1995

Examination Of Nucleoside Transport In The Mammalian Nervous System

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EXAMINATION OF NUCLEOSIDE TRANSPORT IN THE MAMMALIAN NERVOUS SYSTEM

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Submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
January, 1995

ISBN 0-315-99261-1

ABSTRACT

In rabbit and guinea pig cortical synaptosomes, the nucleoside transport inhibitors nitrobenzylthioinosine and dipyridamole were used to test the hypotheses that the nitrobenzylthioinosine-sensitive and -resistant [^{3}H]dipyridamole binding sites are associated with the *es* and *ei* nucleoside transporters, respectively. In addition, the hypothesis that the *es* and *ei* nucleoside transporters in rabbit cortical synaptosomes differ in their selectivity for compounds besides nitrobenzylthioinosine was tested. Nitrobenzylthioinosine-sensitive [^{3}H]dipyridamole binding and [^{3}H]nitrobenzylthioinosine binding involved the same site on the *es* transporter. The relative proportions and inhibitor sensitivities of nitrobenzylthioinosine-resistant [^{3}H]dipyridamole binding could not be correlated with nitrobenzylthioinosine-resistant nucleoside transport. This, and other data, suggested that the nitrobenzylthioinosine-resistant [^{3}H]dipyridamole binding site(s) involved membrane components distinct from those associated with functional, *ei* nucleoside transporters. In addition, none of the substrates examined in the rabbit were selective for one transporter subtype over the other.

R75231 is a newly developed mioflazine derivative which has extremely tight binding characteristics *in vitro* and *in vivo*. The hypothesis that R75231 bound to the *es* nucleoside transporter in an irreversible manner was tested. In rabbit synaptosomes, R75231 was shown to bind extremely tightly and to be a "mixed" inhibitor of [^{3}H]nitrobenzylthioinosine binding. Binding of [^{3}H]R75231 to human erythrocyte ghost membranes was reversible, but the rate of dissociation depended upon the displacer used. R75231 and mioflazine slowed the rate of dissociation of [^{3}H]R75231, and caused an initial increase of site-bound [^{3}H]R75231. These and other

results, indicate that R75231 binding to the nucleoside transporter is a reversible, complex reaction involving multiple interacting sites exhibiting positive cooperativity.

The hypothesis that the nucleoside transporter characteristics changed upon the differentiation of LA-N-2 cells was tested. Undifferentiated cells accumulated [^{3}H]formycin B by the *es* nucleoside transport system exclusively. Cell differentiation, induced by growth in serum-free medium, increased the initial rate of [^{3}H]formycin B transport 25%. However, there was no concomitant change in the number of [^{3}H]NBMPR binding sites. The enhanced uptake in the differentiated cells appeared to be due to an increased expression of *ei* nucleoside transporters.

ACKNOWLEDGEMENTS

I would like to thank Dr. J.R. Hammond for his guidance and support, both personal and financial, during the course of my graduate work. I would also like to thank him for his encouragement and patience when I decided to pursue another degree before I finished this one.

I would also like to thank my advisory committee, Dr. Cook, Dr. Lo and especially Dr. Rylett for their input, advice, and support. Chapter 4 would not have been possible without Dr. Rylett's help with the LA-N-2 cell line. Thanks also to the many professors in the department who made this work a little easier whether it be through their practical assistance or social conversations. Specifically, I would liked to have thanked Dr. Marnie Moffat for her help with my comprehensive exams and for her friendly conversation in the coffee room. She is missed.

Thanks to Cathy Cassidy, partly for her technical assistance, but mostly for keeping me company in the lab and listening to me babble on. Thanks also to Colin and Brian for finding, making, or "borrowing" various items that I needed for my research.

I would like to thank friends and fellow students who provided distractions and advise: Sharon, Charles, Mike, Chris, Rob, Kim, Leah, Henry, Gord, Gus, Rob, Nancy, Jacqui, Joanne, Julie. Thanks also to fellow graduate students at Lakehead who allowed me to co-misscrate with them.

To my family, I would like to thank them for their support throughout this degree. I was always told to make sure that I got a good education. I hope this qualifies.

For personal funding, I would like to acknowledge the Ontario Graduate Scholarships.

Finally, I wish to thank Caroline. Without her love, advice, listening abilities, completion complex, and ability to keep things in perspective, I don't think I would have

finished this degree. At least half this Ph.D. belongs to her. Hakuna Matata.

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ABBREVIATIONS:

A_{1-3}: adenosine receptor subtypes

ADA: adenosine deaminase

ADO: adenosine

AMP: adenosine monophosphate

ANOVA: analysis of variance

ATP: adenosine triphosphate

AZT: 3'-azido-3'-deoxythymidine

B_e: bound radioligand at equilibrium

B: bound radioligand at time t

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CGS 21680: 2-[*p*-(2-carboxyethyl)-phenethylamino]-5'N-ethylcarboxamido adenosine

CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

cif, cit, cib: sodium-dependent nucleoside transporter subtypes

CNS: central nervous system

CPA: N^6-cyclopentyladenosine

ddC: 2',3'-dideoxycytidine

dilazep: N,N'-bis[3-(3,4,5-trimethoxybenzoyloxy)propyl]-homopiperazine

DMSO: dimethylsulfoxide

dpm: decays per minute

DPR: 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine

DPR: dipyridamole

ei:	NBMPR-resistant
es:	NBMPR-sensitive
INO:	inosine
k_{ob}:	observed rate constant
k_{+1}:	association rate constant
k_{-1}:	dissociation rate constant
[L]:	concentration of radioligand
LDH:	lactate dehydrogenase
min:	minute
mioflazine:	3-(aminocarbonyl)-4-[4,4-bis(4-fluorophenyl)butyl]-N-(2,6-dichlorophenyl)-1-piperazineacetamide 2HCl
N1-5:	sodium-dependent nucleoside transporter subtypes
NBMPR:	nitrobenzylthioinosine; 6-(4-nitrobenzylmercapto) purine
NBTGR:	2-Amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-ß-D-ribofuranosylpurine
NECA:	5'N-ethylcarboxamido adenosine
P_2:	crude synaptosomal fraction
P_v:	synaptosomal fraction containing a larger percentage of synaptosomes than the P_v fraction
PMA:	phorbol 12-myristate 13-acetate
R75231:	2-(aminocarbonyl)-4-amino-2,6-dichlorophenyl)-4-[5,5-bis(4-fluorophenyl)-pentyl]-1-piperazineacetamide·2HCl ribonucleoside)
SAH:	S-adenosylhomocysteine
SF-N2:	serum-free defined medium
t:	time
w/v:	weight/volume

CHAPTER ONE: REVIEW

The nucleoside adenosine has been shown to produce a wide range of physiological activities. As well as being a reactant/product in the metabolism of adenosine triphosphate (ATP) and nucleic acids (Arch and Newsholme, 1978), adenosine is important in the regulation of blood flow, bronchial airway tone, and central nervous system (CNS) activity (Williams, 1987; Phillis, 1991). Adenosine produces these effects via interactions with cell surface receptors (Londos and Wolff, 1977; Van Calker et al., 1979). Transport proteins, located in the plasma membrane, play an important role in the control of extracellular adenosine concentration. These nucleoside transporters can release adenosine into the extracellular milieu (Fredholm et al., 1980, 1983; Jonzon and Fredholm, 1985) or transport adenosine out of the extracellular space and into the cell, where it is subsequently metabolized (Arch and Newsholme, 1978; Wu and Phillis, 1984). In addition, some animal cells that are deficient in *de novo* purine synthesis use nucleoside transporters to acquire purines which have been formed in other tissues (Plagemann et al., 1988). Factors which affect the nucleoside transporter will modulate both extracellular and intracellular nucleoside concentrations, and therefore, could be expected to have a significant physiological impact (Wu and Phillis, 1984; Decker et al., 1988; Plagemann et al., 1988).

The overall objective of this thesis is the examination of nucleoside transporters in the mammalian nervous system. A review of nucleoside transport follows. It includes descriptions of the transporter subtypes, the probes for the transporter, the regulation of transporters, and the therapeutic potential of transport substrates and inhibitors. Because adenosine is the most important physiological substrate of the nucleoside transporter, a description of adenosine, including its receptor subtypes and physiological

effects, is also included.

1.1 Adenosine

1.1.1 Biosynthesis and Degradation

There are two primary mechanisms responsible for the formation of adenosine: dephosphorylation of adenosine monophosphate (AMP) and hydrolysis of S-adenosylhomocysteine (Geiger and Nagy, 1990, Meghji, 1991). The relative importance of each mechanism appears to depend on the circumstances, for example, tissues undergoing hypoxia, and the type of tissue (Ohisalo, 1987). See Fig 1.1 for adenosine formation and metabolic pathways.

AMP is dephosphorylated by the enzyme 5′-nucleotidase (EC 3.1.3.5) which exists as both an ecto-5′-nucleotidase, linked to the plasma membrane with its catalytic site located outside the cell, and a cytosolic enzyme (Stanley et al., 1980; Misumi et al., 1990). Under hypoxic conditions, ATP is degraded and adenosine levels increase. It appears that in many cases the ecto-5′-nucleotidase is not responsible for this increase (Pons et al., 1980; Daval and Barberis, 1981; Schutz et al., 1981; Maire et al., 1984; Meghji et al., 1985; Rubio et al., 1988; Meghji et al., 1989). Since the active site of this enzyme is extracellular, these studies provide evidence for the importance of intracellular adenosine production by cytosolic 5′-nucleotidase and/or S-adenosylhomocysteine hydrolase (EC 3.3.1.1) (Meghji, 1991). The ecto-5′-nucleotidase appears to produce adenosine primarily as part of an ecto-enzyme cascade which rapidly hydrolyses ATP to adenosine (Stefanovic et al., 1976; Pearson et al., 1980; Maire et al., 1984; Meghji, 1991). The source of extracellular ATP for this cascade includes release from nerve terminals (Silinksky, 1975; Potter and White, 1980; Lew and White, 1987) and damaged cells (Meghji, 1991).

The first step in the inactivation of adenosine's extracellular effects is the transport of adenosine from the extracellular fluid into the cell (Arch and Newsholme, 1978;

Wu and Phillis, 1984;). This transport is primarily via a nucleoside transporter and will be discussed in more detail later. Once inside the cell, adenosine may be phosphorylated by adenosine kinase (EC 2.7.1.20) or deaminated by adenosine deaminase (ADA) (EC 3.5.4.4). The K_m values for adenosine kinase range from <0.4 to 10 μM (Arch and Newsholme, 1978; Phillips and Newsholme, 1979; Brosh et al., 1990) and in most preparations the K_m values of ADA are 10-100 fold higher (Arch and Newsholme, 1978). Metabolism of adenosine to S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase is another possible, although minor, inactivation pathway (Reddington and Pusch, 1983).

In brain tissue, adenosine (0.3-16 μM) appears to metabolized primarily via phosporylation rather than deamination (Santos et al., 1968; Shimizu et al., 1972; Bender et al., 1981; Reddington and Pusch, 1983; Lee and Jarvis, 1988*b*). However, the dominant pathway in the brain may vary according to brain tissue cell type. For example, the most prominent pathway of adenosine metabolism (10 μM) in mouse astrocyte primary cultures was the synthesis of nucleotides, whereas the production of deaminated metabolites dominated in cultured neurons (Matz and Hertz, 1989).

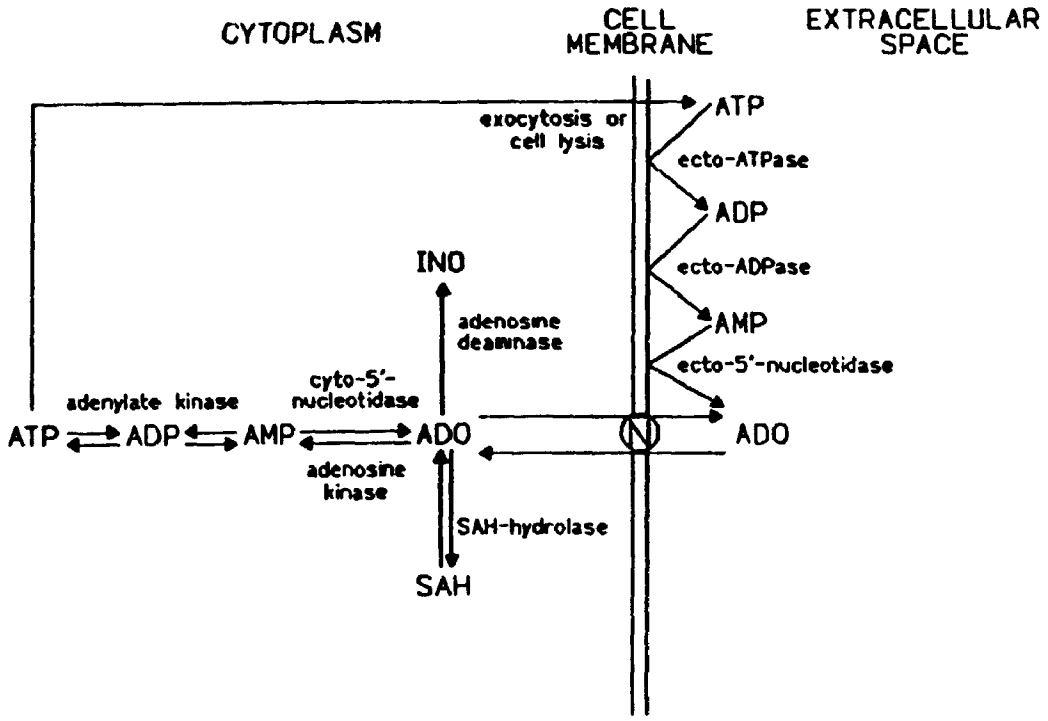


Figure 1.1 Pathways of adenosine formation and metabolism. ADO, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate, ATP, adenosine triphosphate; INO, inosine; N, nucleoside transporter, SAH, S-adenosylhomocysteine (adapted from Meghji, 1991).

1.1.2 Adenosine Receptor Subtypes

Purinoceptors were initially classified into receptors sensitive to adenosine (P_1) and those sensitive to ATP (P_2) (Burnstock, 1983). The P_1 receptors were further subdivided into A_1 (or R_i) and A_2 (or R_a) receptors, which were associated with the inhibition and activation of adenylate cyclase activity, respectively (Londos and Wolff, 1977; Van Calker et al., 1979). Because effector systems other than adenylate cyclase, such as potassium channels, calcium channels, and phosphatidyl inositol phosphate turnover, can be affected by adenosine (Riberio et al., 1986; Cooper and Caldwell, 1990; Olsson and Pearson, 1990; Olah and Stiles, 1992), A_1 and A_2 receptors are currently classified based on their structural and pharmacological profiles (Hamprecht and Van Calker, 1985; Kenakin et al., 1992; Fredholm et al., 1994). Receptors of the A_1 subtype are preferentially activated by adenosine analogues with N^6-substitutions such as N^6-cyclopentyladenosine (CPA) (Bruns et al., 1986, Williams et al., 1986). A_2 receptors are activated by analogues with 5′-substitutions such as 5′N-ethylcarboxamido adenosine (NECA) which is not A_2 selective (Bruns et al., 1986), and 2-[*p*-(2-carboxyethyl)-phenethylamino]-5′N-ethylcarboxamido adenosine (CGS 21680) which is highly A_2 selective (Jarvis et al., 1989). In general, the pharmacological profile of the A_1 and A_2 receptor can be defined by the agonist potency order CPA > NECA > CGS 21680 and CGS 21680 > NECA > CPA, respectively (adapted from Schwabe, 1991). These profiles are only a generalization, which varies depending on species and the subtypes of A_1 and A_2 receptors that are involved. For a more detailed summary of adenosine receptor pharmacology, including subtypes of A_1 and A_2 receptors, see Fredholm et al., 1994.

An A_3 receptor, which affects calcium channels in cardiac and nervous tissues, has also been proposed (Ribeiro and Sebastiao, 1986). However, the existence of the A_3 receptor, as designated by Ribeiro and Sebastiao, has not been accepted.

It is further complicated by the proposal of a different A_3 receptor which has been cloned recently (Meyerhof et al., 1991; Zhou et al., 1992; Linden et al., 1993; Salvatore et al., 1993). This later A_3 receptor, which has a widespread distribution is humans, is the official A_3 receptor as sanctioned by the IUPHAR Committee on Receptor Nomenclature and Drug Classification (Fredholm et al., 1994). Finally a non cell-surface, adenosine binding site (P-site), which is activated by high adenosine concentrations, is located on the catalytic subunit of adenylate cyclase (Daly, 1982). The physiological importance of the P-site is uncertain (Johnson et al., 1990).

As has been the case with other receptor families, the adenosine receptors are being subcatagorized. Proposed subtypes of the A_1 receptor (A_{1a} and A_{1b}) (Gustafs .n et al., 1990; Linden, 1991) and A_2 receptor (A_{2a} and A_{2b}) (Bruns et al., 1986; Lupica et al., 1990) have been described. Molecular cloning of adenosine receptors has begun (Maenhaut et al., 1990; Libert et al., 1991, Reppert et al., 1991; Mahan et al., 1991; Meyerhof et al., 1991; Stehle et al., 1992; Zhou et al., 1992; Linden et al., 1993; Salvatore et al., 1993) and may resolve some of the confusion over adenosine receptor subtype designations, as well as add a few (several?) more subtypes to the list.

1.1.3 Physiological Effects

The study of adenosine as an endogenous regulator of physiological processes has its origin in 1929, when Drury and Szent-Gyorgyi demonstrated that adenosine injected into mammals could produce a variety of effects. These effects included bradycardia, impaired conduction from auricle to ventricle, inhibited auricular fibrillation, shortened absolute refractory period in the atrium, dilation of coronary vessels, inhibition of intestinal movement, and sedation (Drury and Szent-Gyorgyi, 1929). Since this first work, which focused on the cardiovascular system, the investigation of

adenosine's physiological effects has expanded to include virtually every major system in the body including respiratory, renal, gastrointestinal, immunological, and CNS (Ohisalo, 1987; Williams, 1987; Belardinelli et al., 1989; Jarvis and Williams, 1990; Cook, 1991; Spielman, 1991). Some of the effects of adenosine on the systems of the body will be described briefly, with the effects on the CNS being emphasized.

In the heart, as in all tissues except kidney and liver (Osswald, 1983; Buxton, 1988), adenosine is a vasodilator (Berne, 1963; Williams, 1987; Collis, 1989; Collis, 1991a). It also has negative chronotropic, dromotropic, and ionotropic effects on the heart (Belardinelli et al., 1983; Fredholm and Sollevi, 1986; Belardinelli et al., 1989; Collis, 1991*b*). The vasodilation effects of adenosine appear to be due primarily to interaction with A_2 receptors on the vascular smooth muscle (Ramagopal et al., 1988; King et al., 1990; Collis, 1991a). However, there is some suggestion that part of the vasodilation is due to adenosine mediated release of endothelium derived relaxing factor (Nees et al., 1985), possibly via A_1 receptors (Kurtz, 1987).

Adenosine, in rat, causes respiratory depression when given centrally or peripherally (Wessberg, 1985). Adenosine has also been shown to cause bronchoconstriction when administered to asthmatics, but not in non-asthmatics (Church and Holgate, 1986). This induced bronchoconstriction may be due to release of histamine from mast cells via an adenosine receptor which does not appear to be A_1 or A_2 (Church and Hughes, 1985).

Adenosine has many effects in the kidney including regulation of blood flow, hormone release, and tubular reabsorption (Spielman, 1991). Infusion of adenosine into the renal artery of dogs causes a decrease in glomerular filtration rate via afferent arteriolar vasocontriction and efferent arteriolar vasodilation (Osswald et al., 1978) apparently mediated by A_1 and A_2 receptors, respectively

(Murray and Churchill, 1985; Rossi et al., 1988). Adenosine has also been shown to inhibit and stimulate renin release via A_1 and A_2 receptors, respectively (Williams, 1987).

1.1.4 CNS Effects of Adenosine

The mammalian CNS has the highest concentration of adenosine receptors of any tissue studied (Jarvis and Williams, 1990). Using radiolabelled adenosine analogues, a heterogeneous localization of adenosine receptors in the CNS has been found (Bruns et al., 1986; Fastbom et al., 1986; Lee and Reddington, 1986; Reddington et al, 1986; Tetzlaff et al., 1987; Weber et al., 1988; Jarvis and Williams, 1989, Fastborn and Fredholm, 1990). The highest density of A_1 receptors is in the molecular layer of the cerebellum, the hippocampus, medial geniculate, and superior colliculus; whereas, the A_2 receptors are localized to the striatum, nucleus accumbens, and olfactory tubercle.

It is widely accepted that adenosine acts as an inhibitory neuromodulator in the CNS (Phillis and Wu, 1981; Stone, 1981; Dunwiddie, 1985; Williams, 1989). This action of adenosine is a result of its ability to inhibit evoked electrical potentials and spontaneous firing in neurons (Phillis et al., 1974; Kostopoulos and Phillis, 1977; Siggins and Schubert, 1981; Segal, 1982; Stone, 1982; DiCori and Henry, 1984; Dunwiddie et al., 1984), and inhibit the release of various neurotransmitters, including acetylcholine (Harms et al., 1979; Jackisch et al., 1984; Richardson et al., 1987), noradrenaline (Jonzon and Fredholm, 1984; Jackisch et al., 1985), dopamine (Harms et al., 1979; Michaelis et al., 1979), serotonin (Harms et al., 1979; Feuerstein, et al., 1985), glutamate (Corradetti et al., 1984), and aspartate (Corradetti et al., 1984; Bowker and Chapman, 1986). The depression of synaptic transmission by adenosine appears to be mediated primarily by A_1 receptors at both pre- and post-synaptic sites (Reddington et al, 1982; Fredholm et al., 1983; Dunwiddie and Fredholm, 1984; Snyder, 1985; Fredholm and Dunwiddie, 1988;

Ribeiro, 1991; Stone and Bartrup, 1991). Proposed mechanisms by which adenosine acts as an inhibitory neuromodulator include decreasing calcium flux into the cell (Wu et al., 1982; Macdonald et al., 1986; Schubert et al., 1986; Madison et al., 1987; Gross et al., 1989) and increasing potassium channel conductance (Tussell and Jackson, 1985; Macdonald et al., 1986; Madison et al., 1987; Scholfield and Steel, 1988; Gerber et al., 1989). Recent work has suggested that activation of A_2 receptors may also be involved in inhibition of neurotransmitter release (Phillis, 1990). However, in most cases, A_2 receptor activation promotes release (Dolphin and Archer, 1983; Spignoli et al., 1984; Stone and Bartrup, 1991).

