analysis of these systems measured under zero-trans conditions at 100 mM $NaNO_3$ or KNO_3 and 22-24°C indicated high-affinity transport sites obeying Michaelis Menten kinetics with K_m values of 6.7 ± 0.5 and 28 ± 3 μM and V_{max} values of 70 ± 1 and 15 ± 4 pmol/mg protein/s (means ± S.E.) for Na^+- and K^+-dependent uridine uptake, respectively (Fig. 6.1). These values are similar to those reported earlier for Na^+-dependent uridine uptake in rat renal brush border membrane vesicles [19], and are one order of magnitude lower than the K_m values for the facilitated diffusion nucleoside transporters found in other cell types [40]. No previous data has been reported for K^+-dependent uridine uptake.

The effect of Na^+ on the kinetics of uridine transport by renal brush border membrane vesicles was to increase the affinity of uridine for zero-trans influx (Fig. 6.2) as the cis Na^+ concentration increased, but the V_{max} was unaltered (Fig. 6.3). This result suggests that the Na^+-dependent renal uridine transporter is an ordered system under zero-trans conditions where Na^+ binds first to increase the affinity of the carrier for

uridine [32]. The intestinal brush border Na^+-dependent D-glucose transporter and the renal brush border Na^+-dependent succinate carrier are also believed to function in the same manner [15, 38]. In contrast, K^+ had no effect on K_m (Fig. 6.2) but increased V_{max} (Fig. 6.3) suggesting that the order of binding for the K^+-dependent carrier is random.

The Na^+:uridine and K^+:uridine coupling stoichiometry was determined by the "activation method" on iodide-clamped membrane vesicles [9, 35, 36]. The data (Figs. 6.9 and 6.10) are consistent with the notion of a coupling ratio of 1:1 and 3:2 for Na^+:uridine and K^+:uridine, respectively. This difference further suggests that Na^+- and K^+-dependent uridine transport represent separate transport systems.

The transport inhibition studies, which were designed to investigate the substrate specificity, show that selected purine and pyrimidine nucleosides were inhibiting uridine transport competitively (Table 6.2). However, only pyrimidine nucleosides, adenosine and some of the adenosine analogues were potent inhibitors of uridine transport (Table 6.1). The similarity of the K_i values for inhibition of Na^+-dependent uridine transport with the K_m values for transport of adenosine and thymidine [19, 20, Table 6.1] suggest the existence of a pyrimidine-adenosine specific transport system in rat

renal proximal tubules. Modification of adenosine at the 2 position or substitution of the ribose with deoxyribose did not have profound effects on the ability of the molecule to inhibit uridine uptake (Table 6.1). However, modification at the 8 position resulted in a decreased affinity of the compound for the carrier system, as shown by the low potency of 8-azidoadenosine as an uptake inhibitor (K_i values of 550 and 1200 μM for Na^+- and K^+-dependent systems, respectively) and by its low transport activity (Appendix 6.1). In contrast to adenosine, other purine nucleosides such as inosine, 2'-deoxyinosine, guanosine and 2'-deoxyguanosine were weak inhibitors of Na^+- and K^+-dependent uridine uptake. Inhibition by these nucleosides is unlikely to be due to secondary effects, that is, transport by a different system that eliminates the electrochemical gradient thereby decreasing uridine uptake, as D-glucose known to be transported in a Na^+-dependent manner had no effect on uridine influx. However, the K_i values for inosine and guanosine as inhibitors of Na^+-dependent uridine transport (300 and 92 μM) did not equate with the K_m values for their uptake (2 and 3.5 μM, respectively) [19]. Moreover, the K_i values determined in this study do not correspond with their K_i values as inhibitors of Na^+-dependent adenosine transport (2.2 and 3.9 μM, respectively) [19, Appendix 6.2]. These results suggest the existence of a second different active nucleoside

transport system with a high affinity for purines. Further studies will be required to investigate the detailed specificity of such a Na^+-dependent purine system. Present findings contrast with those of Darnowski et al. [7] who demonstrated that Na^+-dependent uridine transport by murine splenocytes was inhibited by purine nucleosides including inosine and guanosine but pyrimidine nucleosides had no effect. It thus seems likely that there may exist a number of active nucleoside transporters with distinct substrate specificities.

Na^+- and K^+-dependent uridine transport is electrogenic (Fig. 6.6), and there is a net transfer of positive charge across the membrane, presumably carried by the cation(s). The stimulation of uridine transport in the vesicles by lipid soluble anions can be explained, therefore, by their passive movement which would then minimize charge separation. However the greatest effect was seen with nitrate which is not the most permeable anion tested (permeability $I^->SCN^->NO_3^->Cl^->SO_4^{2-}$). This suggests that there may be a more direct effect of NO_3^- on the transport system(s). Indeed, anion-gradient activated uridine transport was shown to exhibit a concentration dependence and conform to simple Michaelis Menten kinetics with apparent $K_{nitrate}$ values of 42 and 160 mM for Na^+- and K^+-dependent systems, respectively (Figs. 6.12 and 6.13). A chloride-gradient also stimulated uridine uptake but at a lower affinity

($K_{chloride}$ > 200 mM) compared with nitrate. In conditions where the anion was equilibrated across the membrane (in=out), no stimulation of uridine transport was observed. This suggests the possible involvement of anions in uridine transport in rat renal brush border membrane vesicles.

Although the K^+-dependent system is hypothesized to be responsible for the secretion of nucleosides (Section 5), no difference in substrate specificity between the Na^+- and K^+-dependent systems was observed. It is possible that the K^+-dependent system is a contaminant from the basolateral membrane, that functions as part of the reabsorption process by moving nucleosides across the basolateral membrane into the blood. Alternatively, the K^+-dependent system can still be involved in the secretion of nucleoside but depends heavily on the translocation step that moves nucleoside across the basolateral membranes in the event of reabsorption. That is, substrates that are not readily transported across the basolateral membrane could therefore be subjected to intracellular metabolism and/or secretion via the K^+-dependent system. Such an active secretion of nucleosides by the kidney is possible as studies have shown that nucleoside such as 2′-deoxyadenosine is found in excess in the urine [17]. This active renal secretion of 2′-deoxyadenosine was later demonstrated to be unique and independent of the organic cation secretory system

which was responsible for the secretion of 2'-deoxytubercidin [23]. Clearly, nucleoside transport across the renal basolateral membrane should be characterized to understand the overall renal handling of nucleosides.

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Table 6.1. Inhibition of the Na^+- and K^+-dependent uridine transport in rat renal brush border membrane vesicles by various compounds.

Inhibitors	K_i (μM)	
	Na^+-dependent	K^+-dependent
Adenosine	3.8 ± 0.4	83 ± 2
2'-deoxyadenosine	3.5 ± 0.1	65 ± 10
2-chloroadenosine	7.3 ± 0.7	42 ± 4
8-azidoadenosine	530 ± 18	1200 ± 30
2'-deoxyuridine	7.9 ± 1.0	101 ± 11
Thymidine	4.8 ± 0.6	54 ± 10
Inosine	300 ± 44	2000 ± 500
2'-deoxyinosine	320 ± 30	1610 ± 350
Guanosine	88 ± 3	350 ± 21
2'-deoxyguanosine	110 ± 6	750 ± 110
Uracil	-----[a]	-----[a]
D-ribose	-----[a]	-----[a]
D-glucose	-----[a]	-----[a]
Dilazep	34 ± 2	90 ± 1
NBMPR	-----[b]	-----[b]
Dipyridamole	-----[b]	-----[b]
Phloridzin	60 ± 8	100 ± 9

[a] no inhibition at 1 mM inhibitor concentration.
[b] no inhibition at 30 μM inhibitor concentration.

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The uptake of uridine was initiated by addition of brush border membrane vesicles to medium containing (final concentrations) 5 μM [^{3}H]uridine, 100 mM of either $NaNO_3$ or KNO_3 and test compound. For dilazep, NBMPR, dipyridamole, and phloridzin, vesicles were preincubated with these inhibitors before addition of [^{3}H]uridine. Initial rates of uridine uptake at 22°C were taken at 2 and 5 s for Na^+- and K^+-dependent systems, respectively. K_i values were calculated from the equation $K_i = IC_{50}/(1+L/K_m)$, where K_m values for Na^+- and K^+-dependent uridine influx were taken as 6.7 and 28 μM respectively, and L = 5 μM uridine. The values are the means $\pm$ S.E. of at least 3 separate experiments conducted in triplicate.

Table 6.2. Kinetic parameters for the inhibition of the Na^+- and K^+-dependent uridine transport in rat renal brush border membrane vesicles by various nucleosides.

	Na^+-dependent		K^+-dependent	
Nucleosides	Inhibition	K_i (μM)	Inhibition	K_i (μM)
Adenosine	Competitive	3 ± 1	Competitive	80 ± 6
Inosine	Competitive	300 ± 10	Competitive	1300 ± 160
Guanosine	Competitive	92 ± 4	Competitive	320 ± 30
8-Azido-adenosine	Competitive	500 ± 30	Competitive	1200 ± 60
Thymidine	Competitive	3 ± 1	Competitive	40 ± 14

[^{3}H]Uridine concentrations were 3, 6 and 12 μM for Na^+-dependent system and 12.5, 25 and 50 μM for K^+-dependent system. Other experimental conditions were as described in the legend of Table 6.1. K_i values and the type of inhibition were determined by Dixon and Lineweaver-Burk plots. Values are means ± S.E. of two separate experiments conducted in triplicate.