Because of the ubiquitous distribution of adenosine and its receptors (Goodman and Snyder, 1982; Snowhill and Williams, 1986), and the lack of understanding regarding the mechanisms of many CNS drugs, the elucidation of adenosine's pathophysiological role in the CNS has been difficult (Williams, 1987). Nonetheless, there are several CNS activities in which adenosine has been implicated. These include, antiepileptic (Burley and Ferrendelli, 1984; Dragunow and Goddard, 1984; Rosen and Berman, 1985; Dragunow and Faull, 1988; Chin, 1989; Dragunow, 1991), antianxiety (Phillis and Wu, 1981; Phillis and O'Regan, 1988), hypnotic/sedative (Proctor and Dunwiddie, 1984; Radulovacki et al., 1984), antinociceptive (Ho et al., 1973; Yarbrough and McGuffin-Clineschmidt, 1981; Jurna, 1984) antidepressant (Sattin et al., 1978; Newman et al, 1984; Ahlijanian and Takemori, 1986), and antipsychotic (Heffner et al., 1985; Holloway and Thor, 1985) activities.

Perhaps the most interesting of adenosine's activities is its role as a retaliatory metabolite (Newby, 1984). Under conditions of energy demand, such as seizures, hypoxia, and ischaemia, adenosine is formed as a result of ATP catabolism (Newby, 1984; Dragunow, 1988). The increased adenosine has a number of neuroprotective effects: 1/ Inhibition of excitatory amino acid release which, at the elevated levels

found during a metabolic insult, can lead to cell damage or death as a result of massive calcium influx. Adenosine can also act at excitatory amino acid receptors to directly inhibit the calcium influx due to activation by excitatory amino acids (Phillis and Wu, 1981; Dragunow, 1988); 2/ Increased glycogenolysis in astrocytes (Magistretti et al., 1986); 3/ Increased cerebral blood flow through local vasodilation and inhibition of clot formation (Phillis et al., 1984); 4/ Decreased neuronal activity (Dragunow, 1988; Dragunow, 1991). These combined effects may help to regain cell homeostasis and reduce cell death following a metabolic insult (Dragunow and Faull, 1988; Dragunow, 1991).

The receptor mediated effects of adenosine will be greatly influenced by the magnitude and duration of receptor activation. This, in turn, is directly influenced by the extracellular adenosine concentration. The extracellular adenosine levels can rise up to 200-fold from resting levels of 0.1-1 μM during periods of ischemia or hypoxia. (Winn et al., 1981; Zetterstrom et al., 1982, Newman, 1983; Park et al., 1987; Phillis et al., 1987; Rudolphi, 1991). Control of the extracellular adenosine concentration involves the release and the reuptake of the nucleoside. Possible mechanisms of adenosine release include: 1/ calcium-dependent release of ATP, which is subsequently metabolized to adenosine; 2/ calcium-dependent adenosine release; 3/ calcium-independent adenosine release; 4/ release of adenosine and ATP as a result of cell lysis (Shimizu et al., 1974; Fredholm and Vernet, 1979; Fredholm et al., 1980, 1983; Jhamandas and Dumbrille, 1980; MacDonald and White, 1985; Hoehn and White, 1990a, 1990b; Meghji, 1991; Shank, 1992; Craig and White, 1993). The relative importance of neurons and glia to the extracellular adenosine levels is still unresolved (Stone et al., 1990).

1.2 Nucleoside Transport

1.2.1 Introduction

Nucleoside transporters play a major role in the control

of extracellular adenosine levels. These transporters can facilitate the release of adenosine produced in the cell, thereby increasing the extracellular adenosine concentration (Fredholm et al., 1980, 1983; Jonzon and Fredholm, 1985). Conversely, when adenosine is produced outside the cell, by ATP degradation for example, the action of nucleoside transport decreases the extracellular adenosine concentration by transporting the adenosine into the cell (Phillis and Wu, 1981; Morgan and Marangos, 1987; Geiger and Fyda, 1991). Therefore, substances which modify the function of these transporters, for example transport inhibitors, will alter the extracellular adenosine concentration and, consequently, adenosine receptor mediated events will be affected (Wu and Phillis, 1984; Deckert et al., 1988).

1.2.2 Adenosine Transport and Uptake

Functional characteristics of nucleoside transporters can be studied directly by examining the transport of radiolabelled nucleosides across the cell membrane. Once across the cell membrane, the nucleoside can then be metabolized by intracellular enzymes, as described earlier. This metabolism can be problematic when trying to measure the kinetics of adenosine transport (Paterson et al., 1981; Deckert et al., 1988; Plagemann et al., 1988, Geiger and Nagy, 1990). For example, when measuring the accumulation of adenosine in a cell, the rate limiting step may be the phosphorylation of adenosine to AMP by adenosine kinase. Therefore, the kinetic values obtained will reflect those of adenosine kinase rather than the nucleoside transporter (Deckert et al., 1988; Paterson et al., 1988; Geiger and Nagy, 1990). As a result, it is important to distinguish between the processes of "transport" and "uptake". Transport is the translocation of unmodified substrate across a cell membrane via a selective saturable carrier, whereas uptake is the accumulation of substrate and metabolized substrate inside a cell regardless of mode of entry and metabolism of the

substrate (Paterson et al., 1981; Paterson et al., 1987; Plagemann et al., 1988).

In order to accurately measure the kinetics of nucleoside transporters, the influence of nucleoside metabolism must be minimized. This can be achieved by using short incubation times or cells which are deficient in intracellular nucleoside metabolism. Some cell types lack the necessary nucleoside metabolizing enzymes; alternatively, metabolism may be inhibited as a result of ATP depletion or the use of inhibitors of metabolizing enzymes such as 5'-iodotubercidin and 2'-deoxycoformycin, for adenosine kinase and adenosine deaminase, respectively (Paterson et al., 1985; Deckert et al., 1988; Plagemann et al., 1988; Geiger and Fyda, 1991). In addition, nucleosides which are not metabolized as rapidly as adenosine can also be used for transporter characterization. Examples of these substrates include: uridine, a pyrimidine nucleoside (Plagemann et al., 1988; Geiger and Fyda, 1991); formycin B, an analogue of inosine (Vijayalakshmi and Belt, 1988; Plagemann and Woffendin, 1989; Crawford et al., 1990a; Crawford and Belt, 1991); and L-adenosine, the stereo-enantiomer of the physiological D-adenosine (Gati et al., 1989; Gu et al., 1991; Gu and Geiger, 1992; Casillas et al., 1993).

1.2.3 Transporter Heterogeneity

Adenosine flux across the cell membrane occurs primarily by three processes: 1/ passive diffusion; 2/ non-concentrative, facilitated diffusion (Paterson et al., 1987; Plagemann et al., 1988); 3/ concentrative, ion-dependent transport (Vijayalakshmi and Belt, 1988; Jarvis et al., 1989; Johnston and Geiger, 1989; Plagemann et al., 1990, Williams and Jarvis, 1991, Dagino et al., 1991a, 1991b). At physiological adenosine concentrations, the rate of passive diffusion is slow and of minor importance compared to nucleoside transporter mediated translocation (Plagemann et al, 1988; Geiger and Nagy, 1990). The term nucleoside

transport will be used here to describe nucleoside flux across a membrane which is mediated by the facilitated and/or active transport systems.

1.2.3.1 Facilitated Transporters

1.2.3.1.1 Kinetic Properties

The valid analysis of a transporter's kinetic properties requires the measurement of initial transport rates (Paterson et al., 1981; Plagemann et al., 1988). This is crucial when measuring nucleoside transport because of the rapid rate of transport in many cells, and the bidirectional and non-concentrative nature of facilitated nucleoside transport. *Zero trans* entry describes the transport of substrate from one side of the membrane to the other where its concentration is zero. In an experiment of this type, the substrate level into the cell begins at zero, but soon rises due to influx of substrate. Under these conditions, the apparent rate of accumulation decreases over time due to substrate backflow, making it difficult to measure initial transport rates. However, initial velocities can be calculated by measuring a time course of substrate transport into a cell and then fitting an integrated rate equation to the data. From this analysis, the initial rate of transport at time = 0 can be calculated (Plagemann et al., 1988). Using kinetic analyses which measured initial transport rates in metabolically challenged (kinase-deficient or ATP-depleted) mammalian cells, the affinity of natural nucleosides for the facilitated nucleoside transporter follows a trend of adenosine > thymidine > uridine > cytidine (Plagemann et al., 1988).

1.2.3.1.2 NBMPR sensitivity

Major progress in the characterization of the facilitated nucleoside transporter has been made largely due to the development of selective, high-affinity inhibitors of nucleoside transport. Amongst the first of these were the N^6-thiopurines such as NBMPR (nitrobenzylthioinosine; 6-(4-

nitrobenzylmercapto) purine ribonucleoside) (Pickard et al., 1973; Cass et al., 1974; Plagemann and Wohlheuter, 1980; Paterson et al., 1981, 1987, Plagemann et al., 1988). NBMPR has been shown to potently inhibit nucleoside transport (K_i 0.1-10 nM) in many cell types as a result of its binding to proteins associated with the nucleoside transporter (Plagemann and Wohlheuter, 1980; Paterson et al., 1987; Deckert et al., 1988; Plagemann et al, 1988). However, it became apparent that many types of cells possessed nucleoside transport that could only be inhibited by μM concentrations of NBMPR (Wohlhueter et al., 1979; Paterson et al., 1980; Jarvis and Young, 1982; Belt, 1983*a*, 1983*b*; Plagemann and Wohlhueter, 1984, 1985; Belt and Noel, 1985). These two equilibrative systems will be distinguished here as NBMPR-sensitive (*es*) and NBMPR-resistant (*ei*) (Vijayalakshmi and Belt, 1988). Cell types may exhibit only *es* transport (Jarvis and Young, 1982, 1986; Plagemann and Wohlhueter, 1985), only *ei* transport (Wohlhueter et al., 1979; Belt, 1983; Belt and Noel, 1985; Plagemann and Wohlhueter, 1985), or both systems (Plagemann and Wohlhueter, 1984, 1985; Aronow et al., 1985; Jarvis and Young, 1986; Lee and Jarvis, 1988; Parkinson and Clanachan, 1989). In most systems studied, the *es* and *ei* transporters have a broad nucleoside substrate specificity (Plagemann and Wohlhueter, 1980, 1985; Belt, 1983*a*, *b*; Belt and Noel, 1985; Jarvis and Young, 1986). As well, both *es* and *ei* transporters have been reported to have similar substrate affinities (Lee and Jarvis, 1988*a*; Plagemann and Wohlhueter, 1984; Belt, 1983). An exception to this occurs in at least one system, where significant differences in substrate selectivity were found, with guanosine, 2′-deoxyguanosine, cytidine, and 2′-deoxycytidine having a higher affinity for the *es* transporter (Hammond, 1991*a*, 1992). There are also some reports that the *ei* transporter is more sensitive to inhibition by sulfhydryl-reactive reagents such as p-mercuribenzenesulfonate (Belt and Noel, 1985; Tse et al., 1985*b*; Jarvis and Young, 1986).

There are methodological difficulties in directly

measuring transport. These include, rapid separation of cells from incubation medium, intracellular metabolism, and efflux of substrate (Paterson et al., 1981; Plagemann et al., 1988). Consequently, the use of high-affinity radioligands to study the nucleoside transporter has gained popularity. [^{3}H]NBMPR has proven to be a useful tool for predicting the capacity of compounds to inhibit nucleoside transport (Cass et al., 1974; Jarvis and Young, 1980), for quantifying the numbers of transporters per cell (Plagemann et al., 1988; Cass, 1994), for localizing transporters in tissue sections (Geiger and Nagy, 1984, 1985; Bisserbe et al., 1985), and for photoaffinity labelling a protein which is the nucleoside transporter or one which is tightly coupled to the transporter (Young et al., 1984; Hammond and Johnstone, 1989; Jarman et al., 1989).

The binding site for NBMPR is located on the external side of the membrane (Young and Jarvis, 1983; Jarvis, 1986; Jarvis and Young, 1986). The NBMPR binding site and the substrate binding site seem to overlap (Jarvis and Young, 1980; Jarvis et al., 1982, 1983; Koren et al., 1983; Wohlhueter et al., 1983). However, the binding of NBMPR also appears to involve an interaction between the nitrobenzyl group and a hydrophobic domain of the transporter, or a closely associated molecule (Jarvis et al., 1983; Wohlhueter et al., 1983; Young and Jarvis, 1983*b*). In fact, it is the lipophilic nitrobenzyl group of NBMPR that is believed to be responsible for its approximately 10^6-fold higher affinity for the nucleoside transporter, when compared to natural nucleosides (Jarvis et al., 1983; Wohlhueter et al., 1983; Young and Jarvis, 1983*b*). Since the NBMPR-binding site does not separate from the transporter upon solubilization in detergent (Tse et al., 1985*a*; Hammond and Johnston, 1989), the hydrophobic domain of the [^{3}H]NBMPR binding site is likely part of the transporter protein itself, or a covalently bound molecule.

Based on the finding that the nucleoside transporter fits

a simple carrier model with a single, apparently non-cooperative, substrate binding site, it has been assumed that NBMPR binds to the nucleoside transporter with a simple 1:1 relationship (Plagemann et al., 1988). However, it is becoming evident that in some systems the interaction of NBMPR, and other inhibitors, with the nucleoside transporter is not a simple one but involves more complex interactions, such as multiple binding sites and allosteric interactions. Specifically, pseudo-Hill coefficients greater than or less than one were found for inhibition of [^{3}H]NBMPR binding by lidoflazine analogues and dipyridamole, respectively (Wu and Phillis, 1982*b*; Hammond and Clanachan, 1985; Plagemann and Wohlhueter, 1985; IJzermann et al., 1989). In addition, various agents, including nucleosides and nucleoside transport inhibitors, have been shown to modify the rate of dissociation of [^{3}H]NBMPR from its binding sites (Jarvis et al., 1983; Koren et al., 1983; Wohlhueter et al., 1983; Hammond and Clanachan, 1985; Ogbunude and Baer, 1989; Hammond, 1991*b*).

1.2.3.1.3 Dipyridamole sensitivity

Dipyridamole (DPR), an inhibitor of facilitated nucleoside transport, does not distinguish between the *es* and *ei* transporters in most cell types and species (Deckert et al., 1988; Plagemann et al., 1988). However, as was the case with NBMPR, both DPR-sensitive and DPR-resistant nucleoside transporters have been observed (Hammond and Clanachan, 1985; Jarvis, 1986; Marangos and Deckert, 1987; Woffendin and Plagemann, 1987). Transporter sensitivity to DPR appears to be primarily species dependent, with rats, mice, and hamsters possessing DPR-resistant transporters (IC_{50} for inhibition of transport ≈ 100-1000 nM), and humans, pigs, and guinea pigs possessing DPR-sensitive transporters (IC_{50} ≈ 10-100 nM) (Jarvis, 1986; Shi and Young, 1986; Woffendin and Plagemann, 1987; Deckert et al., 1988; Plagemann et al., 1988). Examples of tissue dependence have also been demonstrated (Plagemann et al., 1988).

[^{3}H]DPR has been shown to bind, with high affinity, to the nucleoside transport protein (Deckert et al., 1988; Plagemann et al., 1988). Since DPR could inhibit both the *es* and *ei* transporters of many species (except rat, mouse and hamsters) at low concentrations (Plagemann and Wohlhueter, 1984; Deckert et al., 1988), it was hoped [^{3}H]DPR would be a more general probe for the nucleoside transporter. As expected, [^{3}H]DPR ($K_D \approx 10$ nM) labelled two- to ten-fold as many sites as [^{3}H]NBMPR in guinea pig, and human tissues (Bisserbe et al., 1986; Marangos and Deckert, 1987; Woffendin and Plagemann, 1987*a*; Jones-Humble and Morgan, 1994), with a portion of these sites being resistant to inhibition by NBMPR.

Despite the apparent usefulness of [^{3}H]DPR as a probe for the *ei* transporter, [^{3}H]DPR is not without problems. As discussed earlier, there are DPR-resistant nucleoside transporters which will not be labelled by [^{3}H]DPR due to their low affinity for this ligand. In addition, it has never been demonstrated definitively, that the NBMPR-resistant [^{3}H]DPR binding site is directly associated with the *ei* nucleoside transporter. This fact becomes more important in light of the ability of [^{3}H]DPR to bind to 3-5 times as many sites as [^{3}H]NBMPR in human erythrocytes (Woffendin and Plagemann, 1987*a*), cells with no *ei* transporters (Plagemann et al., 1988). These results and others, such as discrepancies in the calculation of K_D for [^{3}H]DPR binding depending on the method used to calculate it (Woffendin and Plagemann, 1987*a*), may be due to technical difficulties including high non-specific tissue binding (Jarvis, 1986; Woffendin and Plagemann, 1987*a*), binding to glass and plastic assay tubes (Shi and Young, 1986; Marangos and Deckert, 1987), and chemical instability of [^{3}H]DPR (Woffendin and Plagemann, 1987*a*).

1.2.3.1.4 Other Inhibitors

Many substances have been found to have some inhibitory effect on the nucleoside transporter. These substances often

bear no obvious structural similarities, but are usually lipophilic (Plagemann et al., 1988). Groups of substances with members which have been shown to inhibit nucleoside transport include calcium channel blockers, benzodiazepines, epipodophyllotoxins, substituted piperazines, and adenosine receptor agonists and antagonists (Phillis and Wu, 1982; Deckert et al., 1988; Plagemann et al., 1988; Wright et al., 1990; Geiger and Fyda, 1991; Marangos and Miller, 1991; Van Belle and Janssen, 1991). As was the case with NBMPR and DPR, the sensitivity of nucleoside transport to inhibition by various other transport inhibitors tends to be dependent on species and cell type (Plagemann et al., 1988).

In addition to [^{3}H]NBMPR and [^{3}H]DPR, two more probes for the nucleoside transporter have been produced, [^{3}H]dilazep and [^{3}H]R75231. [^{3}H]Dilazep, whose affinity for the *es* transporter is similar to that of NBMPR, may prove to be a useful ligand for examining this transporter (Plagemann et al., 1988; Gati and Paterson, 1989). [^{3}H]Dilazep binding to S49 mouse lymphoma cells has been resolved into two populations of binding sites, which are associated with the nucleoside transporter (Gati and Paterson, 1989). Both of these sites may be located on a single membrane protein (Gati and Paterson, 1989).

Finally, [^{3}H]R75231, a lidoflazine derivative, has unique binding characteristics, potentially making it useful both as a probe for the nucleoside transporter, and clinically. Studies have shown that R75231 binds to the nucleoside transporter extremely tightly *in vitro* (Masuda et al., 1991; Van Belle and Janssen, 1991; IJzerman et al., 1992), and has long-lasting inhibitory effects *in vivo* (Baer et al., 1991; Van Belle et al., 1991). In addition, R75231 and other lidoflazine derivatives inhibit [^{3}H]NBMPR binding with pseudo-Hill coefficients greater than one (IJzerman et al., 1989). These results suggest that [^{3}H]R75231 binds to the nucleoside transporter in a different manner than any of the other available probes.

Table 1.1: Summary of radioligand probes used for the facilitated nucleoside transporters.

Radioligand Probe	Transporter Subtype		K_D (nM)
	es	*ei*	
[^{3}H]NBMPR	+	-	0.1-1[a]
[^{3}H]DPR	+	+[b]	1-10[c]
[^{3}H]dilazep	+	-	0.2, 10[d]
[^{3}H]R75231	+	-	0.3[e]

[a] summarized from Deckert et al., 1988; Plagemann et al., 1988; Geiger and Nagy, 1990

[b] the ability of [^{3}H]DPR to label the *ei* transporter is not certain (see section 1.2.3.1.3)

[c] from Jarvis, 1986; Shi and Young, 1986; Marangos and Deckert, 1987; Woffendin and Plagemann, 1987. However, K_D values which were higher (114 nM) and lower (0.2 nM) than this range have been observed (Woffendin and Plagemann, 1987; Jones-Humble and Morgan, 1994).

[d] high and low affinity binding sites were found (Gati and Paterson, 1989)

[e] from IJzerman et al., 1992

1.2.3.1.5 Molecular Properties

The *es* transporter has been examined in more detail than the other nucleoside transporters due to the high affinity probe NBMPR. The [^{3}H]NBMPR binding protein of human erythrocytes (*es*) is an integral membrane glycoprotein with a molecular weight of 50-60 kD. This protein is either the transport protein itself or a protein which is tightly coupled to the transport protein (Wu et al., 1983; Young et al., 1984; Tse et al., 1985a; Kwong et al., 1988). [^{3}H]DPR can also be used to photolabel a protein (molecular weight 50-60 kD) which appears to be involved in nucleoside transport (Woffendin and Plagemann, 1987a).

Structural conservation of the *es* transporter has been demonstrated between human, pig, rabbit and rat using polyclonal antibodies (Kwong et al., 1992) and between human, pig, rat and guinea pig using peptide mapping (Kwong et al., 1993). This later study also showed structural conservation between *es* transporters in erythrocytes, liver, and lung of these species (Kwong et al., 1993). Nonetheless, there are *es* structural differences between species as a result of glycosylation (Crawford et al., 1990c; Kwong et al., 1993; Cass, 1994). In the case of pig vs. human erythrocytes, there are also structural differences in the form of an additional protein domain in the pig transporter (Kwong et al., 1993). Although the *es* transporter has not been sequenced yet, cDNA fragments encoding polypeptides which are recognized by polyclonal antibodies for the human *es* transporter, have been isolated from cultured human choriocarcinoma BeWo cells (Cass, 1994). Confirmation of the *es*-related identity of these cDNA fragments is being pursued (Cass, 1994).

Purification of the equilibrative nucleoside transporter has proven difficult. Most purification attempts have used erythrocytes as the source and have involved strategies such as ion-exchange chromatography (Jarvis and Young, 1981; Tse et al., 1985a; Kwong et al., 1987), immunoaffinity chromatography (Kwong et al., 1988; Kwong et al., 1992), and affinity columns

(Agbamyo et al., 1990). A major problem in purification attempts, except those using pig erythrocytes which lack glucose transporters, is contamination with glucose transport proteins. In human erythrocytes, the glucose transporters are approximately 25-fold more numerous than the nucleoside transporter and have a similar molecular weight (Plagemann et al., 1988; Cass, 1994). Properties including, functioning as facilitated transporters, similar molecular weights before and after glycosylation, and similar location of trypsin cleavage sites (Plagemann et al., 1988), suggest that the glucose transporter (GLUT1) and nucleoside transporter of human erythrocytes (*es*) are structurally related. Despite this, both transporters differ substantially in their substrate selectivity. In human erythrocytes, sugars at concentrations greater than 100 mM have no effect on uridine transport and 50 mM uridine has no effect on 3-O-methylglucose transport (Plagemann and Woffendin, 1987).

The structural aspects required for the binding of the substrate to the *es* transporter have not been studied in detail; however, some generalizations can be made. The ribose moiety of nucleosides seems to be important for substrate recognition as nucleobases are usually not recognized by the transporter (Plagemann et al., 1988). In contrast, the nucleobase moiety seems to be less important for substrate recognition, since all natural purine and pyrimidine nucleosides, as well as many nucleoside analogues, are transported (Plagemann and Wohlhueter, 1980; Paterson et al., 1981; Zimmerman et al., 1989; Belt et al., 1993; Cass, 1994). The affinity of nucleosides generally increases with their lipophilicity, suggesting that a hydrophobic domain on the transporter is involved in substrate binding (Plagemann et al., 1988). Finally, the nucleoside transporter does not accept ionized substrates (Plagemann et al., 1988).

Due to the lack of a specific probe for the *ei* transporter, very little is known about this protein. Functional similarities between *es* and *ei* transporters,

discussed previously, suggest they may be structurally related; however, cell mutation studies have suggested that these transporter proteins may be genetically distinct (Belt and Noel, 1988; Crawford et al., 1990*b*). It is not known if differences between *es* and *ei* transporters are a result of the transporters being different gene products or due to a differential expression of modifier proteins.

1.2.3.2 Ion-dependent transport

As is the case for other compounds such as amino acids, glucose, and succinate, adenosine can be transported across the cell membrane by active transport systems as well as by facilitated transport systems (Geiger and Fyda, 1991). Sodium-dependent, concentrative nucleoside transport systems have been described in lymphocytes (Plagemann and Aran, 1990; Plagemann et al., 1990), transformed cell lines (Crawford and Belt, 1991; Dagnino et al., 1991*a*, 1991*b*), neuronal systems (Spector and Huntoon, 1984; Hertz and Matz, 1989; Johnston and Geiger, 1989, 1990; Wu et al., 1992; Wu et al., 1994), fibroblasts and macrophages (Plagemann and Aran, 1990) and epithelial tissues (Williams et al., 1989; Franco et al., 1990; Jakobs et al., 1990; Williams and Jarvis, 1991).

To date, five subtypes of concentrative nucleoside transporters have been proposed based on substrate specificity. The first, designated N1 or *cif*, is selective for purine nucleosides and uridine as substrates and the second, designated N2 or *cit*, is selective for pyrimidine nucleosides and adenosine as substrates (Vijayalakshmi and Belt, 1988; Vijayalakshmi et al., 1992; Williams and Jarvis, 1991; Plagemann et al., 1990). Both of these systems have a coupling stoichiometry of 1:1 (sodium: nucleoside) (Franco et al., 1990; Dagnino et al., 1991*b*; Williams and Jarvis, 1991). In tissues that possess both sodium-dependent and facilitated transporters, the sodium-dependent transporter tends to have an approximately 10-fold higher affinity for its nucleoside substrate than the facilitated transporter (Jarvis et al.,

1989; Williams and Jarvis, 1991).

Although the N1 and N2 transporters have been studied in the most detail, neither have been found in human tissues. Three sodium-dependent nucleoside transporters have been described in human tissues. The N3, or *cib*, transporter, found in the choroid plexus of rabbit (Wu et al., 1992, 1994), and human colorectal and leukaemia cells (Belt et al., 1993; Lee et al., 1991), accepts both purine and pyrimidine nucleosides and has a coupling stoichiometry of 2:1 (sodium:nucleoside). An N4 transporter, found in brush border membranes of human kidneys, has also been proposed (Gutierrez et al., 1992; Gutierrez and Giacomini, 1993). It has a 1:1 stoichiometry and a substrate specificity similar to N2, except it transports guanosine. Finally, a sodium-dependent transporter designated N5, or *cs*, has been described in human leukaemic cells (Paterson et al., 1993). Although, the stoichiometry and substrate specificity have not yet been determined, this transporter can be distinguished by its sensitivity to inhibition by NBMPR and DPR (<10 nM) (Paterson et al., 1993). All of the other sodium-dependent transporters are resistant to inhibition by high concentrations (>10 μM) of NBMPR or DPR (Cass, 1994).

Putative clones for both the N2 (Young et al., 1994) and N3 (Pajor and Wright, 1992; Pajor, 1994) transporters have been produced. The N2 transporter, a 648 amino acid protein, was cloned from rat jejunum (Young et al., 1994). The N3 transporter, cloned from rabbit renal tissue (Pajor and Wright, 1992; Pajor, 1994), is a 672 amino acid protein related to the rabbit intestinal sodium-dependent glucose cotransporter, SGLT1. However, it is interesting to note that the cloned N2 and N3 transporters do not appear to be within the same gene family (Young et al., 1994).

In addition to sodium-dependent transporters, potassium-dependent transporters have been observed in renal brush border (Lee et al., 1988; Jarvis et al., 1989; Williams et al., 1989). Like their sodium-dependent counterparts, the

potassium-dependent transporters are electrogenic, coupled transporters (Lee et al., 1988; Jarvis et al., 1989); however, the reported stoichiometry is different, 3:2 (potassium: nucleoside) (Jarvis et al., 1989; Williams et al., 1989).

Table 1.2: Summary of nucleoside transporter subtypes and their functional properties (adapted from Cass, 1994).

Subtype	Equilibrative		Concentrative					
	es	ei	N1	N2	N3	N4	N5	N_K[a]
Ion-dependence	-	-	Na^+	Na^+	Na^+	Na^+	Na^+	K^+
Inhibitor Sensitivity:								
NBMPR	+	-	-	-	-	-	+	ND[b]
DPR	+	+	-	-	-	-	+	ND
dilazep	+	+/-	-	-	-	-	+	ND
Substrates:								
adenosine	+	+	+	+	+	+	+	ND
uridine	+	+	+	+	+	+	ND	+
guanosine	+	+	+	-	+	+	ND	ND
formycin B	+	+	+	-	+	-	+	ND
thymidine	+	+	-	+	+	+	ND	ND

[a] potassium dependent transporter as described in Lee et al., 1988

[b] ND, not determined

1.2.4 Nucleoside Transport in the CNS

1.2.4.1 Transporter Localization

The distribution of nucleoside transporters in the CNS has been examined using [^{3}H]NBMPR and [^{3}H]DPR with autoradiography and microdissection techniques. High densities of [^{3}H]NBMPR binding sites in rat and guinea pig brain were found in the choroid plexus, hypothalamus, thalamus, and superficial layers of the superior colliculus, whereas relatively lower densities were observed in cerebral cortex, hippocampus, and cerebellar cortex (Marangos et al., 1982; Hammond and Clanachan, 1983; Geiger and Nagy, 1984, 1985; Bisserbe et al., 1985). In rat brain, the density of [^{3}H]NBMPR binding coincides well with the density of ADA (Nagy et al., 1985; Geiger and Nagy, 1986). It was hypothesized that nucleoside transporters would be found in highest density at sites where adenosine receptors were in highest density. This arrangement would result in the most rapid removal of adenosine from the receptor sites, and therefore, rapid receptor inactivation. However, in rat brain, the [^{3}H]NBMPR binding distribution did not correlate well with the profiles for either the adenosine A_1 receptor (Goodman et al., 1983; Bisserbe et al., 1985; Geiger, 1986), or the A_2 receptor (Lee and Reddington, 1986; Jarvis et al., 1989). A possible explanation for this result was the presence of nucleoside transporter subtypes which were not labelled by [^{3}H]NBMPR.

With the hope of labelling more of the nucleoside transporters than was possible with [^{3}H]NBMPR, [^{3}H]DPR was used in autoradiographic examinations of guinea pig brain. As was the case with [^{3}H]NBMPR, high levels of [^{3}H]DPR binding were observed in the choroid plexus, hypothalamus, and superficial layers of the superior colliculus (Bisserbe et al., 1986; Deckert et al., 1987). However, unlike [^{3}H]NBMPR, the density of [^{3}H]DPR binding was also high in the cerebral cortex, the central grey matter, and the interpeduncular nucleus (Deckert et al., 1987). In spite of [^{3}H]DPR's ability to label approximately 2.5 times as many sites in guinea pig brain as

[^{3}H]NBMPR (Deckert et al., 1987), again, there was no discernable relationship between adenosine receptor density and nucleoside transporter density.

1.2.4.2 Substrate Transport Studies

In comparison to erythrocytes and tumour cells, there has been comparatively little examination of nucleoside transport in the CNS. Preparations used include synaptosomes from rat (Bender et al., 1980, 1981; Lee and Jarvis, 1988*a*; Shank and Baldy, 1990; Sweeney et al., 1993) and guinea pig (Barberis et al., 1981; Lee and Jarvis, 1988*b*; Shank and Baldy, 1990), synaptoneurosomes from rat (Morgan and Marangos, 1987; Gu et al., 1991; Gu and Geiger, 1992) guinea pig (Morgan and Marangos, 1987) and human (Gu et al., 1993), brain tissue slices from guinea pig (Shimizu et al., 1972; Davies and Hambley, 1986) and mouse (Banay-Schwartz et al., 1980), cultured astrocytes (Hertz, 1978; Thampy and Barnes, 1983; Bender and Hertz, 1986) and neurons (Bender and Hertz, 1986; Hertz and Matz, 1989; Ohkubo et al., 1991), astrocytoma cells (Lewin and Bleck, 1979), rat brain capillaries (Wu and Phillis, 1982*a*), choroid plexus (Spector, 1982; Spector and Huntoon, 1984; Wu et al., 1992, 1994), and dissociated brain cell bodies (Geiger, 1987, 1988; Geiger et al., 1988; Johnston and Geiger, 1989, 1990; Gu et al, 1991; Gu and Geiger, 1992). Unfortunately, much of the early research on nucleoside transport (before late 1980's) was complicated by the presence of nucleoside substrate metabolism; therefore, uptake was examined rather than transport. Because of this and other methodological, tissue, and species differences, it is difficult to make generalizations about kinetic and inhibition data for the CNS nucleoside transporters. For example, the reported K_m (or K_t) for adenosine transport in CNS tissues has varied from 0.9 μM to 313 μM (Bender et al., 1981; Johnston and Geiger, 1989).

In spite of these problems, there are some consistent qualitative results. Nucleoside flux studies have been able

to discern multiple transporter subtypes, and generalizations about the inhibitor sensitivity of some species can be made. Both *es* and *ei* facilitated nucleoside transporters have been well characterized in rat and guinea pig CNS tissues (Davies and Hambley, 1986; Morgan and Marangos, 1987; Geiger et al., 1988; Lee and Jarvis, 1988*a*, 1988*b*; Shank and Baldy, 1990). Species dependence of DPR-sensitivity has also been well established, with rat having primarily DPR-resistant transport, whereas guinea pig has both DPR-sensitive and -resistant transport (Davies and Hambley, 1986; Geiger et al., 1988; Lee and Jarvis, 1988*a*, 1988*b*; Shank and Baldy, 1990).

In the CNS, active nucleoside transport has been observed in choroid plexus (Spector, 1982; Spector and Huntoon, 1984; Wu et al., 1992, 1994), and dissociated brain cell bodies (Johnston and Geiger, 1989, 1990). In both instances, the nucleoside transporters were high-affinity, inward-directed, sodium-dependent, co-transporters. The sodium dependent transporter in choroid plexus has been defined as N3 (Wu et al., 1992, 1994). The transporter in dissociated brain cell bodies has not been sufficiently characterized to determine its subtype (Johnston and Geiger, 1989). As yet, there have been no reports of active nucleoside transport in synaptosomes.

1.2.4.3 Radioligand Binding Studies

[^{3}H]NBMPR has been used extensively to examine nucleoside transporters in the CNS (Deckert et al., 1988; Plagemann et al., 1988; Geiger and Nagy, 1990; Geiger and Fyda, 1991). In the majority of cases, [^{3}H]NBMPR has been demonstrated to bind to homogeneous, high affinity (K_D < 1nM), low capacity, (B_{max} < 200 fmol/mg protein) sites which are associated with the *es* transporter (Morgan and Marangos, 1987; Geiger et al., 1988; Lee and Jarvis, 1988*b*). Consistent with findings in non-CNS tissues, [^{3}H]NBMPR binding in rat and mouse CNS preparations (Marangos et al., 1982; Hammond and Clanachan, 1984; Marangos, 1984; Geiger et al., 1985; Verma and Marangos, 1985; Lee and

Jarvis, 1988a) is resistant to inhibition by DPR in comparison to guinea pig and human (Hammond and Clanachan, 1984; Marangos, 1984; Verma and Marangos, 1985; Lee and Jarvis, 1988*b*). Another species dependent difference is the biphasic competition curves found for the inhibition of [^{3}H]NBMPR binding by DPR in rabbit and dog cerebral cortical membranes (Hammond and Clanachan, 1985). This indicates DPR-sensitive and -resistant [^{3}H]NBMPR binding sites in the same species. These sites may represent DPR-sensitive and -resistant nucleoside transporters which are both sensitive to inhibition by NBMPR.

Although most studies report a single class of [^{3}H]NBMPR binding sites in CNS tissues, there have been reports of both high and low affinity [^{3}H]NBMPR binding sites. In rabbit cerebral cortical membranes, the K_D and B_{max} were 0.4 nM and 72 fmol/mg protein and 13.8 nM and 981 fmol/mg protein, respectively (Hammond and Clanachan, 1985).

[^{3}H]DPR binds with high affinity to guinea pig brain (K_D from 3.5-10 nM) to about 2.5 times as many sites as labelled by [^{3}H]NBMPR (Marangos et al., 1985; Bisserbe et al., 1986; Deckert et al., 1987; Marangos and Deckert, 1987). These additional sites may represent *ei* nucleoside transporters, but as stated above, this has not yet been demonstrated. In contrast to guinea pig, binding of [^{3}H]DPR to the nucleoside transporter could not be observed in rat brain, presumably as a result of the low affinity of DPR for the transporters in this species (Marangos and Deckert, 1987).

1.2.5 Regulation of Transport

From research focused on the regulation of nucleoside transport, it is apparent that the rate of cellular growth and division is linked to changes in transporter numbers and/or activity. The number of *es* transporters has been shown to increase during the S-phase of human HeLa carcinoma cells and S1 macrophages (Cass et al., 1979; Meckling-Gill et al., 1993), as well as during periods of rapid proliferation of

human thymocytes and lymphocytes (Smith et al., 1989), and lymphoma and leukaemic blast cells (Wiley et al., 1989). Logarithmically growing S1 macrophages exhibited an increased ratio of *es* to *ei* nucleoside transport when compared to quiescent S1 macrophages (Meckling-Gill et al., 1993). In guinea pig and rat, high levels of [^{3}H]NBMPR binding were apparent during times of neurogenesis (Geiger, 1987; Deckert et al., 1988*b*). In sheep reticulocytes, NBMPR-sensitive transport decreased during maturation into erythrocytes (Johnstone et al., 1987).

Regulation of nucleoside transport is also linked to cellular differentiation. Transformation of rat-2 fibroblasts by v-*fps*, a transforming protein-tyrosine kinase, resulted in an increase in *es* transporter activity without an increase in [^{3}H]NBMPR binding sites (Meckling-Gill and Cass, 1992). Induction of differentiation of HL-60 human promyelocytic cells by N,N-dimethylformamide (Chen et al., 1986), dimethylsulfoxide (DMSO) (Lee et al., 1990; Sokaloski et al., 1991), or phorbol 12-myristate 13-acetate (PMA) (Lee et al., 1991; Sokaloski et al., 1991; Lee, 1994), resulted in a decrease in NBMPR-sensitive transport. DMSO and PMA induced cells also showed an increase in sodium-dependent nucleoside transport (Lee et al., 1990, 1991; Sokaloski et al., 1991). Finally, an increase in sodium-dependent transport was seen in IEC-6 rat intestinal epithelial cells differentiated by low serum content (Jakobs et al., 1990).

Second messengers implicated in the regulation of nucleoside transport include protein kinase A which decreases *es* mediated transport (Sen et al., 1990; Miras-Portugal et al, 1991*b*; Nagy et al., 1991), and protein kinase C which increases sodium-dependent transport and decreases *es* and *ei* mediated transport (Lee et al., 1991; Miras-Portugal et al., 1991*a*, 1991*b*; Sen et al., 1993; Lee, 1994). Exactly how these, or other, second messengers regulate nucleoside transport levels and activity is not known. However, there is evidence for movement of plasma membrane nucleoside

transporters to and from cytoplasmic pools of transporters (Blostein and Grafova, 1987; Liang and Johnstone, 1992; Torres et al., 1992), and for increased transporter synthesis (Fideu and Miras-Portugal, 1992).

1.2.6 Therapeutic Potential

Due to the numerous effects of adenosine in the body, there are various potential therapeutic uses of nucleoside analogues and nucleoside transport inhibitors. It should be noted that the effectiveness of the transport inhibitors will depend on whether the transporters in question are symmetrical, bi-directional, and whether adenosine is formed intracellularly or extracelluarly.

Adenosine itself is used for the treatment of supra-ventricular tachycardia (Dimarco et al., 1983). DPR and other nucleoside inhibitors, including NBMPR, dilazep, etoposide, and BIBW 22, have been investigated, and in some cases used clinically in combination with inhibitors of *de novo* nucleotide synthesis, for cancer and viral chemotherapy (Weinstein et al., 1989; Keane et al., 1990; Lokich, et al., 1991; Cass et al., 1992; Chen et al., 1993; Sato et al., 1993). Some cytotoxic nucleoside analogues used as chemotherapeutic agents, for example chlorodeoxyadenosine and cytosine arabinoside, gain entry into the cell largely by nucleoside transport (Zimmerman et al., 1989; Jamieson et al., 1993; Cass, 1994). When considering a therapy which uses drugs such as these, assessing the quantity of nucleoside transport that a particular tumour possesses is extremely important, because the presence of nucleoside transport is necessary for the drug to have access to the tumour cells (Jamieson et al., 1993). In some cases it may also be possible to use nucleoside transport inhibitors to selectively block the transporters of the host cells, but not the tumour cells, thereby increasing the maximum tolerated drug dosage. This strategy requires that the nucleoside transporters of the host cells have different inhibitor sensitivities than those

of the cancer cells (Kolassa et al., 1982; el Kouni, 1991; Belt et al., 1993). With the knowledge that 3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC) are substrates for N2 transporter, strategies of this type may become useful in the treatment of AIDS (Young et al., 1994). Combinations of nucleoside transport inhibitors and cytotoxic nucleoside analogues can also be used when the analogue is not transported by the nucleoside transporter (Zimmerman et al., 1989; Weinstein et al., 1990). The objective in this case being to interfere with both *de novo* nucleoside synthesis and the "salvage" of nucleosides by the nucleoside transporter, thereby increasing the therapeutic potential.

Other therapeutic uses of nucleoside transport inhibitors stem from adenosine's physiological role as a retaliatory metabolite (Newby, 1984; see section 1.1.4.1). That is, under conditions of physiological stress such as ischemia, increased adenosine levels help target cells to adjust their energy supplies and retaliate against the stress. Nucleoside transport inhibitors, by blocking the uptake of extracellular adenosine, can increase and prolong the effects of endogenous adenosine. The cardioprotective effects (antiarrhythmic effects, arterial dilation, cardiopreservation, inhibition of platelet aggregation, protection from catecholamine stress and ischemia) of nucleoside transport inhibitors, such as dilazep, lidoflazine, and R75231, are being investigated (Haga et al., 1986; Geiger and Fyda, 1991; Masuda et al., 1991, 1992; Chang-Chun et al., 1992; de Haan et al., 1993; Van Belle et al., 1993; Wainwright et al., 1993; Beukers et al., 1994; Bohm et al., 1994). In fact, DPR has been used clinically as a vasodilator and inhibitor of platelet aggregation (Geiger and Fyda, 1991), dilazep has been used as a vasodilator (Geiger and Fyda, 1991), and draflazine, which is the (-)-enantiomer of R75231, is currently undergoing clinical trials as a cardioprotective drug (H. Van Belle, personal communication).