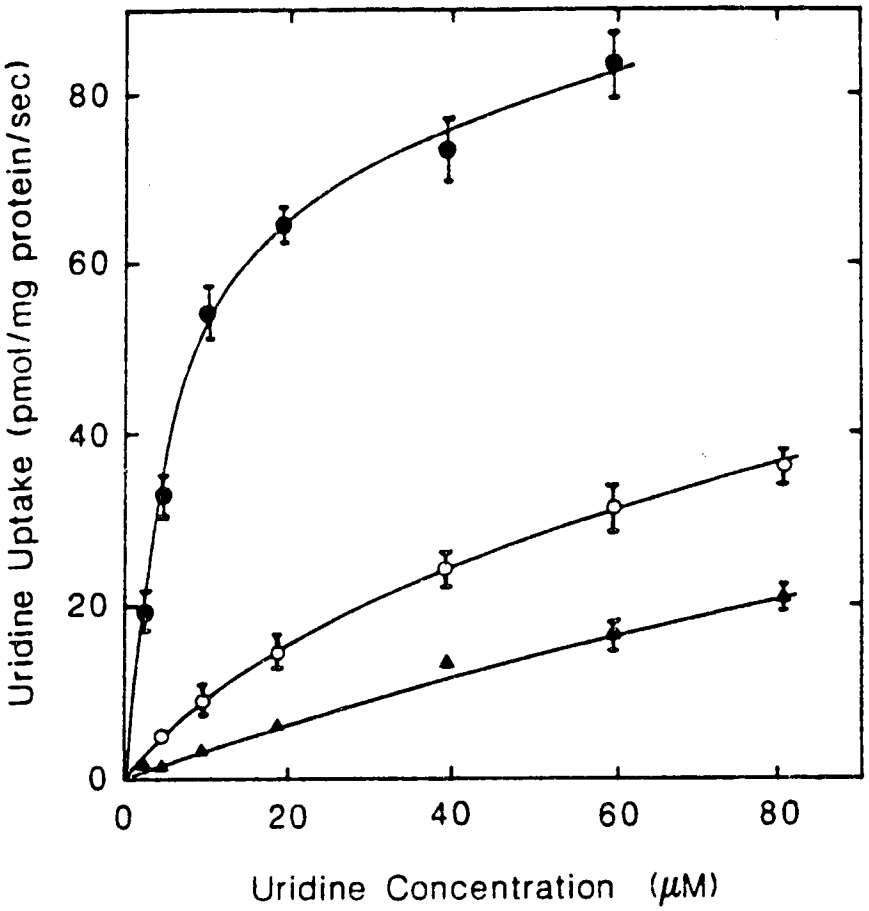

Fig. 6.1. Concentration dependence of Na^+- and K^+-dependent uridine uptake by rat renal brush border membrane vesicles. [^{3}H]Uridine uptake in the presence of either 100 mM $NaNO_3$ (●), KNO_3 (o) or choline chloride (▲) was determined as described in Section 6.2. The approximate initial rates of uridine uptake at 22°C were taken at 2 and 5 s for Na^+- and K^+-dependent systems, respectively. Values are means ± S.E. of triplicate determinations, where absent, S.E. are smaller than symbol. Kinetic constants of saturable uridine uptake were determined by non-linear least squares fit of the Michaelis Menten equation using the computer program HYPMIC [2], and gave K_m values of 6.0 ± 1.2 and 23.6 ± 1.2 μM with V_{max} values of 72 ± 4 and 20 ± 1 pmol/mg protein/s for Na^+- and K^+-dependent systems, respectively (estimates ± S.E.).

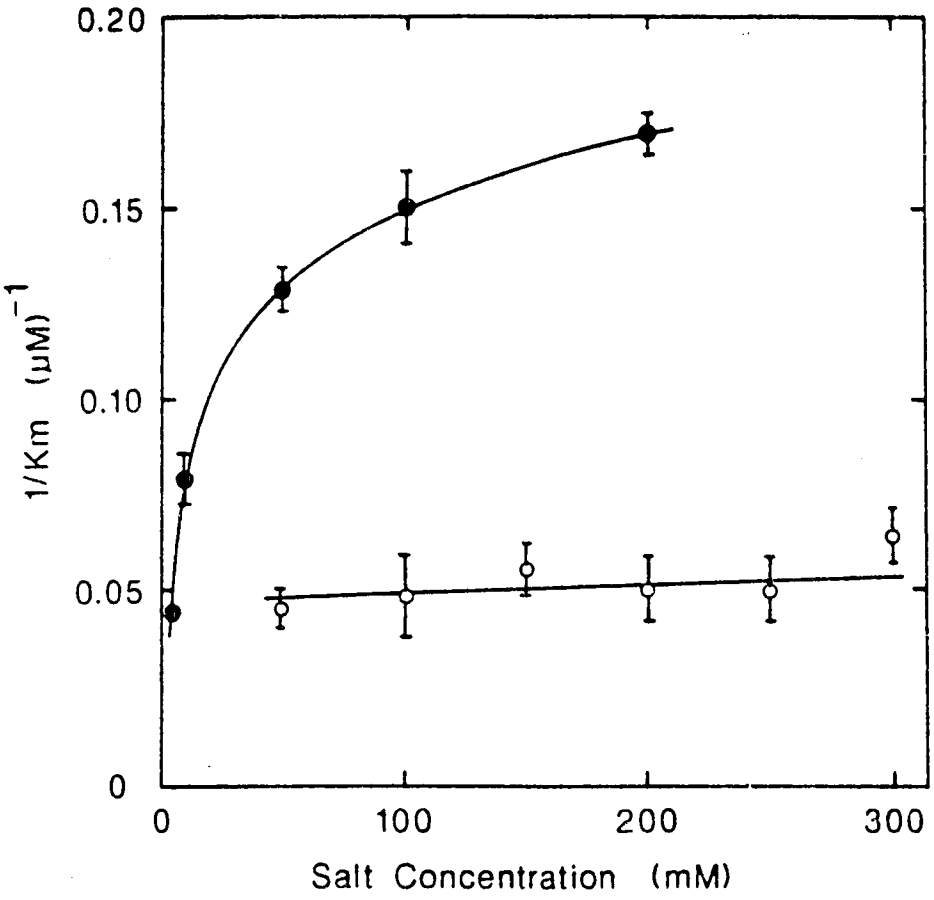

Fig. 6.2. Effect of extravesicular salt concentrations on transport affinity for Na^+- and K^+-dependent uridine uptake by rat renal brush border membrane vesicles. [^{3}H]Uridine uptake was determined in the presence of either $NaNO_3$ (●) or KNO_3 (○). The approximate initial rates of [^{3}H]uridine uptake at 22°C were taken at 2 and 5 s for Na^+- and K^+-dependent systems, respectively. The data shown are corrected for the linear component of transport determined in the absence of $NaNO_3$ and KNO_3. Variable extravesicular Na^+ and K^+ concentrations were obtained by substitution with choline chloride. K_m at each salt concentration was determined using the computer program HYPMIC [2]. Values are means ± S.E. of 3 separate experiments conducted in triplicate, where absent, S.E. is smaller than symbol.

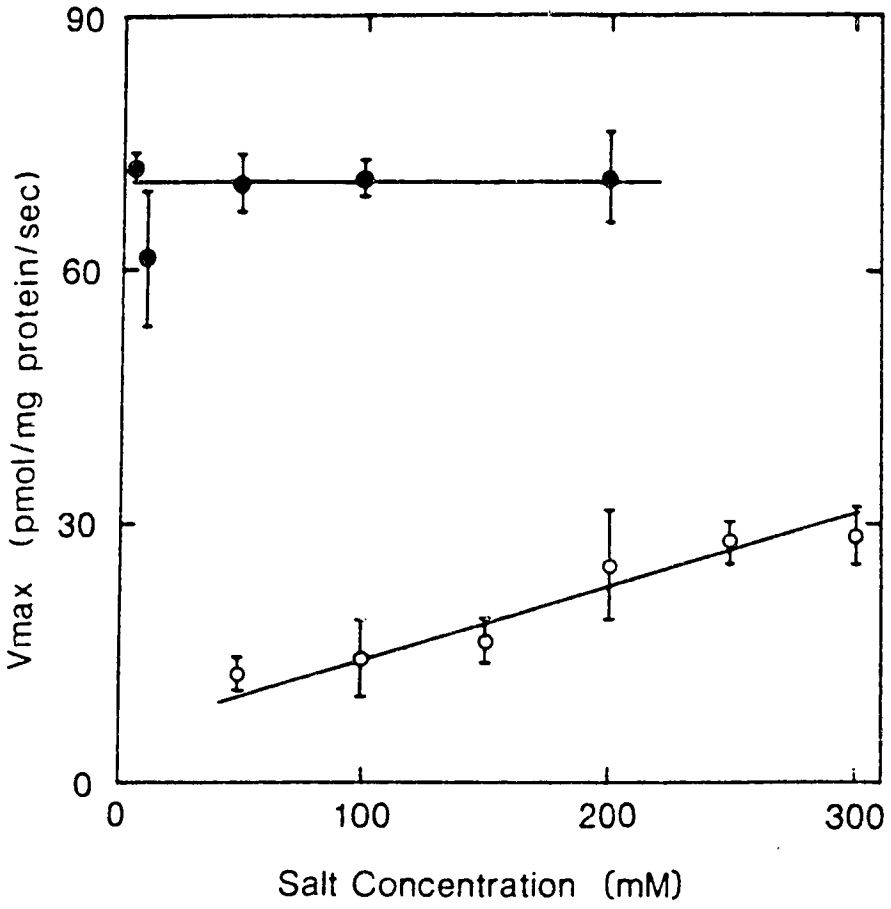

Fig. 6.3. Effect of extravesicular salt concentrations on maximum velocity for Na^+- and K^+-dependent uridine uptake by rat renal brush border membrane vesicles. [^{3}H]Uridine uptake was determined in the presence of either $NaNO_3$ (●) or KNO_3 (o). The approximate initial rates of [^{3}H]uridine uptake at 22°C were taken at 2 and 5 s for Na^+- and K^+-dependent systems, respectively. The data shown are corrected for the linear component of transport determined in the absence of $NaNO_3$ and KNO_3. Variable extravesicular Na^+ and K^+ concentrations were obtained by substitution with choline chloride. V_{max} at each salt concentration was determined using the computer program HYPMIC [2]. Values are means ± S.E. of 3 separate experiments conducted in triplicate.

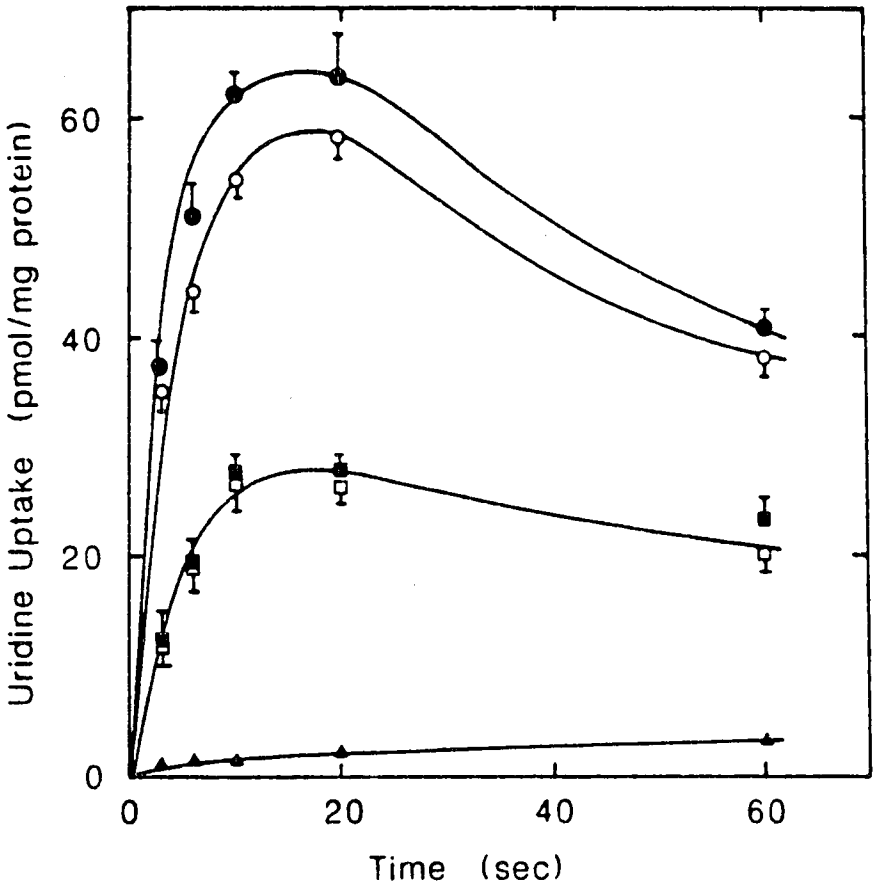

Fig. 6.4. Effect of valinomycin-induced-K^+-clamped transmembrane electrical potential on uridine uptake by rat renal brush border membrane vesicles. Vesicles treated with (o, □) or without (●, ■, ▲) valinomycin (2 μg/mg protein) were preloaded with 100 mM of either KCl (■, □) or choline chloride (●, o, ▲). Uridine uptake was initiated by adding vesicles to the medium containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and 100 mM of either NaCl (●, o, ■, □) or choline chloride (▲). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

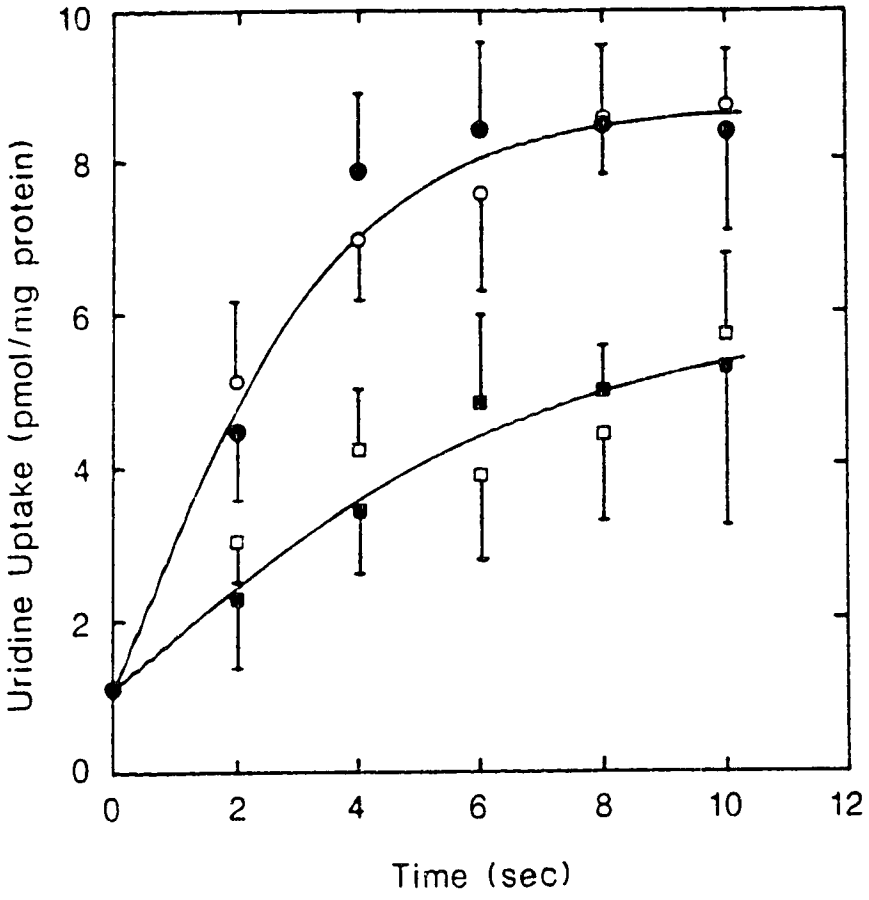

Fig. 6.5. Absence of iodide effects on Na^+- and K^+-dependent uridine uptake by rat renal brush border membrane vesicles. Vesicles were treated with gramicidin D (5 μg/mg protein) (●, ○) or valinomycin (2 μg/mg protein) (■, □). [^{3}H]Uridine (5 μM) uptake by vesicles equilibrated (in=out) with 100 mM of either $NaNO_3$ (●), NaI (○), KNO_3 (■) or KI (□) was measured. Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E..

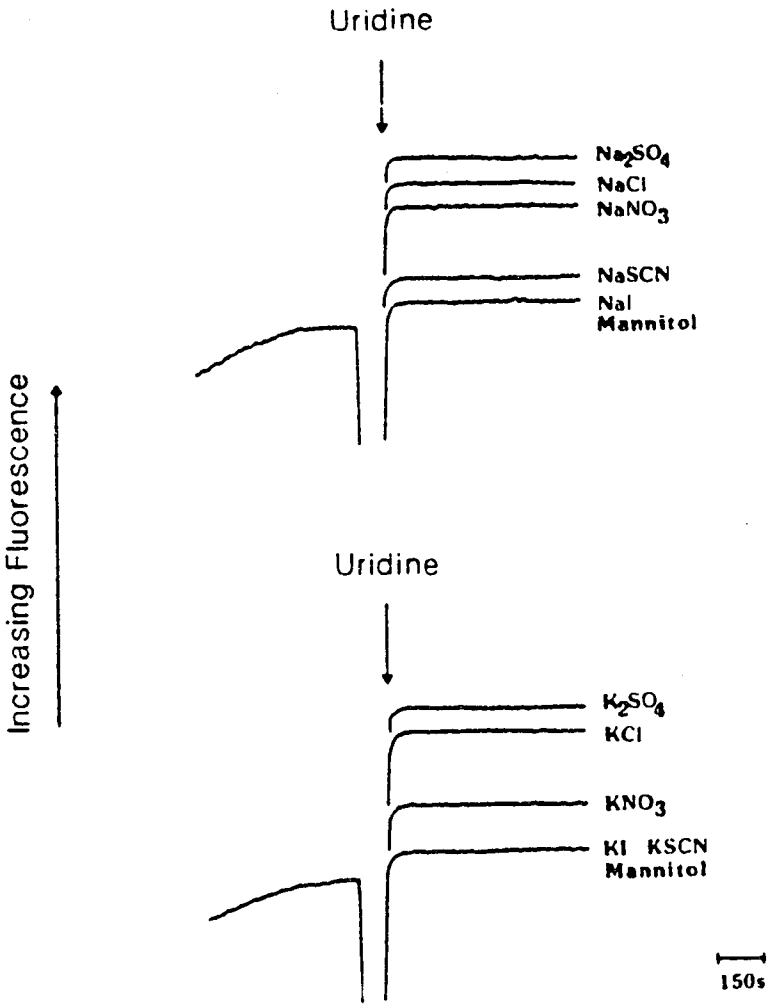

Fig. 6.6. Fluorescence signals for electrogenic uridine influx by rat renal brush border membrane vesicles. Vesicles were suspended in 3 ml of medium containing 300 mM mannitol, 5 mM Tris-HCl (pH 7.4) and 3 μM DiS-C3(5) or substituted 200 mM mannitol with 100 mM of either NaI, NaSCN, $NaNO_3$, NaCl, KI, KSCN, KNO_3, KCl or 50 mM of Na_2SO_4 or K_2SO_4. At the time indicated by the arrow, 100 μl of uridine solution was added into the cuvette to give a final concentration of 100 μM.

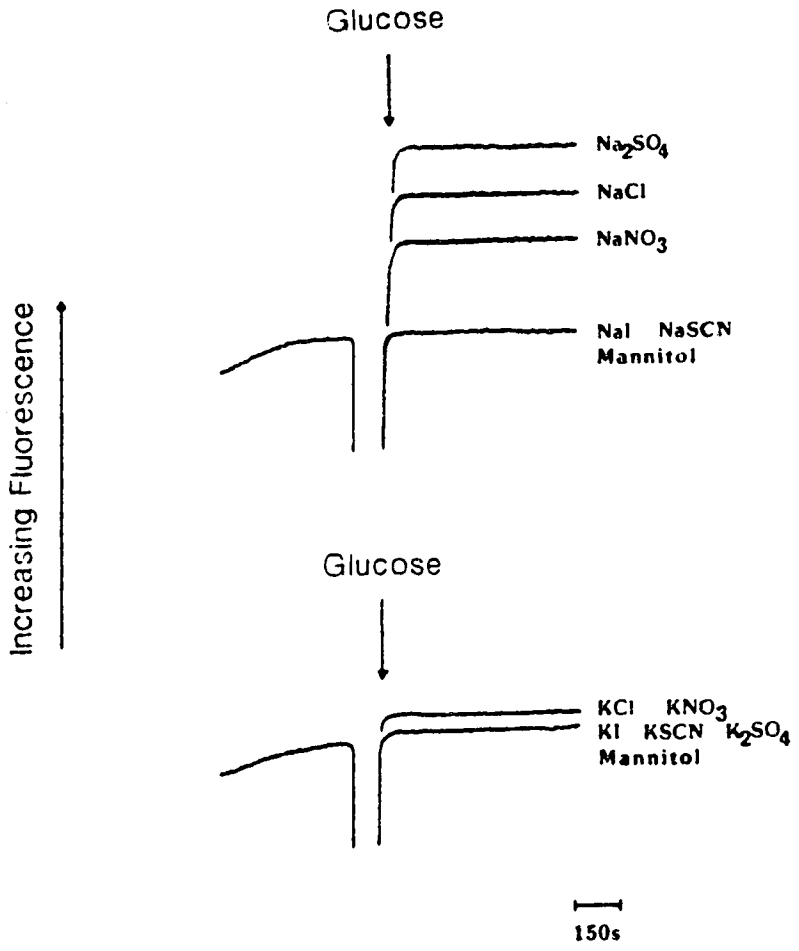

Fig. 6.7. Fluorescence signals for electrogenic D-glucose influx by rat renal brush border membrane vesicles. Vesicles were suspended in 3 ml of medium containing 300 mM mannitol, 5 mM Tris-HCl (pH 7.4) and 3 μM DiS-C3(5) or substituted 200 mM mannitol with 100 mM of either NaI, NaSCN, $NaNO_3$, NaCl, KI, KSCN, KNO_3, KCl or 50 mM of Na_2SO_4 or K_2SO_4. At the time indicated by the arrow, 100 μl of D-glucose solution was added into the cuvette to give a final concentration of 20 mM.