In keeping with its role as a retaliatory metabolite,

adenosine has been proposed as an endogenous neuroprotective agent under ischemic conditions such as stroke (Deckert et al., 1988; Dragunow, 1988; Geiger and Fyda, 1991; Marangos, 1991; Marangos and Miller, 1991). Evidence to support this includes the observations that adenosine is released by trauma such as ischemia (Hagberg et al., 1987; Dux et al., 1990) and that adenosine analogues can prevent ischemia-related neuronal death (Goldberg et al., 1988; Von Lubitz et al., 1988). In addition, the use of adenosine receptor antagonists during ischemia increases the neuronal injury (Rudolphi et al., 1987; Dux et al., 1990; Boissard et al., 1992). Specific mechanisms of neuronal protection by adenosine were discussed in section 1.1.4.1. Adenosine has also been suggested to have endogenous antiepileptic activities (Dragunow, 1988, 1991; Chin, 1991), including the inhibition of the spread of seizures and the termination of seizures (Chin, 1989; Dragunow, 1991). The exact mechanism of anticonvulsant activities has not been determined, but appears to be largely due to activation of A_1 receptors (Dragunow, 1991). The nucleoside transport inhibitors soluflazine and propentofylline have shown neuroprotective effects (Andiné et al., 1990; Dux et al., 1990; Boissard and Gribkoff, 1993; Parkinson et al., 1993), and soluflazine, dilazep, papaverine, and NBMPR 5′-monophosphate have shown anticonvulsant effects (Ashton et al., 1987, 1988; Geiger and Fyda, 1991; Zhang et al., 1993), presumably as a result of increased extracellular adenosine levels.

There is also increasing research, *in vivo* and *in vitro*, into the development and use of nucleoside transport inhibitors such as DPR, NBMPR, papaverine, mioflazine, and dilazep, as antinociception, antianxiety, and sedative drugs (Wauquier et al., 1987; Deckert et al., 1988; Daval et al., 1991; Marangos, 1991; Marangos and Miller, 1991). For example, adenosine A_1 agonists can cause sedation that resembles natural sleep (Radulovacki et al., 1982). In fact, adenosine may be very important as a natural sleep

facilitating agent (Marangos, 1991). The nucleoside transport inhibitors soluflazine and mioflazine have been demonstrated to increase sleep activity in dogs (Wauquier et al., 1987). In addition, the therapeutic action of a number of drugs has been suggested to be mediated, in part, through inhibition of nucleoside transport. These drugs include benzodiazepines, barbiturates, phenothiazines, tricyclic antidepressants, and others (Phillis and Wu, 1982; Deckert et al., 1988, Phillis and O'Regan, 1988; Daval et al., 1991).

Although nucleoside transport inhibitors show great potential as clinically useful drugs, they are not without potential problems. For example, nucleoside transporter blockade will increase the overall adenosine concentrations resulting in general vasodilation. As a result, during a stress such as ischemia much of the increased blood flow that would be diverted to the ischemic area, due to a local increase in adenosine concentrations, will be less as a result of the generalized vasodilation (Braunwald and Sabel, 1988). In addition, increased adenosine levels could result in an increase in the levels of xanthine and hypoxanthine which are products of adenosine metabolism. The increased free radical production from the xanthine and hypoxanthine would exacerbate the damage that was trying to be protected against.

A major barrier to the development of therapeutically useful adenosergic drugs is making them selective, both for the CNS and within the CNS. The objective of this research is to decrease the side effects by increasing the drug's selectivity. The heterogeneity of nucleoside transporters within the mammalian brain may make it possible for drugs to be developed which selectively inhibit a particular nucleoside transporter subtype, and therefore, selectively affect a particular brain region.

Nucleoside transport inhibitors may also have an advantage over adenosine receptor agonists, especially if the drug is to be taken chronically. Transport inhibitors can act to potentiate the effects of endogenous adenosine release.

Therefore, their effect is localized to the release area, and is temporary. That is, the adenosine receptor stimulation will increase upon adenosine release and decrease when adenosine release is terminated. This contrasts with adenosine receptor agonists, which may not be as localized and will stimulate the receptor at a constant level.

1.2.7 Objectives

As discussed, drugs which act by interacting with the nucleoside transport system have great therapeutic potential. However, the full realization of this potential requires a thorough understanding of nucleoside transporters. This will include: developing probes for the transporters, examining transporter subtypes and the effects of inhibitors on each subtype, and studying regulation of transporter numbers and characteristics. This thesis reports work which was divided into 3 phases, all ultimately leading to a better understanding of the nucleoside transport system in neuronal tissues. The first phase examined the assertion that [^{3}H]DPR binds to both *es* and *ei* nucleoside transporters. This study also included a kinetic and pharmacological characterization of nucleoside transport in rabbit cortical synaptosomes. The second phase investigated the interaction of R75231 (and the recently synthesized [^{3}H]R75231) with the nucleoside transporters of rabbit cortical synaptosomes and human erythrocytes. R75231 binds to nucleoside transporters in a unique manner, potentially making it useful both clinically, and as a probe for the nucleoside transporter. The third, and final, phase used a human neuroblastoma cell line to examine changes in nucleoside transport characteristics as a result of cellular differentiation. The results were used to examine the suitability of this cell line as a model to study the mechanisms of nucleoside transporter regulation. In addition, the results may aid in the selection and development of treatment strategies involving cytotoxic nucleoside analogues.

CHAPTER TWO: CHARACTERIZATION OF [^{3}H]DPR BINDING AND NUCLEOSIDE TRANSPORT IN MAMMALIAN CORTICAL SYNAPTOSOMES

2.1 Introduction

Adenosine is an important neuromodulator in the CNS with sedative, antianxiety, anticonvulsant, and antidepressant activities (see section 1.1.4). Inhibition of nucleoside transport can result in increased extracellular adenosine concentrations and, consequently, the potentiation of adenosine receptor mediated events (Wu and Phillis, 1984; Deckert et al., 1988). As a result, there is a growing interest in the use of nucleoside transport inhibitors, including propentofylline, dilazep, and soluflazine, as therapeutic agents in the CNS (see section 1.2.6).

NBMPR (see section 1.2.3.1.2) and DPR (see section 1.2.3.1.3) are both potent inhibitors of facilitated nucleoside transport and have been used as radioligand probes ([^{3}H]NBMPR and [^{3}H]DPR) for nucleoside transport. [^{3}H]DPR and [^{3}H]NBMPR bind to the external side of the *es* nucleoside transporter (Plagemann et al., 1988), and both can be used to photolabel a protein (molecular weight 50-60 kD) which appears to be involved in nucleoside transport (Woffendin and Plagemann, 1987a; Plagemann et al., 1988; Cass, 1994). However, the interactions of DPR and NBMPR with nucleoside transporters are different. The affinity of DPR for nucleoside transporters is species dependent (see section 1.2.3.1.3), whereas that of NBMPR is approximately the same, regardless of species (Plagemann et al., 1988). In addition, *es* and *ei* transporters, which differ >1000-fold in affinity for NBMPR, are inhibited about equally by DPR in many systems (Deckert et al., 1988; Plagemann et al., 1988). In some cell lines, DPR inhibition of [^{3}H]NBMPR binding is not strictly competitive (Plagemann and Wohlhueter, 1985). As well, DPR

has been shown to slow the rate of [^{3}H]NBMPR dissociation from the nucleoside transporter (Jarvis et al., 1983; Wohlhueter et al., 1983; Hammond and Clanachan, 1985; Shi and Young, 1986; Hammond, 1991). Therefore, it appears that although the binding sites for NBMPR and DPR probably overlap (Plagemann et al., 1988), they are not identical.

NBMPR-sensitive and -resistant, as well as DPR-sensitive and -resistant, facilitated nucleoside transport has been identified in the CNS (see section 1.2.4). Because [^{3}H]NBMPR does not label all facilitated nucleoside transporter subtypes, its usefulness as a probe for the nucleoside transport system has been questioned (see section 1.2.3.1.3). DPR does not distinguish between *es* and *ei* transporters of many systems (Plagemann and Wohlheuter, 1984; Deckert et al., 1988; Plagemann et al., 1988); therefore, it was hoped that [^{3}H]DPR would prove to be a more generally applicable probe for the nucleoside transporter (Marangos et al., 1985). Subsequent studies have shown that [^{3}H]DPR labels two- to ten-fold as many sites as [^{3}H]NBMPR in guinea pig brain, human erythrocytes, HeLa cells, and human ependymal tissue, with a portion of these sites being resistant to inhibition by NBMPR (Bisserbe et al., 1986; Marangos and Deckert, 1987; Woffendin and Plagemann, 1987a; Jones-Humble and Morgan, 1994). However, it has never been demonstrated definitively, that the NBMPR-resistant [^{3}H]DPR binding site is associated with the *ei* nucleoside transporter (see section 1.2.3.1.3).

The present study was conducted to better characterize the two [^{3}H]DPR binding components (NBMPR-sensitive and -resistant) in mammalian CNS. Two species, with different proportions of NBMPR-sensitive and -resistant [^{3}H]DPR binding, were used. The NBMPR-sensitive [^{3}H]DPR binding site was examined in rabbit cerebral cortical membranes due to the low proportion of NBMPR-resistant [^{3}H]DPR binding sites. These studies were correlated with [^{3}H]NBMPR binding studies to test the hypothesis that both radioligands were binding to the *es* nucleoside transporter. Guinea pig cerebral cortical

membranes were used to examine the effect of transport inhibitors and substrates on NBMPR-resistant [^{3}H]DPR binding, due to the relatively high proportion of this site in guinea pig. If the NBMPR-resistant [^{3}H]DPR binding was associated with the *ei* transporter, then it was expected that inhibitors of *ei*-mediated transport would inhibit the NBMPR-resistant [^{3}H]DPR binding at similar concentrations. This type of correlation has been shown for [^{3}H]NBMPR binding to the *es* transporter (Plagemann et al., 1988). In addition, if the NBMPR-resistant [^{3}H]DPR binding was associated with the *ei* transporter, then it was expected that a species with a high proportion of NBMPR-resistant [^{3}H]DPR binding (i.e. guinea pig) would have a greater proportion of NBMPR-resistant transport than a species with a low proportion of NBMPR-resistant [^{3}H]DPR binding (i.e. rabbit). Therefore, [^{3}H]uridine transport studies, examining the functional activities of both the *es* and *ei* transporters, were conducted using synaptosomes prepared from either rabbit or guinea pig cortex. These results were compared with the radioligand binding studies.

Finally, a substrate which is subtype selective (for either the *es* or *ei* nucleoside transporter) would be extremely useful for selectively examining transporter subtypes. In Ehrlich ascites-tumour cells, there have been reports of significant differences in substrate selectivity, with guanosine, 2′-deoxyguanosine, cytidine, and 2′-deoxycytidine, having a higher affinity for the *es* transporter (Hammond, 1991, 1992). However, studies with P388 mouse leukaemia cells and rat cerebral cortical synaptosomes, showed that cytidine and guanosine did not distinguish between *es* and *ei* nucleoside transporters (Plagemann and Wohlhueter, 1984; Lee and Jarvis, 1988). This indicates that the characteristics of transporter subtypes may be cell line/species specific. Therefore, a pharmacological profile of the inhibitory effects of various compounds (inhibitors and substrates) on *es* and *ei* nucleoside transporters was conducted in rabbit cortical synaptosomes.

2.1.1 Hypotheses

The NBMPR-sensitive [^{3}H]DPR binding site is associated with the *es* nucleoside transporter.

The NBMPR-resistant [^{3}H]DPR binding site is associated with the *ei* nucleoside transporter.

The *es* and *ei* nucleoside transporters in rabbit cortical synaptosomes differ in their selectivity for compounds besides NBMPR.

2.2 Methods

2.2.1 Materials

[^{3}H]NBMPR (30 Ci/mmol) and [^{3}H]DPR (40 Ci/mmol) were from Moravek Biochemicals, Inc. (Brea, CA). [^{3}H]Uridine (35-50 Ci/mmol) was purchased from ICN Biochemicals (Costa Mesa, CA). Dilazep was generously provided by Asta Werke (Frankfurt, Germany). Mioflazine, soluflazine, and R75231 were gifts from Dr. H. VanBelle, Janssen Research Foundation (Beerse, Belgium). Diazepam (Hoffmann La Roche, Toronto, Canada) and ω-conotoxin (Research Biochemicals Inc., Natick, MA) were obtained from Drs. J.T. Hamilton and R.J. Rylett respectively, University of Western Ontario. 8-Cyclopentyl-1,3-dipropylxanthine (Research Biochemicals Inc., Natick, MA) and nifedipine (Sigma, St. Louis, MO) were obtained from Dr. M.A. Cook, University of Western Ontario. Adenine, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), DPR, NBMPR, nitrobenzylthioguanosine (NBTGR) and uridine were supplied by Sigma (St. Louis, MO). All other compounds were of reagent grade.

2.2.2 Synaptosome Preparation

When neural tissue is homogenized, the cell bodies are sheared from their processes which break up into discrete fragments. The plasma membranes of the cell fragments can reseal to form osmotically active vesicles containing the organelles of the synapse. These vesicles, called synaptosomes, are useful for examining physiological and

pharmacological aspects of synaptic function. Subcellular fractions can be obtained, through centrifugation and density gradients, that have an increased concentration of synaptosomes (Gordon-Weeks, 1987). After the crude synaptosomal fraction (P_2) has been isolated, the synaptosomes can be concentrated using density gradients. The fractions obtained from this process are referred to as myelin (a myelin enriched fraction), P_1 (a synaptosome enriched fraction), and mitochondria (a mitochondria enriched fraction).

Rabbits (New Zealand White, male and female, 1-3 kg) and guinea pigs (albino, male, 250-300 g) were killed by decapitation, or sodium pentabarbitol injection (50 mg/kg, some rabbits). The brains were removed, and the cortices were dissected on ice. There was no significant difference in [^{3}H]NBMPR binding characteristics between rabbits injected with sodium pentabarbitol (K_D=0.26±0.04 nM, B_{max}=115±9 fmol/mg, n=3) and those killed by decapitation (K_D=0.30±0.01, B_{max}=130±30, n=5). Synaptosomes were prepared (at 4°C) by a method described by Gray and Whittaker (1962), as modified by White (1975). The cerebral cortex was minced and homogenized in approximately 10 volumes of ice cold sucrose solution (0.32 M sucrose, 0.1 mM EDTA, 5 mM Hepes, pH 7.4). The homogenate was centrifuged at 1000 g for 10 min. The pellet was washed once and the pooled supernates were centrifuged at 13 000 g for 20 min. For binding assays, the pellet (P_2) was resuspended in sucrose solution and kept at 4°C for use within 24 h, or was frozen at -70°C for use within four weeks. There was no significant difference in [^{3}H]NBMPR binding characteristics between rabbit synaptosomes which had been frozen and then thawed (K_D=0.27±0.01 nM, B_{max}=98±5 fmol/mg, n=5) and those used the same day (K_D=0.30±0.01, B_{max}=130±30, n=5). For the [^{3}H]uridine uptake studies, the P_2 fraction was further purified on discontinuous sucrose gradients (0.8 M and 1.2 M sucrose with 0.1 mM EDTA, 5 mM Hepes, pH 7.4) (80 000 g, 30 min). The 0.8/1.2 M interface was collected and diluted slowly with an equal volume of incubation medium containing

130 mM NaCl, 5 mM KCl, 1 mM $MgSo_4$, 1 mM $CaCl_2$, and 4 mM HEPES, pH 7.4. This dilution was centrifuged (11 000 g, 10 min) and the pellet (P_4) was gently resuspended in incubation medium. This suspension was kept on ice for 2 hours before use in uptake studies.

The activity of the enzyme lactate dehydrogenase (LDH) was assessed in the synaptosomal fractions. This cytoplasmic enzyme, when membrane bound in vesicles, serves as a marker for intact synaptosomes (Johnson and Whittaker, 1963). Non-vesicular LDH was determined by adding a 10 µl aliquot of tissue sample to a semi-micro cuvette containing 0.067 mg/ml NADH, 0.038 mg/ml sodium pyruvate in 50 mM Tris-HCL (pH 7.4). LDH activity was assessed by measuring the changes in absorbance (340 nm) every 10 s for a 2 min period. Total LDH was determined in the same manner but the vesicles in the tissue samples were lysed with 1% Triton X-100 (final concentration). Intravesicular LDH (representing intact synaptosomes) was calculated as: total LDH - non-vesicular LDH.

2.2.3 Radioligand binding

All [^{3}H]NBMPR and [^{3}H]DPR binding studies were conducted at room temperature (≈22°C), using the P_2 fraction resuspended in 50 mM Tris (pH 7.1). In experiments with [^{3}H]DPR, this buffer was supplemented with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) to a final concentration of 0.01% (w/v), unless otherwise indicated. Incubations (final volume of 1 ml) were initiated by the addition of a 50 µl aliquot of the P_2 fraction (0.4-0.6 mg protein) to a glass tube containing the appropriate concentration of radioligand (± inhibitors). In all equilibrium binding experiments, incubations were terminated after 45 min by the addition of 4-5 ml of ice-cold 10 mM Tris (pH 7.1), followed by rapid filtration through a Whatman GF/B filter. Filters were washed once with 5 ml ([^{3}H]NBMPR binding), or 4 times with 4 ml ([^{3}H]DPR binding), of ice-cold

10 mM Tris (pH 7.1). The washes with ice-cold 10 mM Tris did not affect the specific [^{3}H]DPR binding (that which was inhibited by 10 μM dilazep), but reduced the remaining background [^{3}H]DPR binding by ≈4-fold. The radioactivity on the filters was counted by liquid scintillation spectrophotometry. For [^{3}H]NBMPR assays, non-specific binding was determined in the presence of 10 μM nitrobenzylthioguanosine (NBTGR) or 10 μM dilazep (both yielded similar results; see Figure 2.9). [^{3}H]DPR binding assays were conducted in the presence of 1 μM NBMPR (or 10 μM dilazep in rabbit, both yielded similar results; see Figure 2.5) to determine NBMPR-sensitive binding. Non-specific [^{3}H]DPR binding was determined in the presence of 10 μM DPR. NBMPR-resistant [^{3}H]DPR binding was defined as that which could be inhibited by 10 μM DPR, but not 1 μM NBMPR.

2.2.4 [^{3}H]Uridine Uptake

All assays were conducted as described by Lee and Jarvis (1988a) in incubation medium at room temperature (≈22°C). The P_4 fraction was used because it contained the greatest concentration of intact synaptosomes (Table 2.5). Briefly, a 10 μl portion of synaptosomes (approx. 200 μg of protein) and 20 μl of [^{3}H]uridine (final concentration 10 μM, 10 μCi/ml) were added separately to opposite sides of a plastic tube and uptake was initiated by vortex mixing. Synaptosomes were preincubated with inhibitors for 30 s prior to initiation of uptake, and non-mediated uptake was determined using synaptosomes preincubated with 100 μM dilazep. The uptake process was terminated by the addition of 1 ml of stop solution (ice cold incubation buffer with 25 μM DPR and 25 μM NBTGR). The suspension was immediately filtered through a Whatman GF/B filter and washed twice with 5 ml aliquots of ice cold stop solution. The radioactivity on the filters was counted by liquid scintillation spectrophotometry. Blank values for non-specific [^{3}H]uridine binding to the filters, found by conducting the assay in the absence of synaptosomes,

were subtracted from the uridine uptake measurements.

The potential contribution of ion-dependent nucleoside transporters to the accumulation of [^{3}H]uridine by synaptosomes was assessed. The synaptosomes were prepared and uptake of 10 μM [^{3}H]uridine was measured as described above, with the following changes. The 0.8/1.2 M interface was diluted slowly with either the usual incubation media (130 mM NaCl), or "sodium-free" incubation media with isosmotic replacement of sodium chloride by lithium chloride. These dilutions were kept for 30 min on ice to allow the intrasynaptosomal Na^+ concentration to equilibrate with the Na^+ concentration of the media. The dilutions were then centrifuged (11 000 g, 10 min) and the pellets (P_v) were gently resuspended in the appropriate incubation medium ($\pm Na^+$). Nucleoside uptake was measured using synaptosomes ($\pm Na^+$) and [^{3}H]uridine ($\pm Na^+$), such that the desired Na^+ gradient was produced.

Volume estimates for synaptosomes were found by incubating an aliquot of synaptosomes with a combination of [^{14}C]dextran-carboxyl (cell-impermeant) and ^3H_2O layered over a 200 μl cushion of silicone oil/mineral oil (21:4, v/v) in microcentrifuge tubes. At the end of a 3 min incubation, the synaptosomes were pelleted through the oil (60 sec, 12 000 g). A portion of the supernatant was removed for scintillation counting and the remaining supernatant and oil were removed. The pellets were digested in 1 M NaOH, and the radioactivity of the pellet digest was then counted by liquid scintillation spectrophotometry. The intracellular water space (V_i) was determined as:

$$V_i = \frac{\text{Total } ^3\text{H dpm in pellet}}{^3\text{H dpm}/\mu\text{l in supernatant}} - \frac{\text{Total } ^{14}\text{C dpm in pellet}}{^{14}\text{C dpm}/\mu\text{l in supernatant}}$$

2.2.5 Uridine Metabolism

Uridine metabolism was measured using a methodology

similar to that described by Lee and Jarvis (1988a). Briefly, synaptosomes were incubated for 60 s with 10 μM [^{3}H]uridine at room temperature. The incubation was terminated with 1 ml of ice cold stop solution and the synaptosomes were centrifuged at 12,000 g for 30 sec. The supernate was removed and 50 μl of ice cold 7% $HClO_4$ was added to extract the uridine metabolites. The precipitate was removed by centrifugation (12 000 g, 60 s), and an aliquot of the acid extract was neutralized with $NaHCO_3$ and centrifuged (12 000 g, 60 s) to remove further precipitate. The supernates were chromatographed on silica-gel-coated plates impregnated with fluorescent indicator, using butan-1-ol/acetone/acetic acid/ammonium hydroxide/water (7:5:3:3:2 by volume). Standards (uridine, uracil, uridine monophosphate, uridine diphosphate, uridine triphosphate), co-chromatographed with the samples, were localized under ultra-violet light and the corresponding regions on the sample lanes were isolated. Radioactivity was extracted from the plates with water (1 ml) for 1 h before the addition of scintillation fluid (Ready Protein, Beckman Canada).