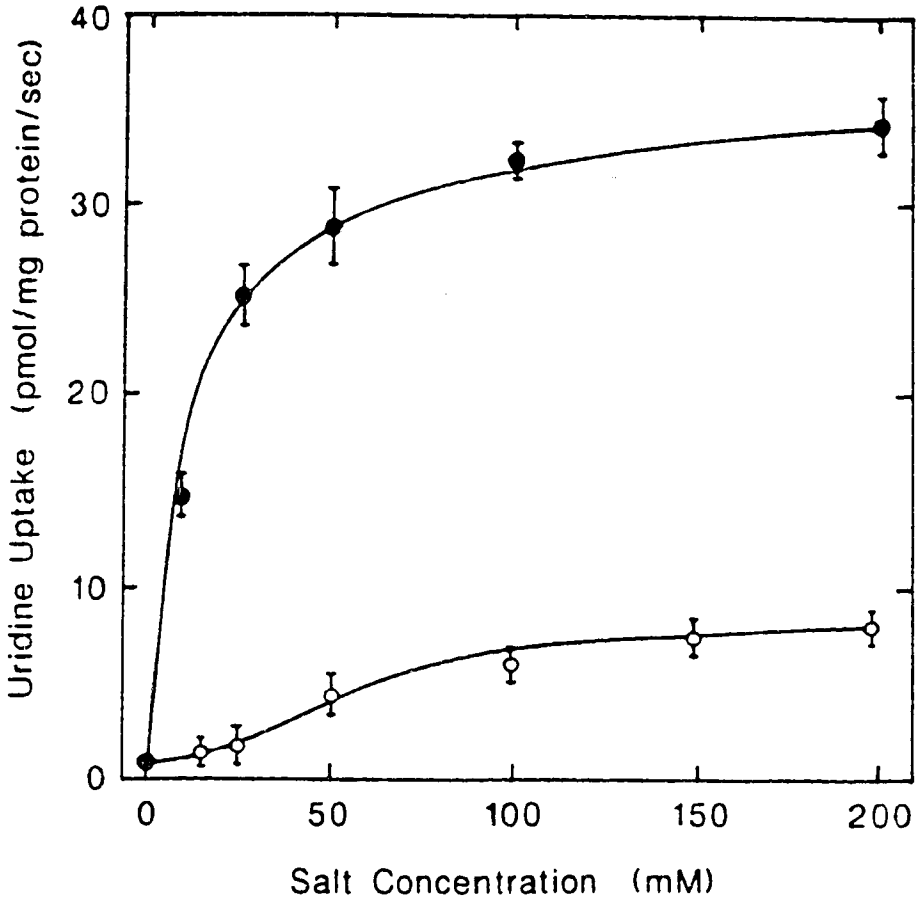

Fig. 6.8. Effect of extravesicular Na^+ and K^+ concentration on uridine uptake by rat renal brush border membrane vesicles (activation method). [^{3}H]Uridine (5 μM) uptake was measured in the presence of variable NaI (●) or KI (○) concentrations from 0 to 200 mM with choline iodide substituted to maintain isoosmolarity. The approximate initial rates of uridine uptake at 22°C were taken at 2 and 5 s for Na^+- and K^+-dependent systems, respectively. Data were corrected for Na^+- and K^+-independent uridine uptake. Values are means ± S.E. of triplicate determinations.

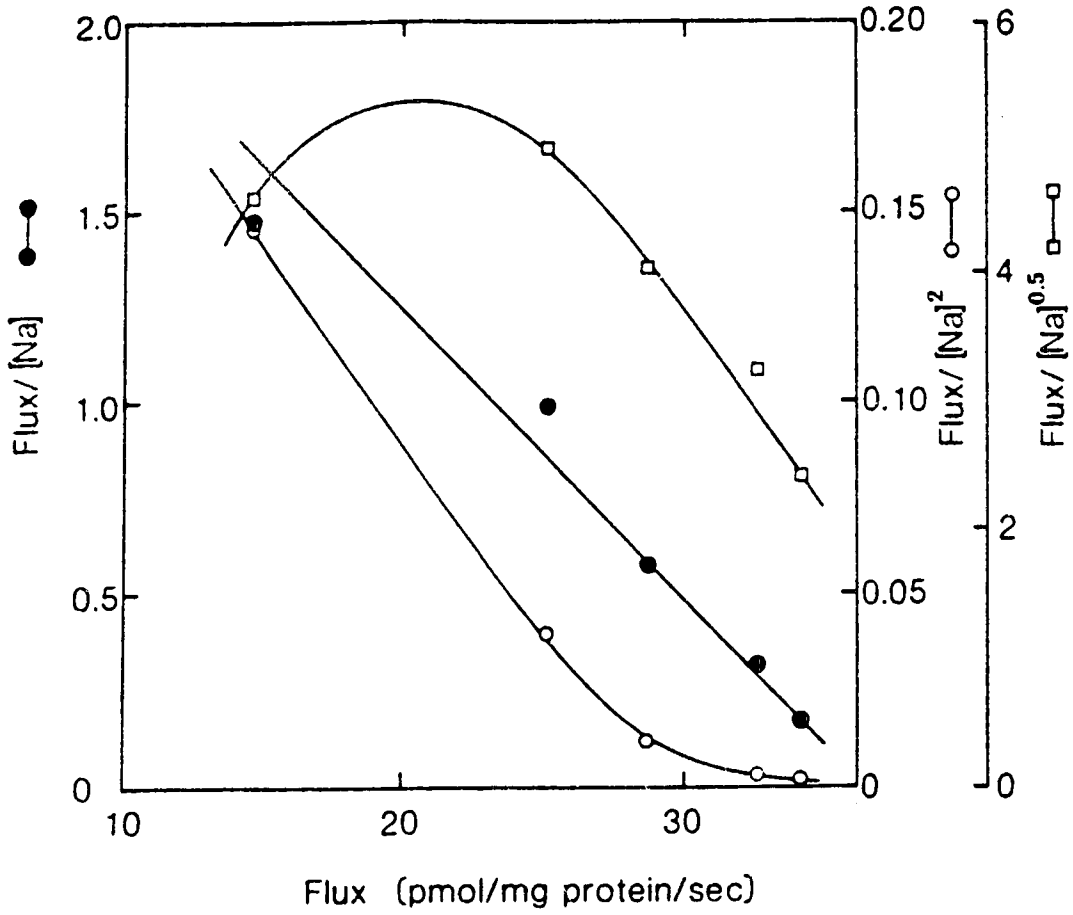

Fig. 6.9. Replot of the data for Na^+-dependent uridine fluxes in Fig. 6.8 according to the Hill equation. Plots of Flux/$[Na]^n$ versus Flux for n = 0.5 (□), 1 (●) and 2 (○). Linearity of the plot for n = 1 is indicative of the involvement of approximately one sodium ion per uridine transport event.

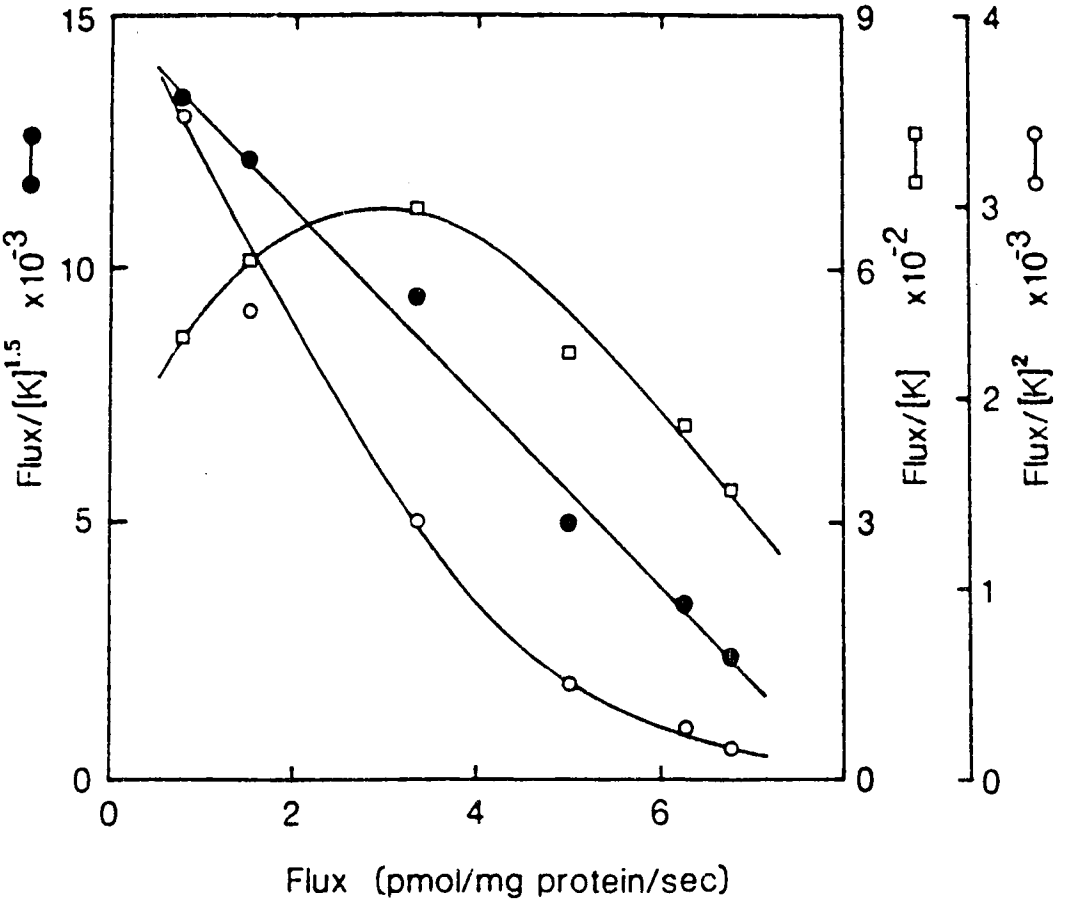

Fig. 6.10. Replot of the data for K^+-dependent uridine fluxes in Fig. 6.8 according to the Hill equation. Plots of the Flux/$[K]^n$ versus Flux for n = 1 (□), 1.5 (●) and 2 (○). Linearity of the plot for n = 1.5 is indicative of the involvement of approximately 1.5 potassium ions per uridine transport event.