2.2.6 Data Analysis

Protein concentrations were determined as described by Lowry et al. (1951) using bovine serum albumin as the standard. Dimethyl sulfoxide, used as a solvent in some cases, did not significantly affect binding or uptake at the concentrations used (≤ 0.1% and 0.33% for binding and uptake, respectively). [^{3}H]NBMPR saturation binding assays were analyzed by the curve fitting program LIGAND (Munson and Rodbard, 1980) to determine the probability of a single-site or two-site binding model (F-test). For radioligand association, the observed rate constant (k_{ob}) was found using the relationship $\ln(B_e/B_e-B)=k_{ob}t$, where B_e was bound radioligand at equilibrium, and B was bound radioligand at time t. The association rate constant (k_{+1}) was then calculated using the relationship $k_{+1}=(k_{ob}-k_{-1})/[L]$, where k_{-1} was the dissociation

rate constant, and [L] was the concentration of radioligand. All K_i values, except those obtained from double reciprocal plot analysis were found using the relationship $K_i=IC_{50}/(1+[L]/K_D)$, where IC_{50} was interpolated from best fit sigmoid curves of percentage inhibition versus log inhibitor concentration, [L] was the concentration of radioligand, and K_D was the equilibrium dissociation constant for the radioligand used. Other values reported were derived from computer-generated algebraic relationships obtained via nonlinear regression analysis (GraphPAD Inplot) of non-transformed experimental data.

The statistical analysis of data was completed as follows.

Unpaired Data:

For a single comparison, a Student's t-test was used. For multiple comparisons, a one-way ANOVA was used followed by Bonferroni t-tests of selected data if significant differences were indicated by the ANOVA.

Paired Data:

For a single comparison, a paired Student's t-test was used. For a multiple comparisons, a repeated measures ANOVA was used followed by Student-Newman-Keuls analysis of selected data if significant differences were indicated by the ANOVA.

Lines of Best Fit:

When the lines of best fit were determined for linear versus hyperbolic and one component versus two component hyperbolic fits. An F-test was completed based on the differences of the sum of squares and degrees of freedom for the two possible curves fits. From the corresponding P value, the line which best fit the data was determined.

In all cases, significance was considered at $P<0.05$ unless otherwise noted.

2.3 Results

2.3.1 [^{3}H]DPR Binding

2.3.1.1 Non-Specific

[^{3}H]DPR was observed to bind to glass, including the glass fibre filters used in the binding assays, and could be displaced from these filters by the structurally dissimilar nucleoside transport inhibitor dilazep (Figure 2.1). The inclusion of 0.01% CHAPS in the assay medium, along with presoaking the filters in 0.01% CHAPS, reduced the filter associated [^{3}H]DPR by ≈15-fold, and eliminated the dilazep displaceable, [^{3}H]DPR filter binding (Figure 2.1). The final assay concentrations of [^{3}H]DPR were determined by taking aliquots from incubation mixtures immediately prior to filtration. In the presence of 0.01% CHAPS, the final [^{3}H]DPR concentration was significantly higher (Student's paired t-test, $P<0.05$) in the assay medium, than that found in the absence of 0.01% CHAPS (0.54±0.06 and 0.49±0.07 nM, respectively; n=5). This was likely due to CHAPS decreasing the binding of [^{3}H]DPR to the glass culture tubes used for the incubations. The presence of 0.01% CHAPS was not high enough to solubilize the membranes and, in a previous study, a 25-fold higher concentration of CHAPS did not affect [^{3}H]NBMPR binding to the nucleoside transporter (Agbanyo et al., 1990). Consequently, all subsequent [^{3}H]DPR binding assays were conducted in the presence of 0.01% CHAPS with prior incubation of the filters in 0.01% CHAPS.

2.3.1.2 Kinetic Analysis

The reversibility of NBMPR-sensitive [^{3}H]DPR binding to rabbit cortical membranes, which possessed primarily NBMPR-sensitive [^{3}H]DPR binding (≈90%; Table 2.1, Figure 2.4), was examined. As shown in Figure 2.2, equilibrium for NBMPR-sensitive [^{3}H]DPR binding was achieved by 20 min and dissociation, after addition of NBTGR (1 μM final, approximately 1000 x K_i), was complete within 15 min. In order to obtain an accurate dissociation constant for [^{3}H]DPR bound to the NBMPR-sensitive site, it was desirable to prevent reassociation of [^{3}H]DPR with a compound which bound competitively with [^{3}H]DPR. Compounds which bind to the

[^{3}H]DPR site in a non-competitive manner could alter the observed dissociation rate. In rabbit cortical synaptosomes, NBMPR appears to be a competitive inhibitor of NBMPR-sensitive [^{3}H]DPR binding (see section 2.4.1). Therefore, NBTGR, whose binding characteristics are virtually identical to NBMPR in a variety of systems (Plagemann et al., 1988; Hammond, 1991) was used to prevent [^{3}H]DPR reassociation. From these experiments (using 1 μM NBTGR to prevent reassociation), dissociation rate (k_{-1}=0.24±0.03 min^{-1}) and association rate (k_{+1}=0.28±0.06 min^{-1}nM^{-1}) constants were found, as described in section 2.2.6. This allowed the calculation of a K_D of 1.1±0.5 nM.

The dissociation rate was dependent on the inhibitor used to prevent reassociation of [^{3}H]DPR. When dilazep or R75231 (0.1 μM or 1 μM final concentration, respectively; approximately 1000 x K_i) were used instead of NBTGR to prevent the reassociation of [^{3}H]DPR (Figure 2.3), significantly slower dissociation rates were found; 0.189±0.009 min^{-1} and 0.18±0.01 min^{-1} for dilazep and R75231 respectively (Student-Newman-Keuls, $P<0.05$).

2.3.1.3 Saturation Analysis

The results of [^{3}H]DPR saturation binding experiments are summarized in Table 2.1. Total specific [^{3}H]DPR binding was defined as [^{3}H]DPR binding which was inhibited by 10 μM DPR. NBMPR-sensitive [^{3}H]DPR binding was defined as that which was inhibited by 1 μM NBMPR. NBMPR-resistant [^{3}H]DPR binding was defined as that which could be inhibited by 10 μM DPR, but not by 1 μM NBMPR (i.e. total specific binding - NBMPR-sensitive binding). Both NBMPR-sensitive and -resistant [^{3}H]DPR binding were observed in rabbit and guinea pig membranes.

RABBIT: In rabbit, mass law analysis of NBMPR-sensitive [^{3}H]DPR binding gave rise to linear Scatchard plots (Figure 2.4, Table 2.1), with a B_{max} value of 150 fmol/mg and Hill coefficients not significantly different from unity (n_H = 0.990±0.003, Student's t-test). The K_D value derived by mass

law analysis (1.4 ± 0.2 nM) was not significantly different from the K_D value found by kinetic analysis (1.1±0.5 nM; see section 2.3.1.2, Student's t-test). The total specific (i.e. DPR-sensitive) binding (Figure 2.4) also satisfied a one-site model better than a two-site model ($P<0.01$), even in preliminary experiments using [^{3}H]DPR concentrations up to 25 nM. Analysis of the total specific [^{3}H]DPR binding yielded a K_D = 1.4 ± 0.3 nM and B_{max} = 170 ± 10 fmol/mg. Approximately 20 fmol/mg of this total specific [^{3}H]DPR binding represents NBMPR-resistant [^{3}H]DPR binding (total [^{3}H]DPR binding B_{max} - NBMPR-sensitive [^{3}H]DPR binding B_{max}).

GUINEA PIG: In contrast to rabbit, mass law analysis of the total specific [^{3}H]DPR binding component (i.e. DPR-sensitive) in guinea pig membranes resulted in curvilinear Scatchard plots (Figure 2.4), which could be split experimentally into two linear components; one was inhibited by 1 μM NBMPR (NBMPR-sensitive) and the other inhibited by 10 μM DPR but not 1 μM NBMPR (NBMPR-resistant). Corresponding K_D and B_{max} values are shown in Table 2.1. The total specific [^{3}H]DPR binding component (i.e. DPR-sensitive) could also be split mathematically by fitting the data to a double-rectangular hyperbola (a two-site model gave a significantly better fit than a one-site model; $P<0.01$; Figure 2.4). This computer-generated curve split resulted in estimated K_D and B_{max} values similar to those for the experimentally derived NBMPR-sensitive and -resistant components (Table 2.1).

2.3.1.4 Inhibition Analysis

Figure 2.5 shows the profiles for NBMPR, DPR, dilazep, and adenosine inhibition of [^{3}H]DPR binding to both rabbit and guinea pig membranes. The final [^{3}H]DPR concentrations were 0.5 nM and 1 nM for rabbit and guinea pig, respectively. As predicted from the saturation binding analysis (section 2.3.1.3), approximately 40% of the binding of [^{3}H]DPR was relatively resistant to inhibition by NBMPR in guinea pig membranes. A lower percentage (≈10%) was resistant to NBMPR

in rabbit, again as predicted from the saturation binding analysis. Therefore, rabbit membranes were used for the pharmacological characterization of the NBMPR-sensitive [^{3}H]DPR binding site (Figures 2.5, 2.6; Table 2.2). For all inhibitors tested inhibition was dose-dependent and, with the exception of dilazep, monophasic. For dilazep, which showed a biphasic inhibition profile, only the high-affinity component (90% of total) of the inhibition curve was considered when calculating the IC_{50} value for dilazep inhibition of NBMPR-sensitive [^{3}H]DPR binding. Dilazep was the most potent inhibitor of NBMPR-sensitive [^{3}H]DPR binding, followed by NBMPR, R75231, NBTGR, diazepam, adenosine, and uridine. K_i values were calculated as described in Methods using the K_D for NBMPR-sensitive [^{3}H]DPR binding. Only R75231 had a pseudo-Hill coefficient significantly different from unity (n_H>1, Student's t-test).

Guinea pig membranes were used for pharmacological characterization of the NBMPR-resistant component of [^{3}H]DPR binding (Table 2.3). These assays used 1 μM NBMPR to block [^{3}H]DPR binding to the NBMPR-sensitive site. Even very high concentrations of the potent nucleoside transport inhibitors mioflazine (10 μM final) and dilazep (100 μM), and the nucleoside transporter substrates adenosine and uridine (10 mM final), did not inhibit more than 45% of the NBMPR-resistant/DPR-sensitive [^{3}H]DPR binding. Since DPR has been reported to interfere with transporters and enzymes other than the nucleoside transporter (Gerlach et al., 1964; Plagemann and Richey, 1974; Asano et al., 1977; Deckert et al., 1988; Plagemann et al., 1987, 1988), inhibitors/substrates of Ca^+ channels, adenosine receptors, phosphodiesterase, and glucose transporters were tested for their effects on the NBMPR-resistant [^{3}H]DPR binding component; none inhibited the binding by more than 45% (Table 2.3). The only potent inhibitor of NBMPR-resistant [^{3}H]DPR binding was non-radiolabelled DPR (Figure 2.7), which had a IC_{50} value of 9±2 nM and pseudo-Hill coefficient of 0.34±0.03.

2.3.2 [^{3}H]NBMPR Binding to Synaptosomal Membranes

Results presented in the previous sections suggest that the NBMPR-sensitive [^{3}H]DPR binding sites may be associated with the *es* nucleoside transporter. Since the *es* transporter can also be labelled by [^{3}H]NBMPR (see section 1.2.3.1.2), [^{3}H]NBMPR binding studies were conducted for comparison with the [^{3}H]DPR binding studies. Mass law analysis of the specific binding of [^{3}H]NBMPR to rabbit and guinea pig membranes (Figure 2.8) indicated a single class of binding sites for both species with Hill coefficients not different from unity (Table 2.4). Guinea pig membranes had a higher affinity and a larger number of binding sites for [^{3}H]NBMPR than did rabbit membranes (Table 2.4).

Inhibition of specific [^{3}H]NBMPR binding to rabbit membranes was observed to be dose-dependent and monophasic in nature for all inhibitors tested (Figure 2.9, Table 2.2). The order of inhibitor potency was the same as that observed for inhibition of NBMPR-sensitive [^{3}H]DPR binding to rabbit cortical membranes (dilazep > R75231 > NBTGR > DPR), and both dilazep and R75231 had pseudo-Hill coefficients significantly greater than unity. Double reciprocal plot analysis showed DPR to be a competitive inhibitor of [^{3}H]NBMPR binding to rabbit membranes, in both the presence (Figure 2.10) and absence of 0.01% CHAPS. Likewise, the presence of 0.01% CHAPS had no significant effect on the B_{max} and K_D values obtained for [^{3}H]NBMPR binding (K_D values of 0.271±0.005 and 0.291±0.009 nM, B_{max} values of 101±7 and 96±7 fmol/mg protein found in the absence and presence of 0.01% CHAPS respectively, ANOVA). However, the K_i value obtained for DPR inhibition of [^{3}H]NBMPR binding in the presence of CHAPS was significantly lower than that found in its absence (K_i=0.52±0.02 nM and 0.78±0.07 nM, respectively). This may have been due to reduced binding of DPR to non-specific sites resulting in higher "free" concentrations in the presence of 0.01% CHAPS (see section 2.3.1.1).

Two specific high-affinity binding sites for [^{3}H]NBMPR

(DPR-sensitive and -resistant) were characterized previously in rabbit cortical membranes (Hammond and Clanachan, 1985). However, in the present study, only a single specific binding component for [^{3}H]NBMPR was observed (Figures 2.8, 2.9). Since Dutch rabbits were used in the previous study and New Zealand White rabbits were used in this study, the disparity may have been due to differences in animal strain. However, when experiments were repeated for the present study (Figure 2.11), both strains of rabbit had similar DPR inhibition profiles for [^{3}H]NBMPR binding. As well, no evidence of DPR-sensitive and -resistant [^{3}H]NBMPR binding (biphasic inhibition profile) sites was found in either strain. The reason for the discrepancy between this study and the previous study by Hammond and Clanachan (1985) has yet to be resolved but may be due to uncontrolled factors such as different feeding condition or animal age.

2.3.3 [^{3}H]Uridine Uptake

Examination of the functional activity of nucleoside transporters, through substrate influx studies, requires intact vesicles such as synaptosomes. Therefore, the presence of intact synaptosomes, as isolated from rabbit cerebral cortex (see section 2.2.2), was verified by examining the activity of occluded LDH in the various fractions (Table 2.5). This cytoplasmic enzyme serves as a marker for synaptosomes (Johnson and Whittaker, 1963). As shown in Table 2.5, the P_v fraction had the greatest concentration of intact synaptosomes. Therefore, the P_v fraction was used for the nucleoside uptake studies.

This study involved comparing the results using the P_2 fraction (binding studies) with those using the P_v fraction (uptake studies). To make these comparisons valid, the characteristics of the nucleoside transporters in the two fractions should be similar. Therefore, the binding characteristics of both fractions were compared (Table 2.6). [^{3}H]DPR binding constants (K_D and B_{max}) were not significantly

different between the fractions (ANOVA); therefore, comparisons between the binding studies and the uptake studies were made. [^{3}H]NBMPR binding (K_D and B_{max} values) in guinea pig cortex has also been shown to be similar for the P_2 and P_v synaptosomal fractions (Hammond and Clanachan, 1984).

Uridine uptake was examined using a stop solution (ice-cold incubation buffer containing 25 μM DPR and 25 μM NBTGR) to terminate all incubations. Since this method is only valid if the stop solution terminates influx immediately and prevents subsequent efflux of the substrate from the synaptosomes, the effectiveness of the stop solution was examined. Rabbit cortical synaptosomes were incubated in 10 μM [^{3}H]uridine for 30 s, and then 1 ml of either room temperature buffer or ice cold stop solution was added to the incubation tube. The contents of the tube were filtered immediately, or after a known time interval, to examine the amount of entrapped uridine that remained (Figure 2.12). There was a large (>50% of total) and immediate uridine efflux with the room temperature buffer; however, there was no significant efflux with the stop solution even after 90 s (Student's t-test). Therefore, the stop solution was used in subsequent assays to terminate [^{3}H]uridine uptake.

The metabolism of [^{3}H]uridine by rabbit cortical synaptosomes was also examined to ensure that it was not interfering with the measurement of nucleoside transport (see section 1.2.2). After a 60 s incubation, 98% of the intracellular radioactivity co-chromatographed with uridine, indicating minimal metabolism of uridine by the synaptosomes. Similar results, showing the effectiveness of ice cold stop solution and the lack of nucleoside metabolism have been observed in both guinea pig and rat cortical synaptosomes (Lee and Jarvis, 1988*a*,*b*).

2.3.3.1 Uridine Uptake in Rabbit Synaptosomes

2.3.3.1.1 Kinetic Characterization

To establish the contribution of the NBMPR-resistant

transport system to the cellular accumulation of [^{3}H]uridine, synaptosomes were incubated with a range of concentration of NBMPR, and then exposed to 10 μM [^{3}H]uridine for 30 s. The resulting biphasic inhibition profile is shown in Figure 2.16. The NBMPR-resistant component comprised 37±4% of the total transporter-mediated uptake. The IC_{50} values calculated from the biphasic inhibition curves were 2.5±1.1 nM and 76±26 μM, for the NBMPR-sensitive and -resistant components, respectively (the IC_{50} for the NBMPR-resistant component is extrapolated from data shown in Figure 2.16). From this inhibition profile, it was concluded that 330 nM to 1 μM NBMPR blocked uridine uptake via the NBMPR-sensitive system without affecting uptake via the NBMPR-resistant system; this range is ≈ 100-fold greater than, and 100-fold less than, the IC_{50} values for NBMPR-sensitive and -resistant [^{3}H]uridine uptake (10 μM), respectively. Therefore, each system could be studied selectively. This range of NBMPR concentrations has been used in previous studies to selectively inhibit NBMPR-sensitive transport in rat and guinea pig synaptosomes (Lee and Jarvis, 1988*a*,*b*).

The time course of [^{3}H]uridine uptake (10 μM) by rabbit cortical synaptosomes is shown in Figure 2.13. Total transporter-mediated uptake was defined as the difference between total uptake and that in the presence of 100 μM dilazep (non-mediated uptake). NBMPR-resistant uptake was defined as mediated uptake that was not inhibited by 1 μM NBMPR. Finally, NBMPR-sensitive uptake was defined as the difference between total uptake and NBMPR-resistant uptake. The initial rate of total [^{3}H]uridine (10 μM) uptake was 0.63±0.07 pmol/s per mg protein. This rate was decreased in the presence of 1 μM NBMPR, by about half, to 0.31±0.08 pmol/s per mg protein. The initial rate of non-mediated uptake was 0.14±0.04 pmol/s per mg protein. Total 10 μM [^{3}H]uridine uptake reached an equilibrium of 17.2 pmol/mg (as predicted by computer generated hyperbolic fit to data). Using an intra-synaptosomal water space estimate of 7.0±0.5 μl (n=7), found

as described in section 2.2.4, the equilibrium [^{3}H]uridine concentration in the synaptosomes was approximately 2.5 μM. This value is lower than the expected 10 μM at equilibrium, but is similar to that observed previously for [^{3}H]uridine uptake (100 μM final) in guinea pig synaptosomes where the equilibrium concentration was 15-25 μM (Lee and Jarvis, 1988*b*). This might have been due to the presence of compartments within the synaptosome, such as synaptic vesicles, which may not be accessible to [^{3}H]uridine.

Time courses of [^{3}H]uridine uptake, similar to those shown in Figure 2.13, were constructed using a range of [^{3}H]uridine concentrations (1-500 μM). In each case, the initial rate of [^{3}H]uridine influx was determined for the total uptake, the uptake in the presence of 1 μM NBMPR, and the uptake in the presence of 100 μM dilazep. The average initial rates of [^{3}H]uridine influx were plotted against the [^{3}H]uridine concentration to determine kinetic constants for both the NBMPR-sensitive and -resistant components (Figure 2.14). Non-mediated [^{3}H]uridine influx, found in the presence of 100 μM dilazep, increased linearly (rather than curvilinearly; F-test, $P<0.01$), with [^{3}H]uridine concentration (Figure 2.14). This is as expected if all mediated [^{3}H]uridine transport was inhibited by this concentration of dilazep. Total mediated [^{3}H]uridine transport, defined as the difference between total uptake and that in the presence of 100 μM dilazep, was saturable (K_m=172 μM, V_{max}=7 pmol/s per mg protein) and could be resolved into two components (Figure 2.14). The NBMPR-sensitive component, defined as the difference between the total uptake and that in the presence of 1 μM NBMPR (Figure 2.14), had a K_m of 320 μM and V_{max} of 4.9 pmol/s per mg protein. The NBMPR-resistant component, defined as the difference between the uptake in the presence of 1 μM NBMPR and that in the presence of 100 μM dilazep (Figure 2.14), had a K_m of 94 μM and B_{max} of 2.7 pmol/s per mg protein.

Experiments were conducted, as described in section 2.2.4, to test for the presence of Na^+-dependent transport in

the synaptosomes (Figure 2.15). Synaptosomes were equilibrated in medium which contained Na^+, or one in which Na^+ was replaced iso-osmotically with Li^+. Li^+ does not drive the Na^+-dependent nucleoside transporter (Williams et al., 1989; Plagemann and Aran, 1990). [^{3}H]Uridine (10 μM), in buffer ± Na^+, was then added to the synaptosomes such that a inward Na^+ gradient was produced. The initial rate of influx found in the presence of an inward Na^+ gradient (0.53±0.06 pmol/s per mg protein) was not higher than the initial rate found in the absence of a Na^+ gradient (0.6±0.1 pmol/s per mg protein). The initial rate of influx found with an inward Na^+ gradient in the presence of 330 nM NBMPR was also measured. NBMPR was included in the assay because inhibition of facilitated transport sometimes allows Na^+-dependent transport to be observed more clearly (Dagnino et al., 1991*a*,*b*; Vijayalakshmi et al., 1992). The initial rate of influx found with an inward Na^+ gradient in the presence of 330 nM NBMPR (0.3±0.1 pmol/s per mg protein) was not higher than that found in the absence of a Na^+ gradient (0.31±0.08 pmol/s per mg protein). Therefore, as in other studies with mammalian cortical synaptosomes (see section 1.2.4.2), no Na^+-dependent nucleoside transport was detected (Figure 2.15).