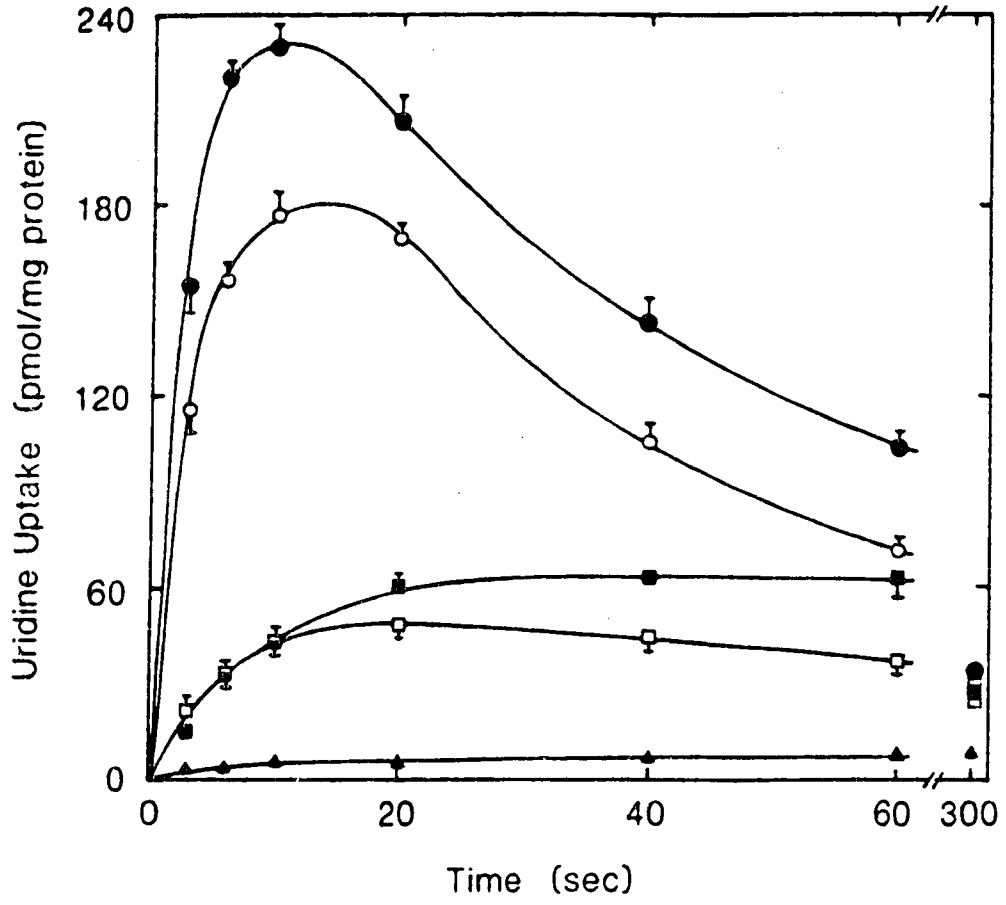

Fig. 6.11. Time course of Na^+- and K^+-dependent uridine uptake by rat renal brush border membrane vesicles. Uridine uptake was initiated by adding vesicles to the media containing (final concentrations) 5 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and 100 mM of either $NaNO_3$ (●), NaI (○), KNO_3 (■), KI (□) or choline chloride (▲). Values are means ± S.E. of triplicate determinations. For clarity, data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

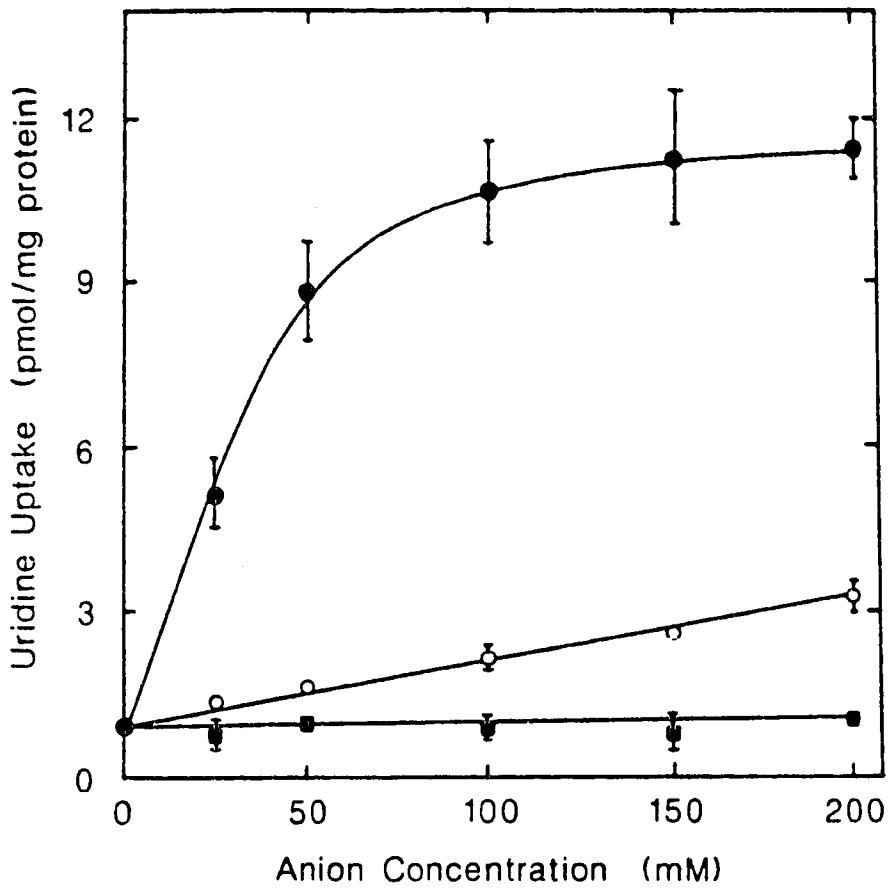

Fig. 6.12. Effect of anion concentration on Na^+-dependent uridine uptake by rat renal brush border membrane vesicles. [^{3}H]Uridine (5 μM) uptake was measured using vesicles treated with gramicidin D (5 μg/mg protein). Variable NO_3^- or Cl^- concentrations from 0 to 200 mM were obtained by substitution with gluconate to maintain isoosmolarity. Na^+ concentration was maintained constant at 200 mM (in=out). The approximate initial rates of uridine uptake were taken at 2 s. The symbols have the following meaning: inward NO_3^--gradient (●), inward Cl^--gradient (o), NO_3^--equilibrated (■). Values are means ± S.E. of triplicate determinations, where absent, S.E. are smaller than symbol.

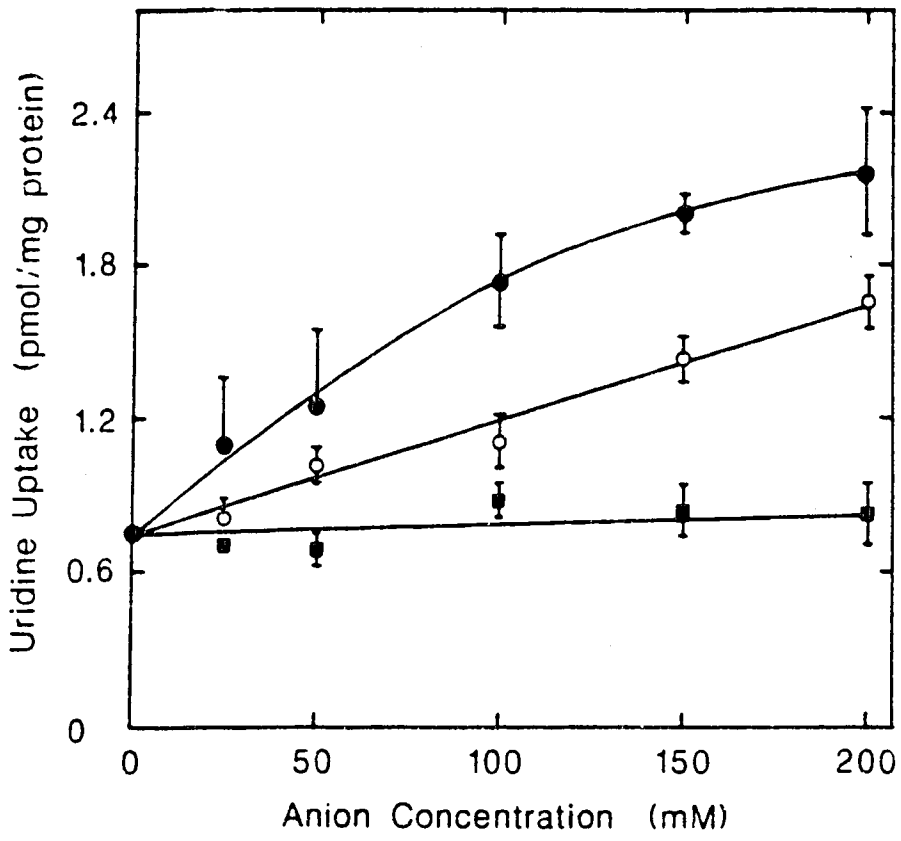

Fig. 6.13. Effect of anion concentration on K^+-dependent uridine uptake by rat renal brush border membrane vesicles. [^{3}H]Uridine (5 μM) uptake was measured using vesicles treated with valinomycin (2 μg/mg protein). Variable NO_3^- or Cl^- concentrations from 0 to 200 mM were obtained by substitution with gluconate to maintain isoosmolarity. K^+ concentration was maintained constant at 200 mM (in=out). The approximate initial rates of uridine uptake were taken at 5 s. The symbols have the following meaning: inward NO_3^--gradient (●), inward Cl^--gradient (o), NO_3^--equilibrated (■). Values are means ± S.E. of triplicate determinations. For clarity, some data are presented with either positive or negative S.E., where absent, S.E. are smaller than symbol.