2.3.3.1.2 Pharmacological Characterization

A variety of nucleoside transport inhibitors and substrates were tested for their abilities to inhibit both the total transporter-mediated uptake, and the NBMPR-sensitive uptake of 10 μM [^{3}H]uridine by rabbit cortical synaptosomes. Dilazep, like NBMPR, had a biphasic inhibition profile which, when fit to a two-site competition model, resulted in IC_{50} values of 3.9±0.6 nM and 17±8 μM for the NBMPR-sensitive and -resistant components, respectively (Figure 2.16). The proportion of dilazep-resistant [^{3}H]uridine uptake was 39±5% of total mediated uptake which is similar to the percentage of NBMPR-resistant uptake (37±4%; Figure 2.16). None of the other inhibitors or substrates tested distinguished between

the two systems to a degree that resulted in biphasic curves. In these cases, selectivity was determined by comparing inhibition profiles in the absence and presence of 330 nM NBMPR, which blocked the NBMPR-sensitive uptake component (Figures 2.17 to 2.20). Of the compounds tested, dipyridamole, R75231, soluflazine, and mioflazine had significantly higher IC_{50} values for inhibition of NBMPR-resistant [^{3}H]uridine uptake when compared with inhibition of total transporter-mediated uptake (Table 2.8, Student's t-test). The other compounds, including the substrates adenosine, guanosine, and cytidine, showed no significant selectivity.

2.3.3.2 [^{3}H]Uridine Uptake by Guinea Pig Synaptosomes

The time course of 10 μM [^{3}H]uridine uptake by guinea pig cortical synaptosomes is shown in Figure 2.21. The initial rate of total [^{3}H]uridine uptake was 0.87±0.07 pmol/s per mg protein and the initial rate of non-mediated uptake was 0.11±0.03 pmol/s per mg protein. These values are similar to those observed in rabbit (0.63 and 0.14 pmol/mg for total and non-mediated uptake, respectively). Inhibition of transporter-mediated, 10 μM [^{3}H]uridine uptake by DPR was monophasic with an IC_{50} of 29±4 nM (Figure 2.22). Inhibition of transporter-mediated, [^{3}H]uridine uptake by NBMPR was biphasic (Figure 2.22) with approximately 25% of the total mediated uptake relatively resistant to inhibition by NBMPR (Figure 2.22). IC_{50} values calculated from the biphasic inhibition curves were 1.1±0.4 nM and 10±3 μM for the NBMPR-sensitive and -resistant components respectively (the IC_{50} for the NBMPR-resistant component was extrapolated from data shown in Figure 2.22).

2.4 Discussion

2.4.1 [^{3}H]DPR Binding

The use of [^{3}H]DPR as a specific probe for the nucleoside transport system has been hampered in past studies by its

tendency to bind to glass and plastic assay tubes and filters (Shi and Young, 1986; Marangos and Deckert, 1987). In the present study, it was found that the [^{3}H]DPR (0.5 nM) which was bound to the glass fibre filters was displaceable by dilazep, a structurally dissimilar nucleoside transport inhibitor, with an IC_{50} of ≈1 μM. Since the filter associated [^{3}H]DPR could be displaced with dilazep, at concentrations within the range used to study [^{3}H]DPR binding to the nucleoside transporter (up to 10 μM), and possibly by other nucleoside transport inhibitors, the filter associated [^{3}H]DPR could potentially be misinterpreted as binding to specific tissue located sites, such as nucleoside transporters. This problem was eliminated by including 0.01% CHAPS, a zwitterionic detergent, in the assay medium and presoaking the filters in 0.01% CHAPS. This methodological refinement also reduced the binding of [^{3}H]DPR to glass tubes, and allowed the assay concentrations of [^{3}H]DPR to be determined accurately. The reduction in non-specific binding of [^{3}H]DPR and the use of a wide range of [^{3}H]DPR concentrations enabled the resolution, directly from mass law analysis data, of the specific (DPR-sensitive) binding of [^{3}H]DPR into two components, which was not possible in a previous study on the binding of [^{3}H]DPR to guinea pig brain membranes (Marangos and Deckert, 1987).

One objective of the present study was to determine if the NBMPR-sensitive and -resistant [^{3}H]DPR binding sites in brain represented *es* and *ei* nucleoside transporters, respectively. Rabbits were chosen for the detailed pharmacological and kinetic examination of the NBMPR-sensitive [^{3}H]DPR binding site, due to the relative lack of NBMPR-resistant [^{3}H]DPR binding in this species. The NBMPR-sensitive [^{3}H]DPR binding and the [^{3}H]NBMPR binding results can be compared to test the hypothesis that, in rabbit, they were both binding to the same site on the *es* nucleoside transporter. These results showed that: 1/ there were a similar number of [^{3}H]NBMPR binding sites and NBMPR-sensitive

[^{3}H]DPR binding sites (130±30 and 150±20 fmol/mg, respectively); 2/ there were similar K_i (or IC_{50}) values for NBTGR, dilazep, and R75231 inhibition of both [^{3}H]NBMPR binding and NBMPR-sensitive [^{3}H]DPR binding (Table 2.2); 3/ there was a similarity between the K_i value for DPR inhibition of [^{3}H]NBMPR binding and the K_D value for NBMPR-sensitive [^{3}H]DPR binding (0.95±0.08 and 1.4±0.2 nM, respectively); and 4/ DPR was a competitive inhibitor of [^{3}H]NBMPR binding (Figure 2.10). These similarities in binding characteristics demonstrate that [^{3}H]NBMPR binding and NBMPR-sensitive [^{3}H]DPR binding is to the same site or, at least, to overlapping sites on the same membrane component. A similar conclusion was attained in studies on [^{3}H]DPR binding to guinea pig and human erythrocytes (Jarvis, 1986) and guinea pig lung membranes (Shi and Young, 1986).

Both R75231 and dilazep were extremely potent inhibitors of [^{3}H]NBMPR binding and NBMPR-sensitive [^{3}H]DPR binding in rabbit (K_i < 1 nM). These inhibitors were unique, amongst those tested, in that they had pseudo-Hill coefficients greater than 1. When used as displacing agents, they also resulted in dissociation rates, for NBMPR-sensitive [^{3}H]DPR binding, that were slower than that found using the reference compound NBTGR. These results, and those of others (see section 1.2.3.1.2), are not compatible with a simple, non-cooperative, binding site model but rather suggest a model involving multiple, interacting, binding sites. In this more complex model of ligand binding to the nucleoside transporter, the binding of a compound to one site (eg. R75231) could affect the binding of another compound to a second site (eg. [^{3}H]NBMPR). This model is examined in more detail in Chapter 3 using the radioligand [^{3}H]R75231.

It has been asserted that the NBMPR-resistant [^{3}H]DPR binding sites in guinea pig brain are associated with *ei* nucleoside transporters (Marangos and Deckert, 1987). The main evidence for this is the similar percentages of NBMPR-

resistant [^{3}H]DPR binding (Marangos and Deckert, 1987) and NBMPR-resistant nucleoside transport in guinea pig brain (Davies and Hambley, 1986), and the high potencies (low K_i values) of recognized nucleoside transport blockers as inhibitors of [^{3}H]DPR binding (Marangos and Deckert, 1987). However, these K_i values were determined for inhibition of DPR-sensitive [^{3}H]DPR binding, which includes both the NBMPR-sensitive and -resistant components. Therefore, these K_i values may not give a true indication of the affinity of these compounds for the NBMPR-resistant [^{3}H]DPR binding site. In addition, endogenous nucleosides were shown to be relatively poor inhibitors of [^{3}H]DPR binding (K_i values > 1 mM) (Marangos and Deckert, 1987). These K_i values for inhibition of [^{3}H]DPR binding are >10 fold lower than the affinity (K_m values) of these same nucleosides for the *ei* transporter (Lee and Jarvis, 1988*a*,*b*; Plagemann et al., 1988). Therefore, it can not be concluded, from these results, that the NBMPR-resistant [^{3}H]DPR binding site is on the *ei* transporter. In fact, the lack of correlation between the K_i values for inhibition of [^{3}H]DPR binding by nucleosides and the affinity of these same nucleosides for the *ei* transporter, suggests that the NBMPR-resistant [^{3}H]DPR binding may be, at least in part, to membrane components distinct from the permeant site of the nucleoside transporter.

This study examined the NBMPR-resistant [^{3}H]DPR binding component, in more detail, to test the hypothesis that this component represents binding to the *ei* transporter. If this hypothesis is correct, a correlation (i.e. similar potencies) between NBMPR-resistant [^{3}H]DPR binding and NBMPR-resistant nucleoside transport is expected. Guinea pig cortical synaptosomes were chosen for examination of NBMPR-resistant [^{3}H]DPR binding, as they possessed a large proportion of this binding component (≈90% of DPR-sensitive [^{3}H]DPR binding). To examine only the NBMPR-resistant [^{3}H]DPR binding, assays were conducted in the presence of 1 μM NBMPR to inhibit binding of

[^{3}H]DPR to NBMPR-sensitive sites. With the exception of non-radiolabelled DPR, none of the nucleoside transport substrates or inhibitors tested were able to inhibit more than 45% of the NBMPR-resistant [^{3}H]DPR binding. For example, uridine (10 mM) had no effect on the NBMPR-resistant binding component, even though the K_m of uridine for the NBMPR-resistant nucleoside transport system was 100-fold lower (≈100 μM). Dilazep (100 μM) inhibited the NBMPR-resistant [^{3}H]DPR binding component by only 20%, even though this concentration of dilazep blocked NBMPR-resistant [^{3}H]uridine influx completely. This analysis shows that the NBMPR-resistant [^{3}H]DPR binding and the NBMPR-resistant transport were not affected similarly by nucleoside substrates and inhibitors. Furthermore, the proportion of NBMPR-resistant [^{3}H]DPR binding (NBMPR-resistant B_{max} : NBMPR-resistant B_{max} + NBMPR-sensitive B_{max}) in guinea pig was ≈7 times that found in rabbit. If NBMPR-resistant [^{3}H]DPR binding and NBMPR-resistant transport are related, it is expected that the proportion of NBMPR-resistant nucleoside transport (NBMPR-resistant V_{max} : NBMPR-resistant V_{max} + NBMPR-sensitive V_{max}) in guinea pig vs. rabbit would also be ≈7. This ratio was actually only 2. These results all suggest that NBMPR-resistant [^{3}H]DPR binding sites are not associated with the *ei* nucleoside transporter.

This assertion is further supported by the relatively high IC_{50} (>1 μM) reported for DPR inhibition of NBMPR-resistant adenosine transport in guinea pig synaptosomes (Lee and Jarvis, 1988b); in the present study, specific binding of [^{3}H]DPR to this site was unlikely to be observed at the radioligand concentration (1 nM) used to examine NBMPR-resistant [^{3}H]DPR binding in guinea pig. A lower IC_{50} (120 μM) for DPR inhibition of NBMPR-resistant adenosine uptake was reported by Shank and Baldy (1990) but this value was found using incubation times of up to 6 minutes. Under such conditions, inhibitor effects reflect interactions with both membrane transport and metabolism (see section 1.2.2). The IC_{50} reported by Lee and Jarvis (1988*b*) determined using

incubation times of 5 seconds is likely to be more representative of the effect of DPR on membrane transport processes. Because of the lack of correlation between NBMPR-resistant [^{3}H]DPR binding and NBMPR-resistant transport, it is unlikely that [^{3}H]DPR is binding to a site on the *ei* nucleoside transporter, or, if it is, then [^{3}H]DPR must be binding in such a way as to be virtually independent of the nucleoside permeant site and other inhibitor binding sites. It is more likely that NBMPR-resistant [^{3}H]DPR binding is to sites not associated with the *ei* transporter. This is supported by the observations that [^{3}H]DPR binds to 5 times as many sites as [^{3}H]NBMPR in human erythrocytes (Woffendin and Plagemann, 1987), a cell which does not possess *ei* transporters (Cass et al., 1974; Jarvis and Young, 1980). As well, only 50% of the nucleoside transport in HeLa cells is NBMPR-resistant (Paterson et al., 1980), but [^{3}H]DPR labelled 10 times as many sites as did [^{3}H]NBMPR (Woffendin and Plagemann, 1987).

It is unknown at this time what the NBMPR-resistant [^{3}H]DPR binding sites are associated with. DPR has been demonstrated to inhibit cAMP phosphodiesterase (Asano et al., 1977; Deckert et al., 1988), adenosine kinase (Deckert et al., 1988; Plagemann et al., 1988), glucose transport (Plagemann et al., 1987a), and phosphate transport (Gerlach et al., 1964), at concentrations ≈100-fold higher than that required to inhibit nucleoside transport. As well, DPR binds to proteins such as α_1-acid glycoprotein and albumin (MacGregor and Sardi, 1991). Some of these potential binding sites for [^{3}H]DPR were investigated experimentally. Based on the lack of inhibition of NBMPR-resistant [^{3}H]DPR binding by nifedipine, ω-conotoxin, DPCPX, and D-glucose, it is unlikely that [^{3}H]DPR was binding to Ca^{2+} channels, adenosine receptors, phosphodiesterase, or glucose transporters. It may be that the NBMPR-resistant [^{3}H]DPR binding site was not located in the membrane but was associated with cytoplasmic components. Support for this conjecture comes from the fact that the 5-fold difference in

[^{3}H]DPR and [^{3}H]NBMPR B_{max} values in whole erythrocytes (Woffendin and Plagemann, 1987*a*) was not observed in erythrocyte membrane preparations (Jarvis, 1986; Woffendin and Plagemann, 1987*a*). Due to the low pseudo-Hill coefficient found for DPR inhibition of NBMPR-resistant [^{3}H]DPR binding (n_H=0.34), it is also probable that this component represents more than one class of binding site.

In conclusion, [^{3}H]DPR is able to bind with high affinity to at least two sites in guinea pig and rabbit cortical synaptosomes. One site appears to be the same as the [^{3}H]NBMPR binding site on the *es* nuclecside transporter. The other site or sites are as yet unidentified but do not appear to be to functional, membrane-located, nucleoside transporters.

2.4.2 Kinetics and Pharmacological Profile of Nucleoside Transport in Rabbit Cortical Synaptosomes

[^{3}H]Uridine transport into rabbit cortical synaptosomes was found to be mediated by two saturable, facilitated, transport systems. As was the case with other cortical synaptosome preparations, no Na^+-dependent transport was detected (Shank and Baldy, 1990; Lee and Jarvis, 1988*a*; Lee and Jarvis, 1988*b*). Because, Na^+-dependent, concentrative transport can be masked, sometimes, by the efflux of substrate via the equilibrative transporter, NBMPR was included in the assay to block this efflux without affecting the Na^+-dependent transporter (Dagnino et al., 1991*a,b*; Vijayalakshmi et al., 1992). However, it should be noted that the block of equilibrative transport was incomplete, due to the presence of NBMPR-resistant transport. As well, although most Na^+-dependent transporters are insensitive to NBMPR (>10 μM), a Na^+-dependent transporter, which can be inhibited by low concentrations of NBMPR and DPR (<10 nM), has recently been found (N5) in human leukemic cells (see section 1.2.3.2). Nonetheless, in the absence of NBMPR, no Na^+-dependent transport was detected in synaptosomes with an inward Na^+

gradient when compared to those without a Na^+ gradient. It should be noted that the "Na^+-free" buffer, used in these assays, likely contained a low (<200 μM), residual, concentration of Na^+ due to contaminants in the other salts used to prepare the buffers. However, it is unlikely that this low concentration of residual Na^+ would be sufficient to drive a Na^+-dependent transporter as it has been determined in other systems that the Na^+-dependent transporters have a K_{im} of about 10 mM (Jarvis, 1989; Jarvis et al., 1989; Williams et al., 1989; Dagnino et al., 1991*b*; Plagemann, 1991; Williams and Jarvis, 1991).

The two systems found in rabbit could be distinguished by their sensitivity to the nucleoside transport inhibitor NBMPR. Table 2.7 shows the K_m and B_{max} values for NBMPR-sensitive and -resistant nucleoside transport in rabbit, rat, and guinea pig cortical synaptosomes. Even though these studies were all done in the same anatomical system and in rodents, there were distinct differences between the species. Rabbit differed from both rat and guinea pig as it had a relatively high proportion of NBMPR-sensitive transport. In addition, the relative substrate affinity of the transporters (i.e. K_m values) differed. In rat and rabbit, *ei* transporters had a higher substrate affinity than *es* transporters did, which was opposite to that seen in guinea pig. As well, the translocation rate for the *es* transporter in guinea pig was much lower than that found in rat or rabbit.

Differences in nucleoside transport characteristics between species are not unexpected. The number of transporters per cell, as well as the subtypes of transporters found in cells, often differs between species (Deckert et al., 1988; Plagemann et al., 1988; see section 1.2.3). For example, the number of *es* transporters per erythrocyte, as measured by [^{3}H]NBMPR binding, is about $1.5x10^4$ for human, $5x10^3$ for pig, 200 for rat, and none for sheep (Plagemann et al., 1988). As well, the effect of many inhibitors, such as dilazep, DPR and lidoflazine, on transport is spec'es

dependent (Plagemann et al., 1988; see section 1.2.3). These species dependent differences may be due to structural differences in the nucleoside transporter. For example, the degree of glycosylation of the *es* nucleoside transporter differs between species (Crawford et al., 1990c; Kwong et al., 1993; Cass, 1994). Pig and human erythrocytes differ as a result of an additional protein domain in the pig transporter (Kwong et al., 1993). As well, nucleoside transport characteristics can be regulated through various second messengers (see section 1.2.5). It is not clear how this regulation differs from species to species.

What is clear, is that the effects produced by a particular transport blocker could be species dependent, as a result of the types and proportions of nucleoside transporters present. For example, in the CNS, a inhibitor selective for the *es* transporter will likely have a greater effect on adenosine mediated events in rabbit than in guinea pig. This is due to the greater proportion of NBMPR-sensitive transport in rabbit (65%) when compared to guinea pig (30%) (Table 2.7). In addition, within a single species, radioligand binding and nucleoside transport studies have demonstrated that the subtypes, densities, and inhibitor sensitivities of nucleoside transporters, vary within different regions of the brain (Morgan and Marangos, 1987; Deckert et al., 1988; Shank and Baldy, 1990; see section 1.2.4.1). Therefore, the development of nucleoside transport inhibitors, to be used clinically in the CNS, should include a detailed characterization of nucleoside transport kinetics and transporter subtype sensitivities. To date, the types of nucleoside transporters and their localization, in the human CNS, has not been established.

In rabbit cortical synaptosomes, inhibition of transporter-mediated [^{3}H]uridine uptake by NBMPR and dilazep both resulted in two components with approximately 60% being inhibitor-sensitive. Similar profiles, representing the inhibition of NBMPR/dilazep-sensitive and NBMPR/dilazep-

insensitive transporters, have been seen in other systems (Lee and Jarvis, 1988*a*; Lee and Jarvis, 1988*b*; Plagemann et al., 1988; Hammond, 1991). None of the other inhibitors or substrates tested could distinguish between the two transporters to this extent; however, many did show transporter selectivity. These included dipyridamole, R75231, mioflazine, and soluflazine, which all had a significantly higher affinity for the *es* transporter. This differs somewhat from studies in Ehrlich cells and rat erythrocytes where mioflazine showed no transporter selectivity and soluflazine was selective for the *ei* transporter (Griffith et al., 1990; Hammond, 1991). None of the substrates tested showed a significant selectivity for either of the transporter subtypes, which is common to that seen in other studies in rat, and mouse (Lee and Jarvis, 1988*a*; Plagemann and Wohlhueter, 1984). However, these results again differ from a study in Ehrlich cells (of murine origin) which demonstrated a selectivity for the NBMPR-sensitive system by cytidine and guanosine (Hammond, 1991). These differences may be attributable to species, or tissue, dependent variability in nucleoside transport characteristics. Finding a substrate which is selective for the *es* or *ei* nucleoside transporter of all species will be difficult. However, detailed structure-activity studies may lead to the design of substrates that have a significant selectivity for one of the facilitated nucleoside transporters.

In conclusion, rabbit cortical synaptosomes possess both *es* and *ei* nucleoside transporters. The relative proportions and kinetic characteristics of these transporters differ from that found in other species. As well, the *es* nucleoside transporter did not show selectivity for any of the substrates examined when compared to the *ei* transporter. The subtypes, quantities, and kinetic and pharmacological characteristics of nucleoside transporters, may all be, at least in part, species dependent. Since nucleoside transport inhibitors are being developed for clinical use in the CNS, these results emphasis

the importance of fully characterizing the nucleoside transport system of human CNS tissue.