7. General Discussion

7.1. Nucleoside transport in mammalian cerebral cortical synaptosomes

Much of the literature concerned with the 'transport' of nucleosides in CNS tissues is difficult to interpret. This is primarily due to inadequacies in the methods which have been employed to measure their uptake which led to difficulties in distinguishing between transport and metabolism. Thus, to date, no reliable information about the kinetic properties of nucleoside transport in CNS tissues has been provided.

In this study, the transport of uridine and adenosine was investigated in rat and guinea pig cerebral cortical synaptosomes respectively using a filtration method with dipyridamole in the stop solution. Under these conditions, the rapid efflux of nucleoside from the synaptosomes was prevented and both uridine and adenosine were not significantly metabolized during the incubation period used to determine the initial rates of their transport.

Of the surprisingly large variety of compounds that inhibit nucleoside transport [1, 29], NBMPR has been the most useful probe of the facilitated diffusion nucleoside transport systems [22, 23]. NBMPR binds tightly to specific sites on the plasma membrane. These are

believed to be the facilitated diffusion nucleoside transport elements of the cell [6, 28]. Occupancy of these sites by NBMPR is related to inhibition of nucleoside transport [6, 17]. However, the dose-response curves for the inhibition of nucleoside transport in rat and guinea pig cerebral cortical synaptosomes by NBMPR were biphasic. Approximately half of the transport activity was inhibited by NBMPR with IC_{50} values of 0.5-0.7 nM, and the remaining activity was insensitive to NBMPR concentrations as high as 1 μM. These two components of nucleoside transport have been defined as "NBMPR-sensitive" and "NBMPR-insensitive", respectively. Similar biphasic dose-response curves were also observed in both species for dilazep inhibition. In contrast, dipyridamole only inhibited adenosine transport in guinea pig cerebral cortical synaptosomes in a biphasic manner. Both uridine transport components in rat cerebral cortical synaptosomes were equally sensitive to dipyridamole inhibition with an IC_{50} value of 3 μM. This low sensitivity of rat nucleoside transporters to dipyridamole has been observed for many different tissues and appears to be a unique characteristic of the rat [10, 14, 34, 38, 40].

Nucleoside influx by NBMPR-sensitive and NBMPR-insensitive components were saturable facilitated-diffusion systems of broad specificity and independent of Na^+. Neither system discriminated between purine and

pyrimidine nucleosides, although adenosine and its analogues had the highest affinity of the nucleosides tested. The transport affinities for both components in rat cerebral cortical synaptosomes were not significantly different (apparent K_m's for uridine 300 ± 51 and 214 ± 23 μM with V_{max}'s 12 ± 3 and 16 ± 3 pmol/mg protein/s at 22-24°C for NBMPR-sensitive and -insensitive components, respectively, means ± S.E.) as compared to guinea pig cerebral cortical synaptosomes where the affinity of the NBMPR-sensitive component was 5-fold higher than that of the NBMPR-insensitive component (apparent K_m's for adenosine 17 ± 3 and 68 ± 8 μM with V_{max}'s 2.8 ± 0.3 and 6.1 ± 0.4 pmol/mg protein/s at 22-24°C, respectively, means ± S.E.).

As mentioned earlier, the inhibition of nucleoside transport by NBMPR is associated with high-affinity binding of NBMPR to the functional specific sites on the cell membrane. The dissociation constants of NBMPR bound at these sites in synaptosomes were about 58 ± 15 and 88 ± 6 pM with B_{max} values of 470 ± 26 and 632 ± 12 fmol/mg protein for rat and guinea pig cerebral cortical synaptosomes, respectively (means ± S.E.). Such binding was competitively inhibited by transported nucleosides. The similarity of the K_i values for inhibition of NBMPR-sensitive transport by nucleosides with the K_i values for inhibiting high-affinity [^{3}H]NBMPR binding suggested that NBMPR is competing with nucleosides for the permeation

site of the NBMPR-sensitive transporter. In addition, the K_d values for [^{3}H]NBMPR binding were similar to the K_i values of NBMPR inhibition of NBMPR-sensitive transport, providing further evidence that NBMPR is binding to a region of the transporter that is essential for transport function.

Benzodiazepines have been proposed to produce some of their central effects through the inhibition of cellular accumulation of adenosine [24-27]. However the low potency for benzodiazepines to inhibit adenosine transport by both the NBMPR-sensitive and NBMPR-insensitive routes in guinea pig cerebral cortical synaptosomes (K_i for diazepam of 36-81 μM) as compared with their affinities for the benzodiazepine binding site in central nervous tissues (K_i for diazepam of 7.4 nM) [21] suggested that benzodiazepines do not exert their sedative effect by blocking adenosine transport.

In conclusion, there are two components of facilitated diffusion nucleoside transport present in the central nervous system. These components appear to be present in a wide range of species, including rat, guinea pig and rabbit, and can be distinguished on the basis of their sensitivity to inhibition by the facilitated diffusion nucleoside transport inhibitors such as NBMPR, dilazep and possibly dipyridamole. The relationship between these two types of transporter is unknown but

they may represent either different functional states of the same protein or products of closely related genes. Thus, conclusions regarding nucleoside transport in mammalian brain based only on NBMPR-binding activity must be viewed with caution.

7.2. Uridine transport in rat renal cortex brush border membrane vesicles

Many analogues of physiological nucleosides have been synthesized as potential therapeutic agents. A large number of these nucleosides have impressive biological activity including cytotoxicity. Nephrotoxicity is most prominent. Thus understanding the mechanism(s) by which the kidney handles high concentrations of nucleosides in the glomerular filtrate could possibly help to understand how these compounds exert their affect on the kidney. In addition, this information would be useful in the study of the pharmacokinetics of these drugs.

An inhibitor-stop filtration method was used to study the transport properties of nucleoside transporters in brush border membrane vesicles prepared from rat renal cortex. Control experiments showed that the rapid efflux of uridine from the vesicles can be prevented by the addition of phloridzin, an inhibitor of Na^+-dependent D-glucose transport, to the stop solution. Uridine was

chosen as a model substrate for this study since this nucleoside was shown not to be metabolized by the membrane vesicles.

The data presented in this study showed that the uptake of uridine was accelerated by the presence of either Na^+ or K^+, and that when an inwardly directed Na^+ or K^+ concentration gradient was imposed uridine was transiently accumulated to concentrations in excess of their extravesicular concentration (the so called "overshoot"). The amplitude of the overshoot was increased by raising either the Na^+ or K^+ concentration outside the membrane or by using more lipophilic anions.

Although evidence for Na^+-dependent uptake of nucleosides by renal brush border membrane vesicles [18-20, 36], isolated epithelial cells [33], murine splenocytes [8], choroid plexus [35], and cultured intestinal IEC-6 cells [13] has been demonstrated, this is the first time that evidence for a K^+-dependent uptake of uridine has been found. The K^+-dependent uptake of uridine cannot be explained due to the action of a membrane potential induced by anion movement, as an inwardly directed gradient of choline chloride failed to produce it. This observation thus raised three possibilities for the Na^+/K^+ interaction in uridine transport: 1. The two cations bind (competitively) to the same site. 2. They bind to different sites of the

same transport system, presumably with or without cooperativity. 3. They bind to different transport systems. The author decided that the third model could best fit the evidence obtained from this study, i.e. that there are two different systems for uridine transport in rat renal brush border membrane vesicles. Analysis of the interaction between Na^+ and K^+ showed that uridine uptake was enhanced when both Na^+ and K^+ were present outside the membrane, ruling out the possibility that Na^+ and K^+ were competing for the same transporter site. Also when only one of the cations was present intravesicularly, uridine uptake was reduced. This is due to the rapid removal of intravesicular uridine via that cation-dependent system as the data showed that uridine transport is bidirectional and Na^+ and K^+ are mandatory for the transport process. Both Li^+ and Rb^+ were also able to elicit active transport of uridine apparently by substituting for Na^+ and K^+, respectively.

Further evidence suggesting that these two uridine transport systems are separate entities came from the stoichiometry studies. The cation:uridine coupling ration was found to be 1:1 and 3:2 for Na^+- and K^+-dependent systems, respectively. The cation:uridine stoichiometry is of more than academic interest because it determines the maximal concentration gradient against which net uridine transport across the proximal tubule cell can occur. For example, if one assumes a typical

intracellular Na^+ concentration of 10 mM and a luminal membrane voltage of -70 mV, the maximal uridine concentration ratio (cell-to-lumen) would be approximately 200 with a stoichiometry of 1:1 but about 40000 if the coupling ratio was 2:1 [32, 37]. Thus, the higher coupling ratio for the K^+-dependent system suggested that this system is capable of transporting uridine against a higher concentration gradient than the Na^+-dependent system.

The uridine influx via both systems was saturable and obeyed Michaelis Menten kinetics. The K_m values were 6.7 ± 0.5 and 28 ± 3 μM with V_{max} values of 70 ± 1 and 15 ± 4 pmol/mg protein/s at 22-24°C for the Na^+- and K^+-dependent systems, respectively, in the presence of 100 mM cation (means ± S.E.). These K_m values were approximately one order of magnitude lower than those for the facilitated diffusion mechanisms found in most cell types [41], and appear to a characteristic unique to concentrative nucleoside transport systems [8, 19].

The kinetics of uridine and cation interactions were further analyzed in an attempt to characterize the molecular details of the transport mechanisms. Results showed that increasing the Na^+ concentration caused an increase in affinity but had little effect on the V_{max} for uridine influx by the Na^+-dependent system. This indicated that the Na^+-dependent transporter is an

ordered system where Na^+ binds first to increase the affinity of the transporter for uridine. A similar mechanism has been proposed for Na^+-dependent D-glucose transporter in both intestinal and renal brush border membrane vesicles [11]. In contrast, the V_{max} but not the K_m for uridine influx via the K^+-dependent system was affected by the K^+ concentration. This suggested that the order of binding of the uridine and the cation for K^+-dependent transporter is random.

Anions have been shown to stimulate Na^+-dependent solute uptake in a variety of cell types including pigeon erythrocytes [12, 39], blood platelets [30, 31], nerve cells [15, 16], fish intestine [3-5], and mammalian kidney and intestine [2, 7, 9]. The stimulatory effect of anions for the above systems is apparently due to the ability of the anion to generate a negative-inside electrical potential and/or take part in the formation of the transport complex. There is no evidence to show that the anion is cotransported with substrate and Na^+. In this study, an unexpected phenomenon was found when the membrane potential was varied using different anions. Nitrate, which is not the most permeable anion tested (permeability $I^->SCN^->NO_3^->Cl^->SO_4^{2-}$) had the highest affect on uridine transport (reactive order $NO_3^->I^->SCN^->Cl^->SO_4^{2-}$). This suggested that there may be a more direct effect of NO_3^- on the transport systems. Indeed, anion-gradient activated uridine transport was shown to

exhibit a concentration dependence and conformed to simple Michaelis Menten kinetics with apparent $K_{nitrate}$ values of 42 ± 13 and 163 ± 54 mM for Na^+- and K^+-dependent systems, respectively (means ± S.E.). A chloride-gradient also stimulated uridine uptake but with a lower affinity ($K_{chloride}$>200 mM). The physiological significance of anion-dependent uridine transport in renal brush border membranes has yet to be determined.

Although the K^+-dependent system is suggested to be responsible for the secretion of nucleosides, no difference in substrate specificity between the Na^+- and K^+-dependent systems was observed. Both systems seem to favour pyrimidine nucleosides and adenosine as substrates and suggested the existence of a pyrimidine-adenosine specific transport system in rat renal brush border membrane. In addition, the K_i values for inosine and guanosine as inhibitors of Na^+-dependent uridine transport (300 and 92 μM) did not correspond with their K_m values for uptake (2 and 3.5 μM) [19] and their K_i values as inhibitors of Na^+-dependent adenosine transport (2.2 and 3.9 μM, respectively) [19], suggesting the existence of a second different active nucleoside transport system with a high affinity for purines.

The K^+-dependent system is not a contaminant from the basolateral membrane that functions as part of the reabsorption process by moving nucleosides across the

basolateral membrane into the blood, as preliminary studies using basolateral membrane vesicles (Appendixes 7.1 an 7.2) showed that neither Na^+ nor K^+ is required for uridine transport across the rat renal basolateral membrane (Appendix 7.3). The high-affinity and "non-selective" transport process across the brush border membrane raised the possibility that, the translocation across the basolateral membrane may be the rate limiting step for reabsorption of nucleosides by the kidney. Substrates which are not readily transported across the basolateral membrane could therefore be subjected to intracellular metabolism and/or secretion via the K^+-dependent system. Thus, further investigation of nucleoside transport across renal basolateral membranes could greatly add to the understanding of the mechanisms by which the kidney handles nucleosides in proximal tubule.

7.3. References

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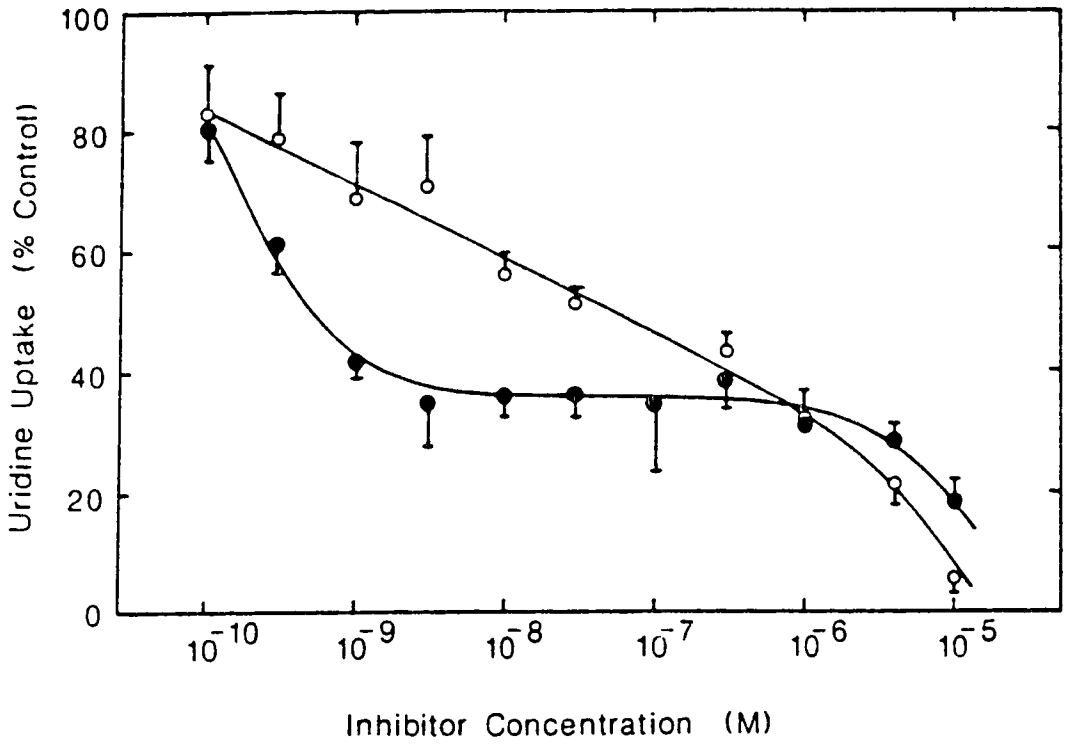
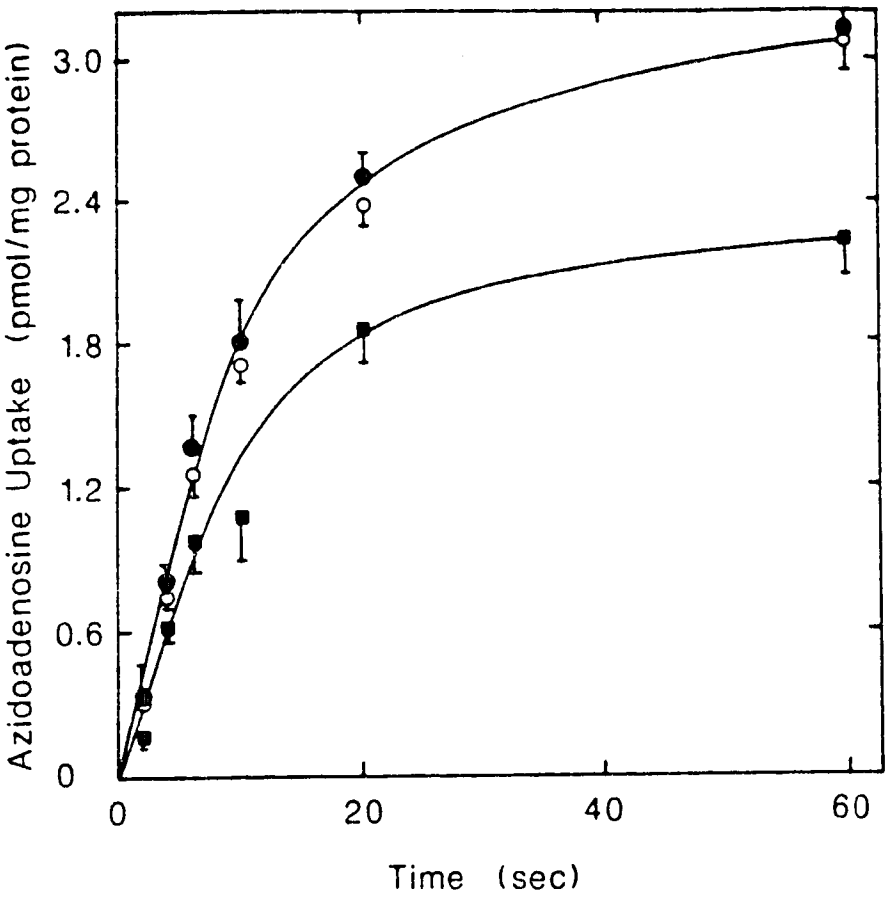
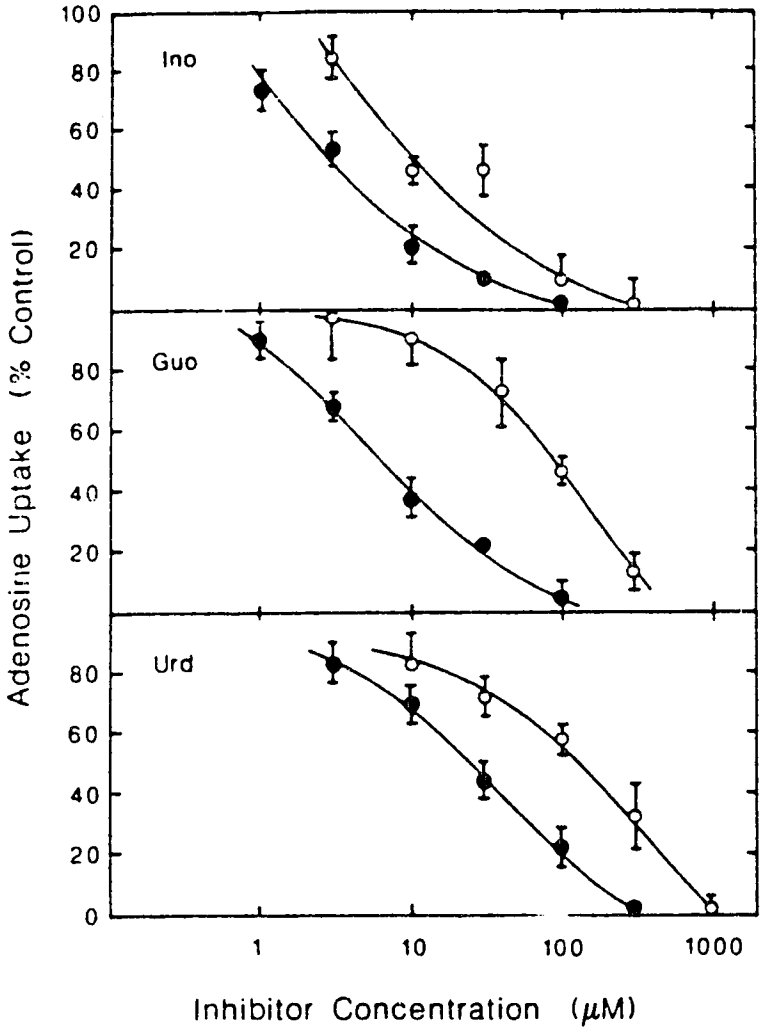


Appendix 4.1. Effect of NBMPR and dipyridamole on uridine transport by rabbit cerebral cortical synaptosomes. Synaptosomes were preincubated with various concentrations of either NBMPR (●) or dipyridamole (o) for 20 min before the addition of 100 μM [^{3}H]uridine. The results are expressed as a percentage of the control transport rate versus the inhibitor concentration. In the case of NBMPR, [^{3}H]NBMPR in the concentration range of 0 to 30 nM was used in parallel experiments to determine the final free concentration of the inhibitor. Values are means ± S.E. of three separate experiments conducted in triplicate. For clarity, data are presented with either positive or negative S.E..