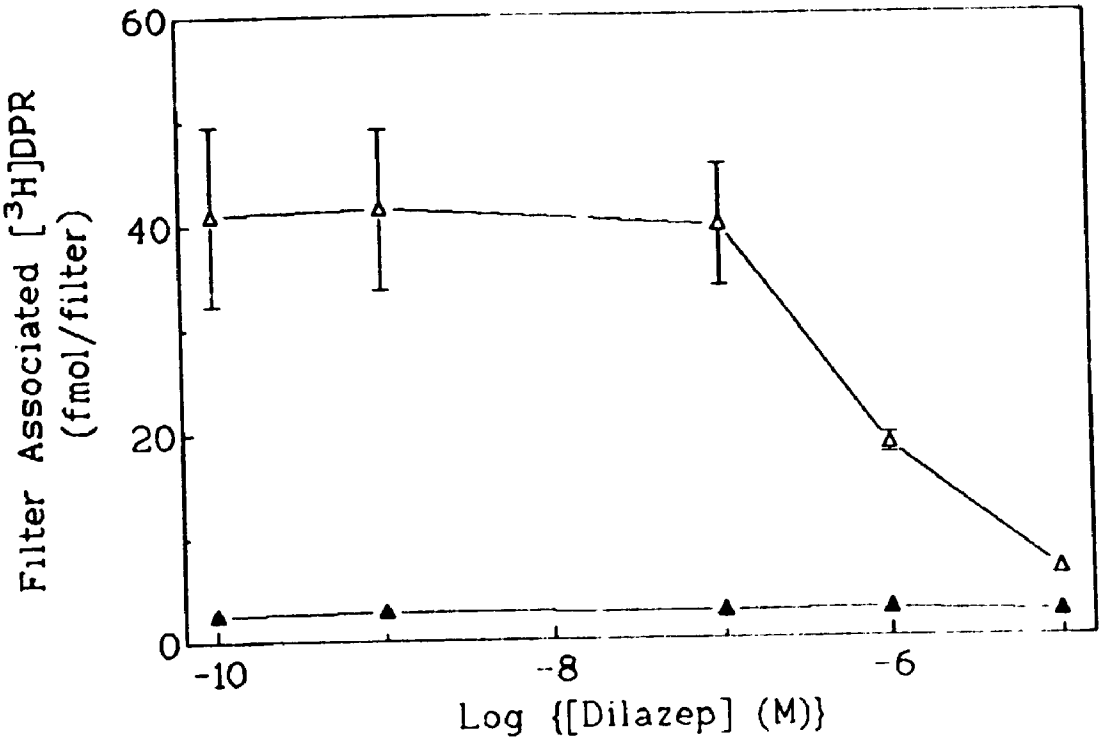


Figure 2.1: The effect of dilazep on [^{3}H]DPR binding to glass fibre filters in the absence (△) and presence (▲) of 0.01% CHAPS in the incubation medium was assessed. Filters were also presoaked (▲ only) for 10 min in a buffer solution containing 0.01% CHAPS. Assays were conducted as described in section 2.2.3, except that no synaptosomal membranes were added to the incubation tubes. The final [^{3}H]DPR concentration was 0.5 nM. Each point represents the mean ± SEM from at least three experiments performed in duplicate.

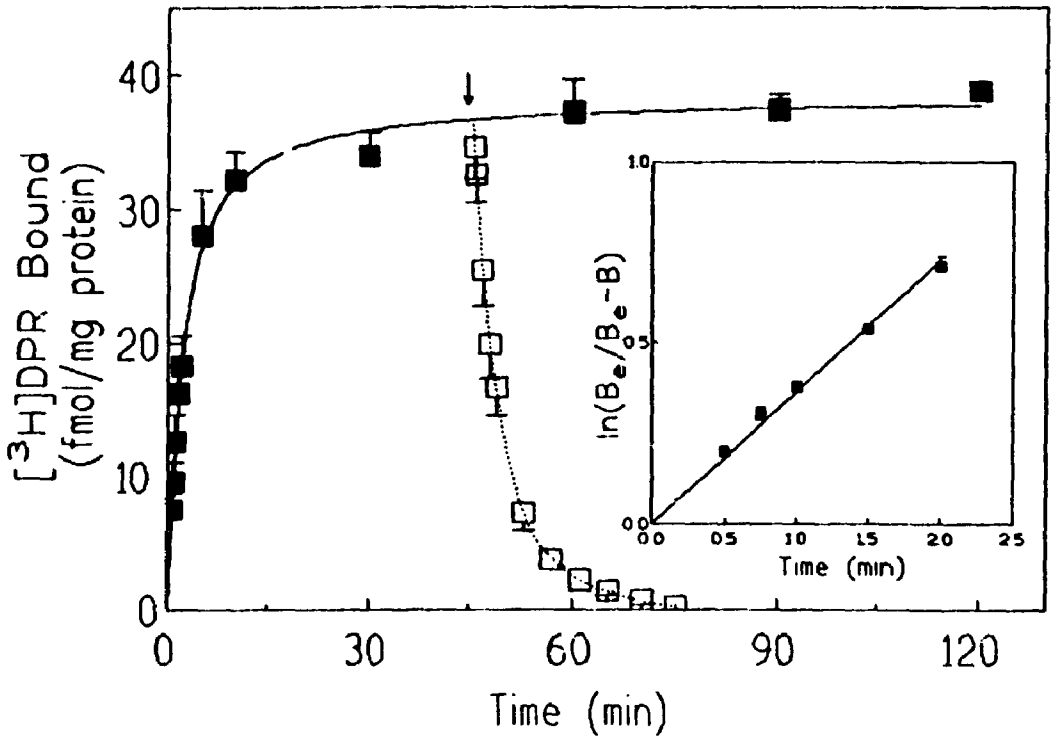


Figure 2.2: Reversibility of NBMPR-sensitive [^{3}H]DPR binding to rabbit cortical synaptosomal membranes. The rate of association (■) of 0.5 nM [^{3}H]DPR with the membranes was observed by filtering the membrane/[^{3}H]DPR suspension after various incubation times (0.5 and 120 min). The inset graph shows the linear transformation of the association data. The slope of these data gave an observed rate constant (k_{ob}) of 0.37±0.01 min^{-1}. To measure the rate of dissociation of [^{3}H]DPR from its specific sites, NBTGR (1 μM, final) was added (↓) after a 45 min incubation and the samples were filtered at various times thereafter (□). Each point represents the mean ± SEM from four experiments.

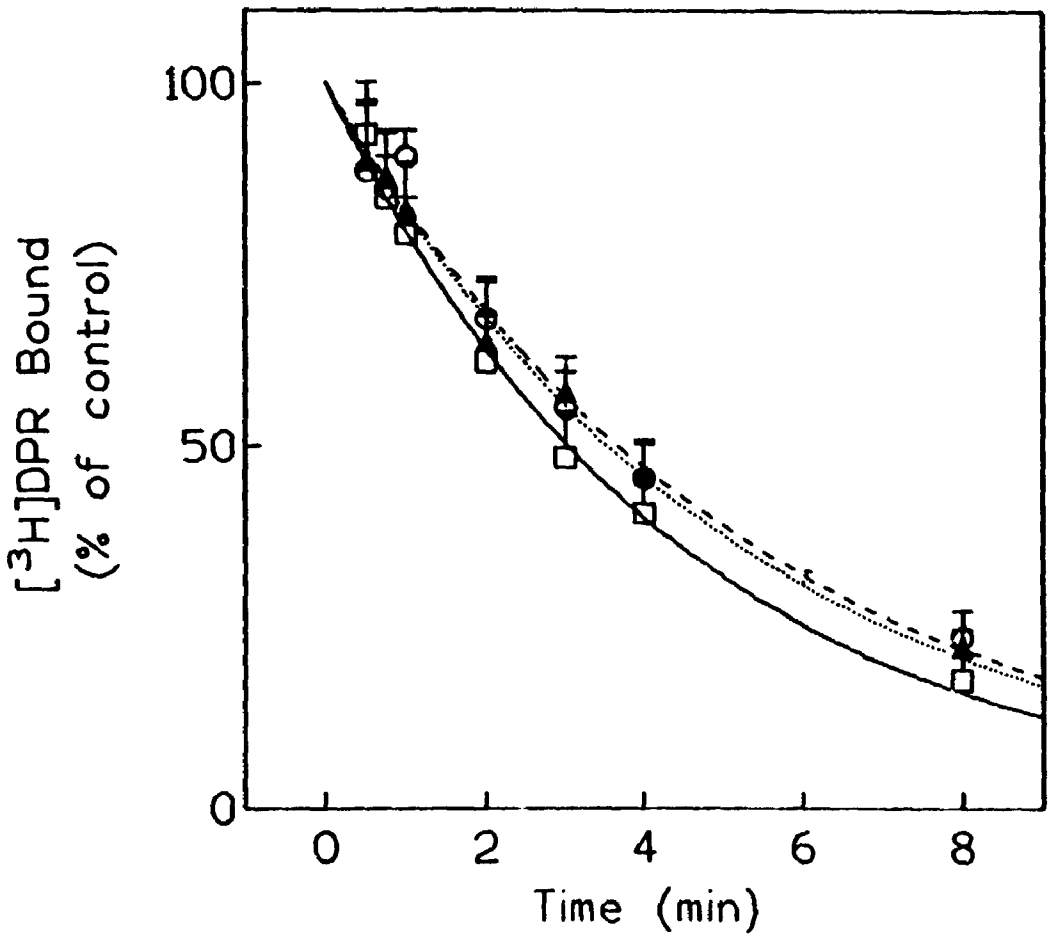
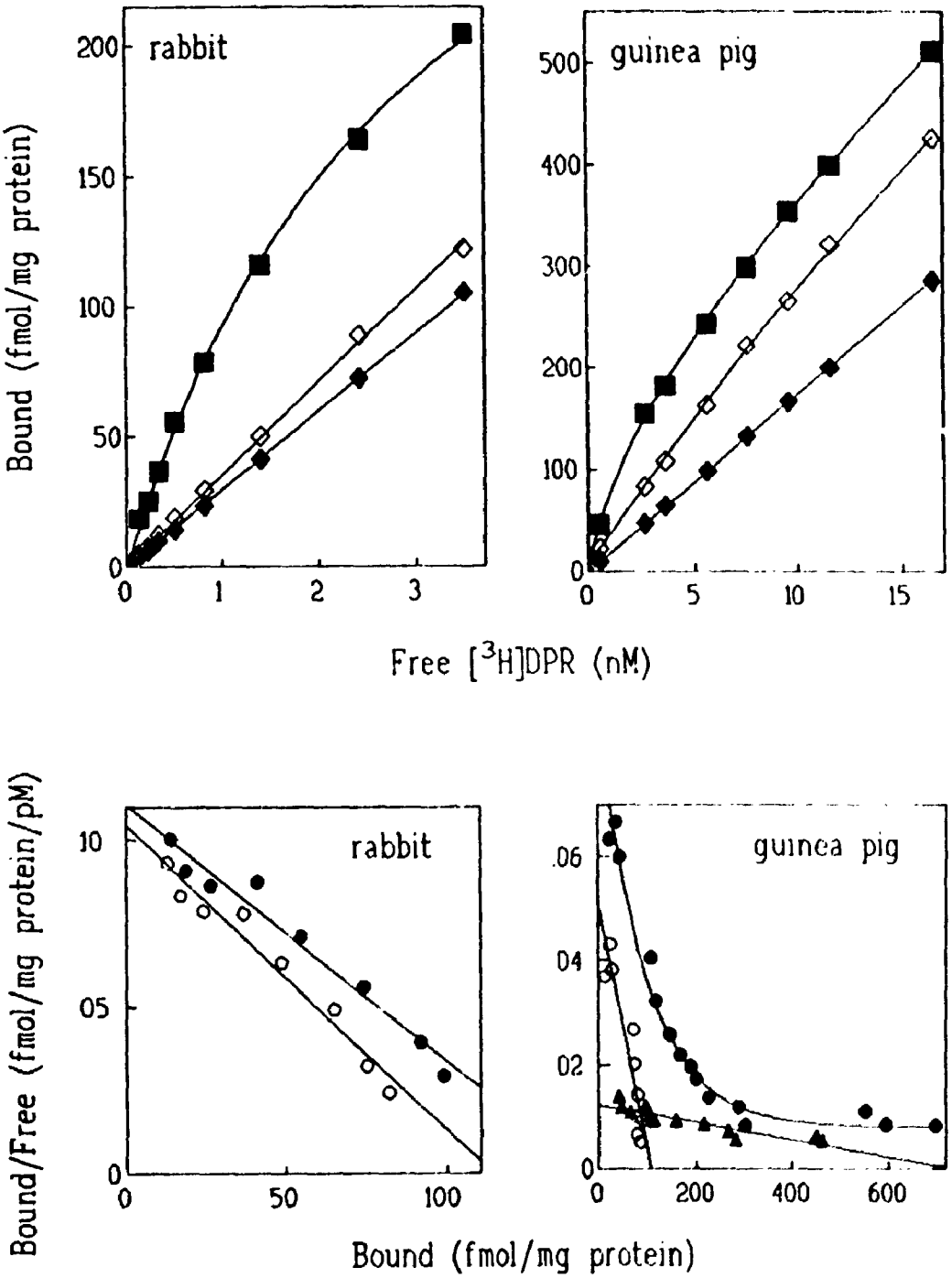


Figure 2.3: Dissociation of NBMPR-sensitive [^{3}H]DPR binding from rabbit cortical synaptosomal membranes. Membranes were incubated with 0.5 nM [^{3}H]DPR for 45 min. To measure the rate of dissociation of [^{3}H]DPR from its specific sites, NBTGR (□, solid line; 1 μM final), dilazep (▲, dotted line; 0.1 μM final), or R75231 (○, dashed line; 1 μM final) were added and the samples were filtered at various times thereafter. Control binding was defined as NBMPR-sensitive [^{3}H]DPR binding observed before the addition of a dissociating agent. For clarity, only the first 8 min of dissociation are shown; however, all inhibitors resulted in 100% dissociation of NBMPR-sensitive [^{3}H]DPR within 30 minutes. Each point represents the mean ± SEM from four experiments.

Figure 2.4: Saturation analysis of [^{3}H]DPR binding to rabbit and guinea pig synaptosomal membranes (upper panels) and the corresponding Scatchard transformations (lower panels). Total binding (■) and binding in the presence of 1 μM NBMPR (◇), or 10 μM DPR (◆, nonspecific) was measured as described in the text. NBMPR-sensitive [^{3}H]DPR binding was calculated as the difference between the total specific binding and the specific binding observed in the presence of 1 μM NBMPR. NBMPR-resistant binding of [^{3}H]DPR was defined as that which could be inhibited by 10 μM DPR but not 1 μM NBMPR. The Scatchard plots show the NBMPR-sensitive [^{3}H]DPR binding (O), NBMPR-resistant binding (▲), and the total specific binding of [^{3}H]DPR (●). To enhance the graphical clarity of the saturation data for guinea pig, the entire range of [^{3}H]DPR concentrations is not shown in the top panel; however, the full range of [^{3}H]DPR concentrations (0.5 - 85 nM) was used to construct the Scatchard plot. These are representative plots from at least four experiments of a similar type done in duplicate. The K_D and B_{max} values derived from these studies are compiled in Table 2.1.


rabbit
guinea pig
Bound (fmol/mg protein)
Free [3H]DPR (nM)
Bound/Free (fmol/mg protein/pM)
Bound (fmol/mg protein)

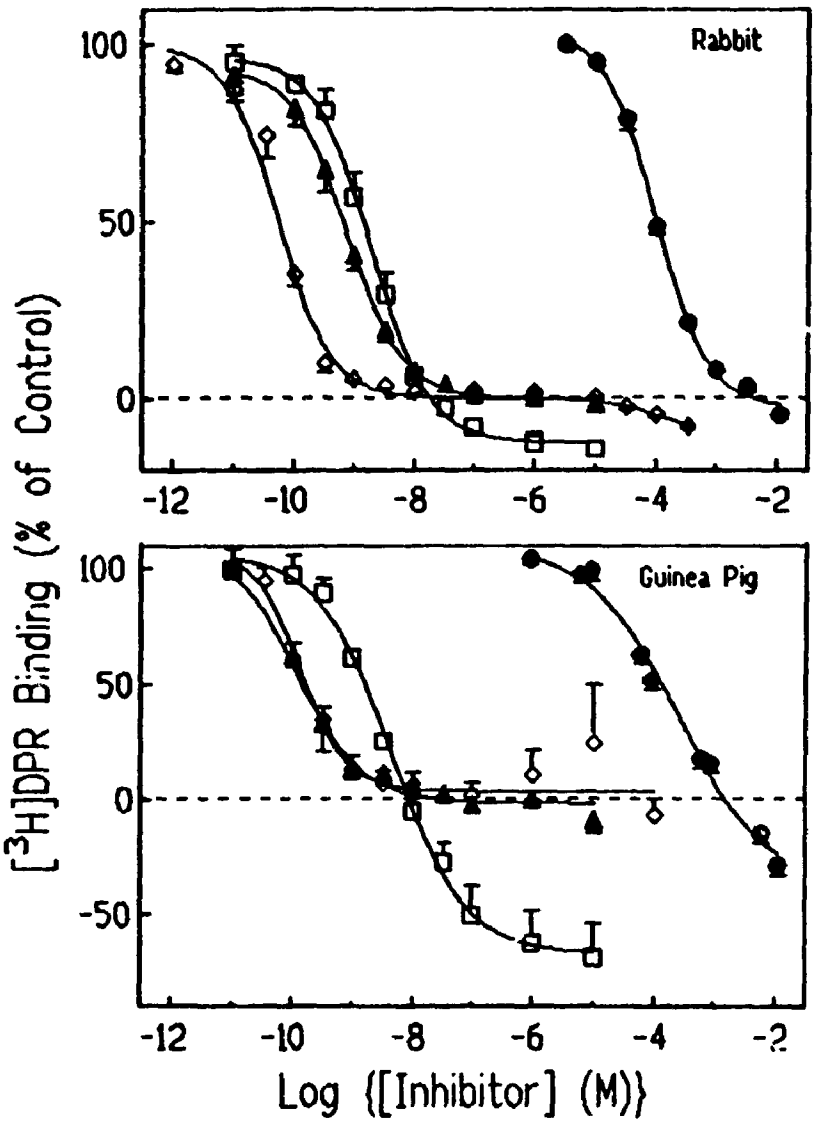


Figure 2.5: Inhibition of [^{3}H]DPR binding to rabbit and guinea pig membranes by dilazep, NBMPR, DPR, or adenosine. Cortical membranes were incubated with 0.5 nM (rabbit) or 1 nM [^{3}H]DPR (guinea pig) in the presence and absence of a range of concentrations of dilazep (◇), NBMPR (▲), DPR (□), and adenosine (●). Control binding was that observed in the absence of inhibitor, and 0% of control (dashed line) was defined as the binding obtained in the presence of 1 μM NBMPR, determined as described in the text. Each point represents the mean ± SEM from at least four experiments conducted in duplicate.

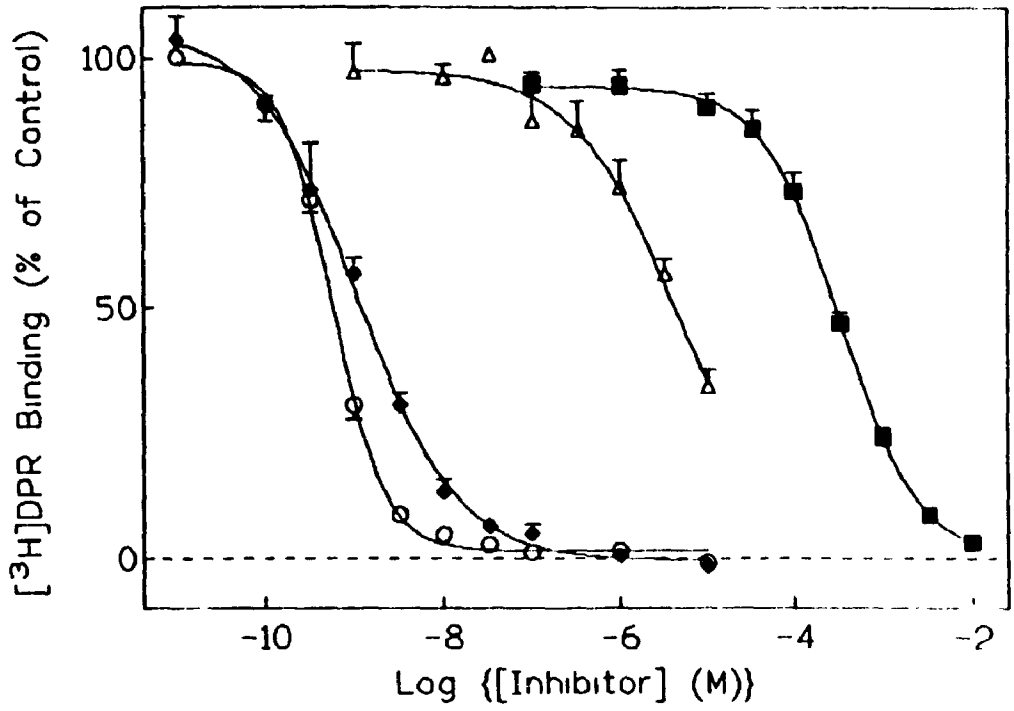


Figure 2.6: Inhibition of [^{3}H]DPR binding to rabbit membranes by R75231, NBTGR, diazepam, or uridine. Cortical membranes were incubated with 0.5 nM [^{3}H]DPR in the presence and absence of a range of concentrations of R75231 (○), NBTGR (◆), diazepam (▲), or uridine (■). Control binding was that observed in the absence of inhibitor, and 0% of control (dashed line) was defined as the binding obtained in the presence of 1 μM NBMPR, determined as described in the text. Each point represents the mean ± SEM from at least four experiments conducted in duplicate.