Appendix 6.1. Time course of 8-azidoadenosine uptake by rat renal brush border membrane vesicles. 8-Azidoadenosine uptake was initiated by adding vesicles to the media containing (final concentration) 5 μM [^{3}H]8-azidoadenosine, 5 mM Tris-HCl (pH 7.4) and 100 mM of either $NaNO_3$ (●), KNO_3 (○) or choline chloride (■). Values are means ± S.E. of triplicate determinations. For clarity reason, data are presented with either positive or negative S.E..

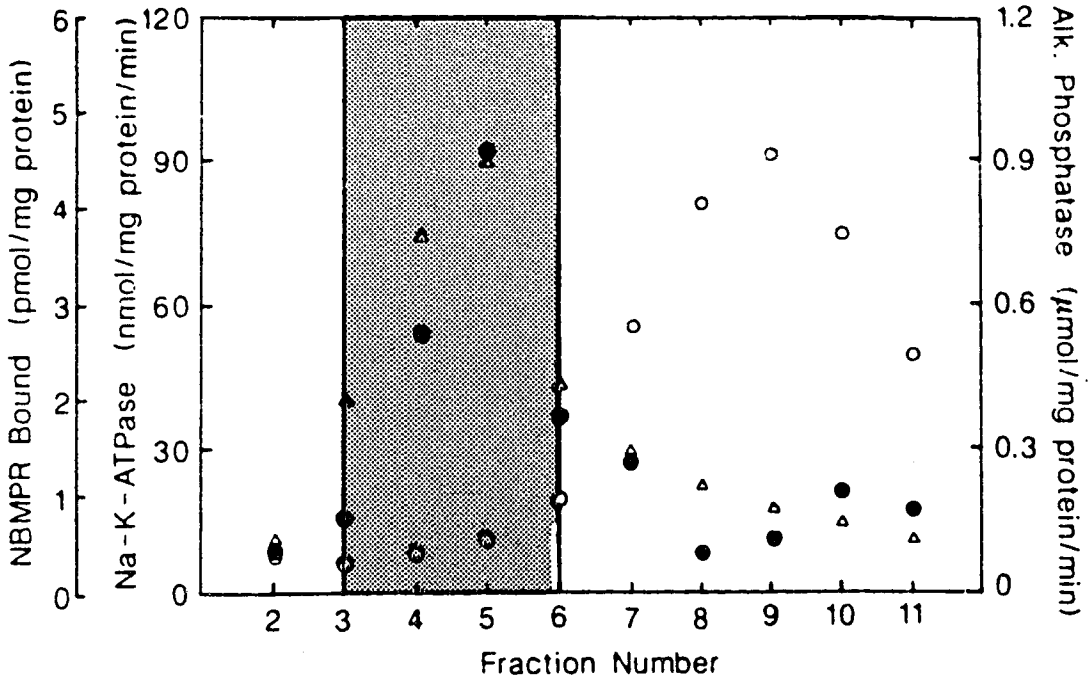


Appendix 6.2. Effect of nucleosides on Na^+- and K^+-dependent adenosine uptake by rat renal brush border membrane vesicles. Adenosine uptake was initiated by adding vesicles to the media containing (final concentration) 5 μM [^{3}H]adenosine, 5 mM Tris-HCl (pH 7.4), 100 mM of either $NaNO_3$ (●) or KNO_3 (○) and graded concentrations of unlabelled nucleoside (inosine, guanosine and uridine). The approximate initial rates of adenosine uptake were taken at 2 and 5 s for Na^+- and K^+-dependent systems, respectively. The data (means ± S.E. of triplicate determinations) shown are corrected for the Na^+- and K^+-independent adenosine uptake. The IC_{50} values for inosine, guanosine and uridine were 3, 6 and 25 μM for Na^+-dependent system, and 13, 75 and 140 μM for K^+-dependent system, respectively.

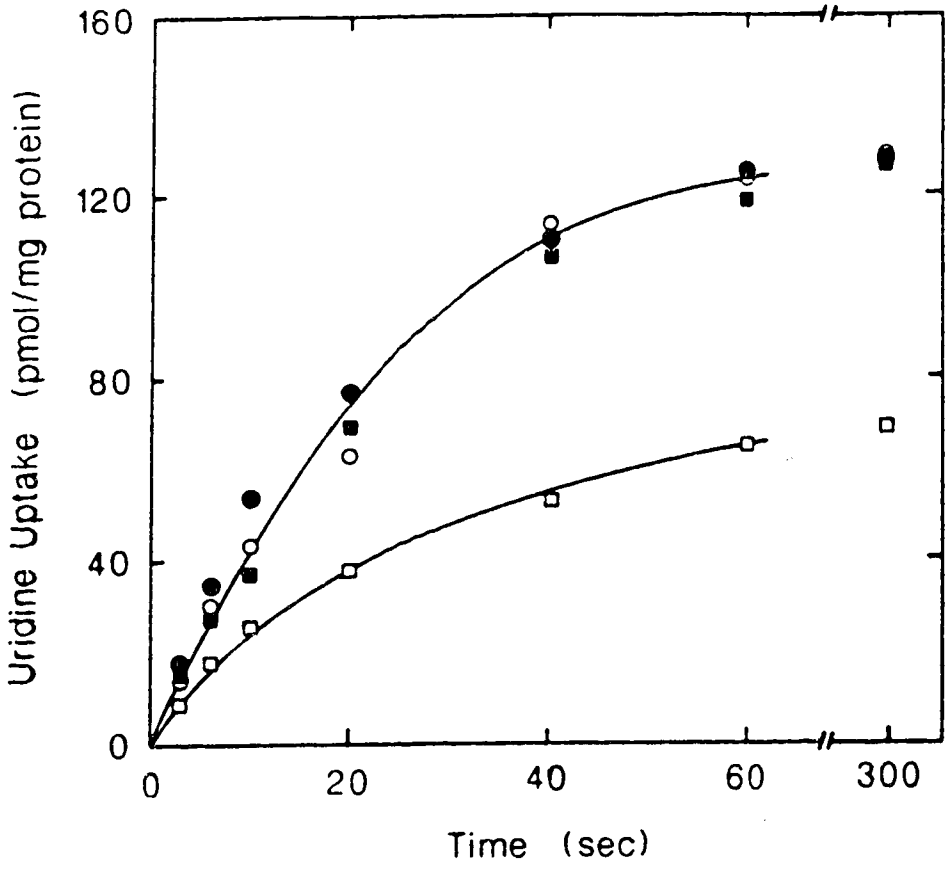
Appendix 7.1. Preparation of rat renal basolateral membrane vesicles.

Two male Sprague-Dawley rats (250-300 g) were anesthetized with sodium pentobarbital intraperitoneal injection. The kidneys were removed, decapsulated and placed into an ice-cold sucrose buffer (250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mM Tris-HCl, pH 7.4). Kidney cortex tissues, after being dissected from the kidney slics (approximately 1 mm thick), were homogenized in 65 ml of sucrose buffer with a Polytron on setting '8' for 3 x 30 second pulses. The homogenates were then centrifuged at 2500 x g for 15 min. The resulting supernatant was collected and recentrifuged at 20500 x g for 20 min. The centrifugation produced a double pellet consisting of a hard brown lower pellet and a fluffy white upper pellet. The fluffy white upper pellet was dislodged from the lower brown pellet by gently swirling the centrifuge tube after adding 5 ml of sucrose buffer. The fluffy white pellet suspension was subjected to glass-Teflon homogenization (5 strokes, 1500 rpm) and mixed with a solution of Percoll/sucrose buffer to a final volume of 35 ml (final concentration of Percoll 12% v/v). The mixture was centrifuged at 48000 x g for one hour to obtain a density gradient. The gradient was separated from top to bottom into 25 fractions of 1.4 ml and fractions 4, 5 and 6 were pooled as basolateral membrane fraction (see Appendix 7.2). The

membrane pool was diluted with vesicle suspension medium (300 mM mannitol and 5 mM Tris-HCl, pH 7.4) to a volume of 35 ml and centrifuged at 48000 x g for 30 min to obtain the basolateral membrane pellet. The purified membrane vesicles were suspended in an ice-cold vesicle suspension medium and used on the same day of preparation.



Appendix 7.2. Distribution of marker enzymes for basolateral and brush border membranes and NBMPR binding proteins on Percoll gradient. The gradient (final volume 35 ml; 12 % v/v percoll) was separated from the top into 25 fractions of 1.4 ml. Only the first 11 fractions of the gradient were tested for the ouabain-sensitive Na^+-K^+-ATPase (●) (basolateral membranes), alkaline phosphatase (o) (brush border membranes), and NBMPR binding (△) activities. Fractions 4, 5 and 6 (shaded area) were collected as basolateral membrane fraction.



Appendix 7.3. Time course of uridine uptake by rat renal basolateral membrane vesicles. Ten μl of the vesicle suspension treated with (□), or without (●,○,■) 1 mM $HgCl_2$ were incubated with 20 μl of media containing (final concentrations) 100 μM [^{3}H]uridine, 5 mM Tris-HCl (pH 7.4) and either 100 mM NaCl (●), 100 mM KCl (○), or 100 mM choline chloride (■,□). Values are means ± S.E. of triplicate determinations.