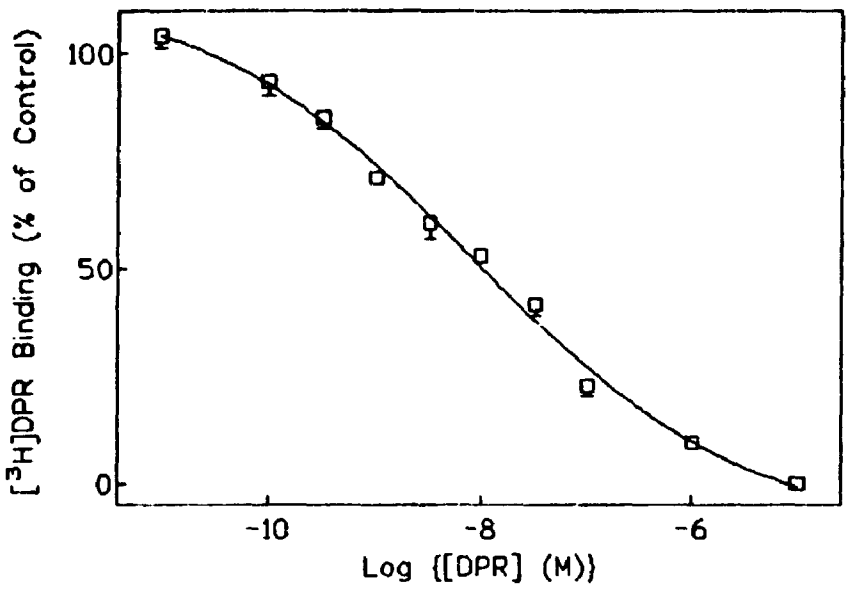


Figure 2.7: Inhibition of the NBMPR-resistant binding of [^{3}H]DPR to guinea pig cortical membranes by DPR. Control binding of [^{3}H]DPR was defined as the DPR-sensitive (10 μM) binding of [^{3}H]DPR (1 nM) which was resistant to inhibition by 1 μM NBMPR. Please note that 100% of control for this figure is equivalent to 0% control in Figures 2.5 and 2.6. Values shown are the means ± SEM from three experiments done in duplicate.

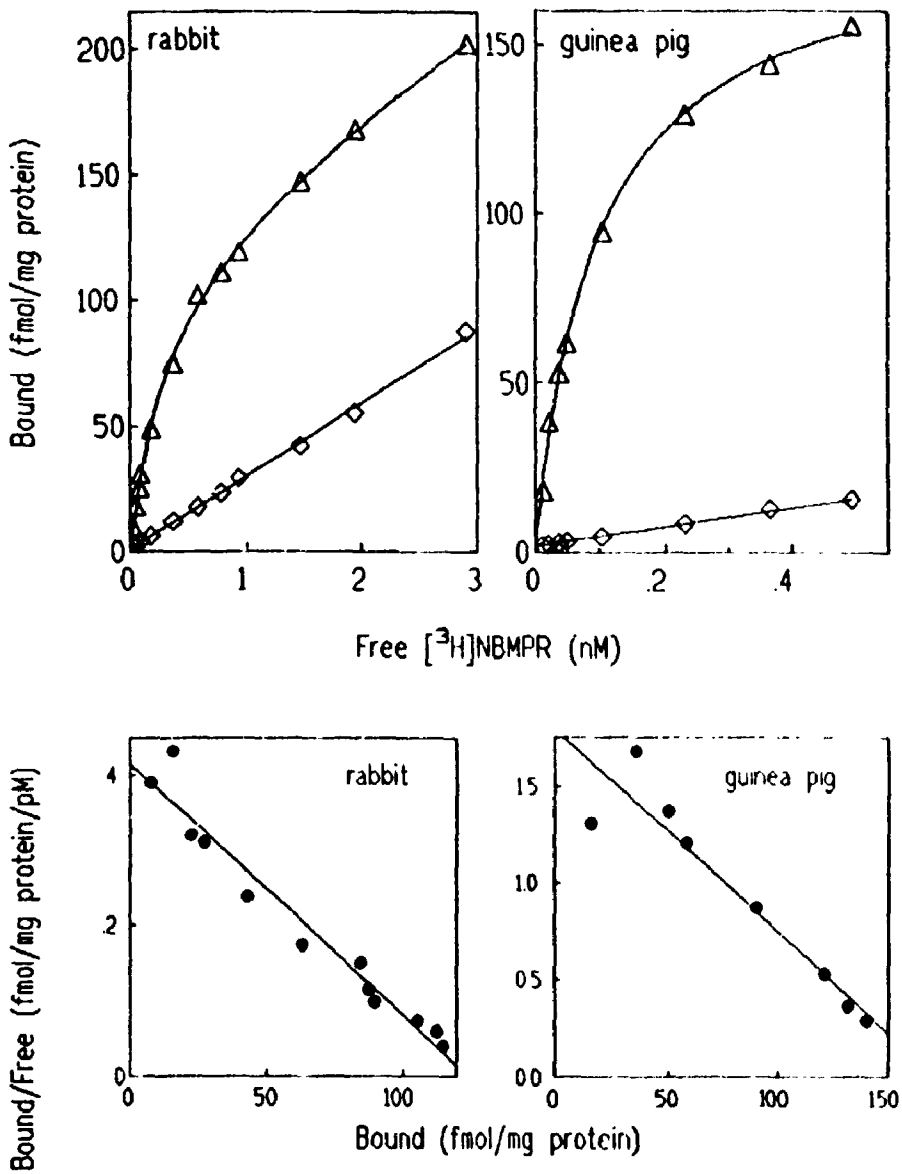


Figure 2.8: Saturation analysis of [^{3}H]NBMPR binding to rabbit and guinea pig synaptosomal membranes (upper panels) and the corresponding Scatchard transformations of the specific binding derived from these data (lower panels). Specific binding (●) was defined as the total binding (△) minus the binding observed in the presence of 10 μM dilazep (◇, nonspecific), determined as described in the text. These are representative plots from at least four experiments conducted in duplicate.

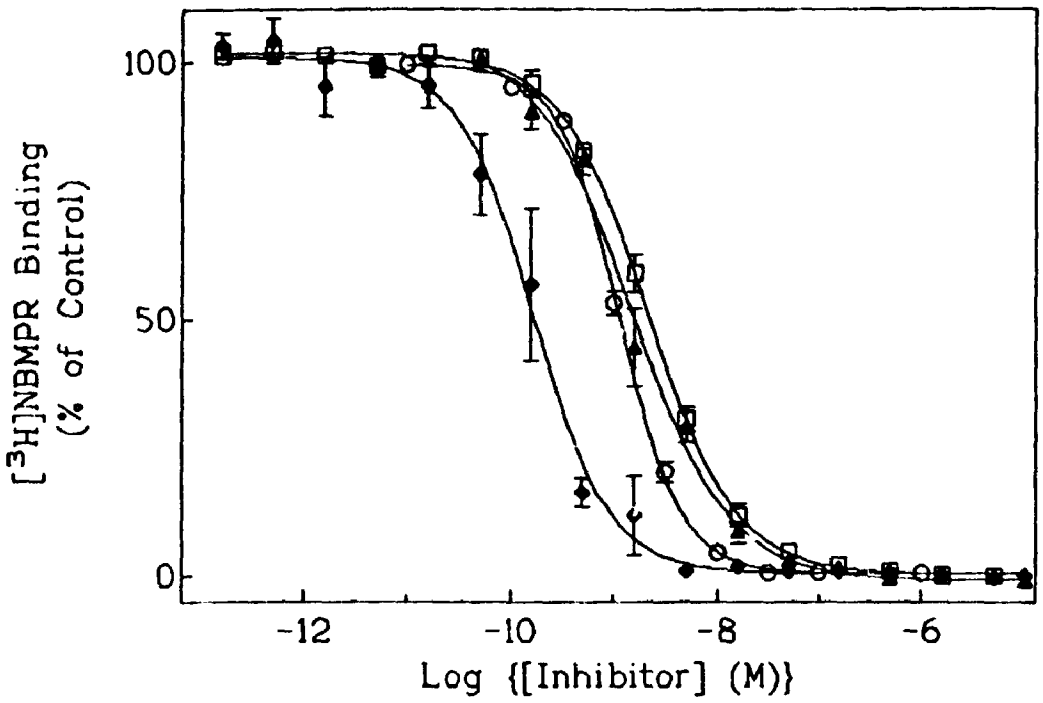


Figure 2.9: Inhibition of [^{3}H]NBMPR binding to rabbit membranes by a series of nucleoside transport inhibitors. Cortical membranes were incubated with 0.4 nM [^{3}H]NBMPR in the presence and absence of a range of concentrations of dilazep (♦), R75231 (○), NBTGR (◆), and DPR (□). Control binding was that observed in the absence of inhibitor, and 0% of control was defined as the binding obtained in the presence of 10 μM dilazep, determined as described in the text. Each point represents the mean ± SEM from three or four experiments conducted in duplicate.

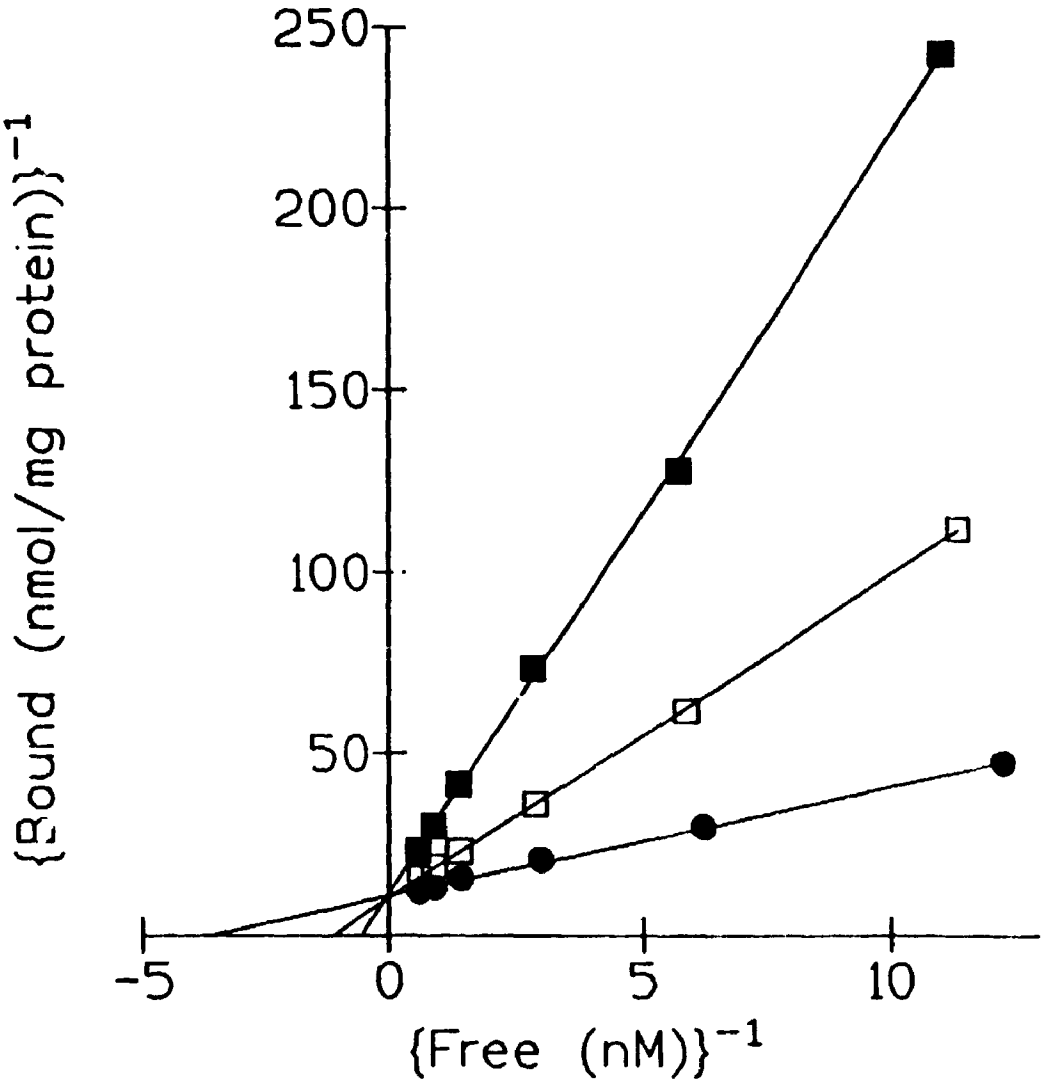


Figure 2.10: Double reciprocal plot analysis of DPR inhibition of the specific binding of [^{3}H]NBMPR to rabbit cortical membranes in the presence of 0.01% CHAPS. The specific binding of 6 concentrations (0.1 - 1.8 nM) of [^{3}H]NBMPR was measured in the presence of 0 nM (●), 1 nM (□), or 3.3 nM (■) DPR. This is a representative plot from four experiments of a similar type done in duplicate.

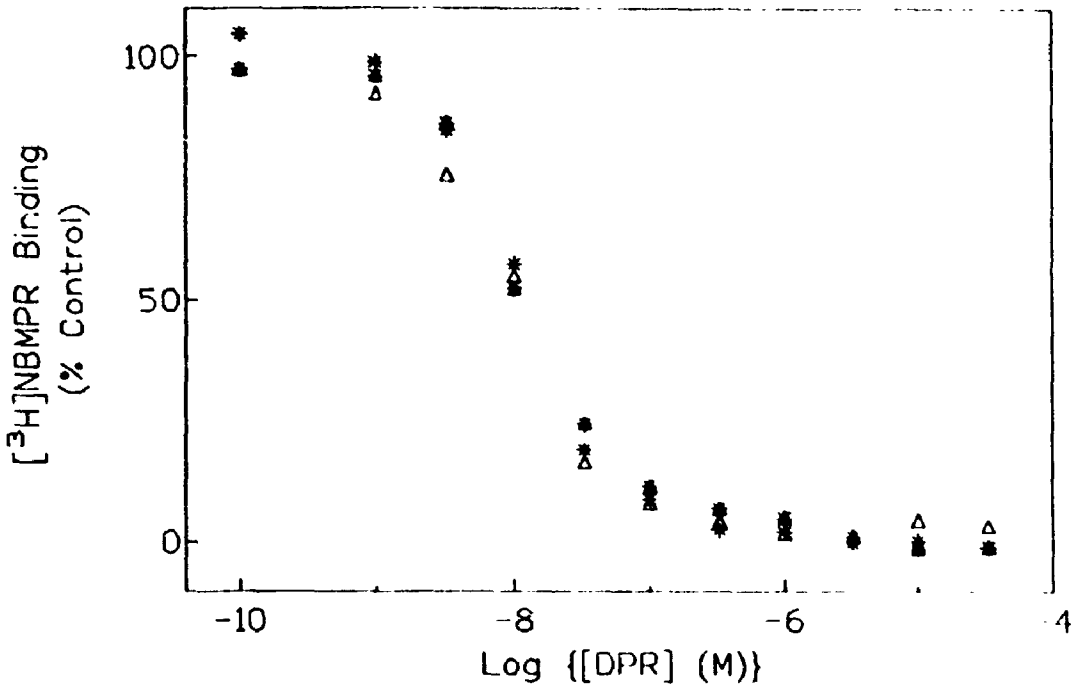


Figure 2.11: Inhibition of [^{3}H]NBMPR binding to cortical membranes of New Zealand White or Dutch rabbits by DPR. Cortical membranes from New Zealand White (▵) or Dutch (*) rabbits, were incubated with 2 nM [^{3}H]NBMPR in the presence and absence of a range of concentrations of DPR. Control binding was that observed in the absence of inhibitor, and 0% of control was defined as the binding obtained in the presence of 10 μM dilazep, determined as described in the text. The experiment was conducted twice in duplicate. Each point shown is the average of a duplicate.

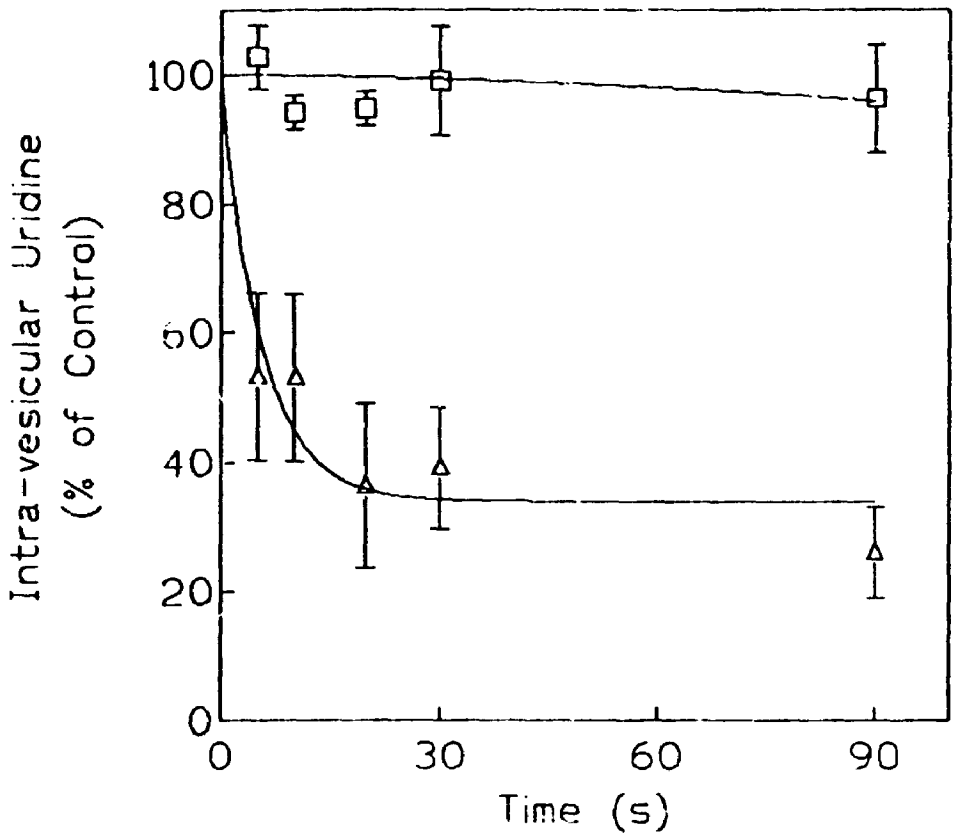


Figure 2.12: The capacity of the stop solution (ice-cold incubation buffer with 25 μM DPR and 25 μM NBTGR) to prevent efflux of uridine from rabbit cortical synaptosomes. After 30 s of [^{3}H]uridine uptake (final concentration, 10 μM), 1 ml of either room temperature buffer (△) or stop solution (□) was added to the incubation tube. This mixture was then filtered immediately or after a known time interval to examine the efflux of uridine. The uridine retained in the synaptosomes is expressed as a percentage of the uridine retained when the stop solution was used and the synaptosomes were filtered immediately. Each point represents the mean ± SEM from four experiments.

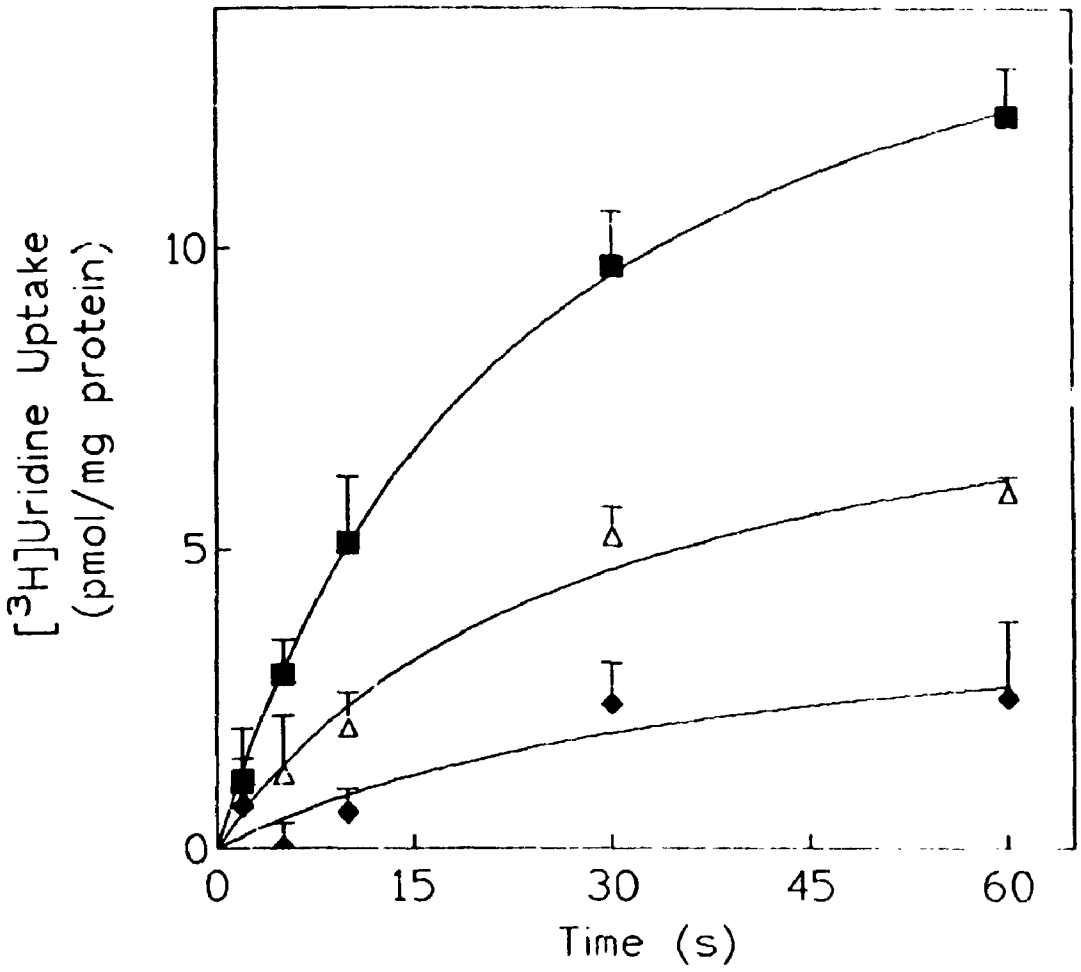


Figure 2.13. Time courses of [^{3}H]uridine uptake by rabbit cortical synaptosomes. Synaptosomes were preincubated in buffer (■), 1 μM NBMPR (▲), or 100 μM dilazep (◆) before initiation of [^{3}H]uridine uptake (final concentration, 10 μM). Each point represents mean ± SEM from four experiments performed in duplicate.

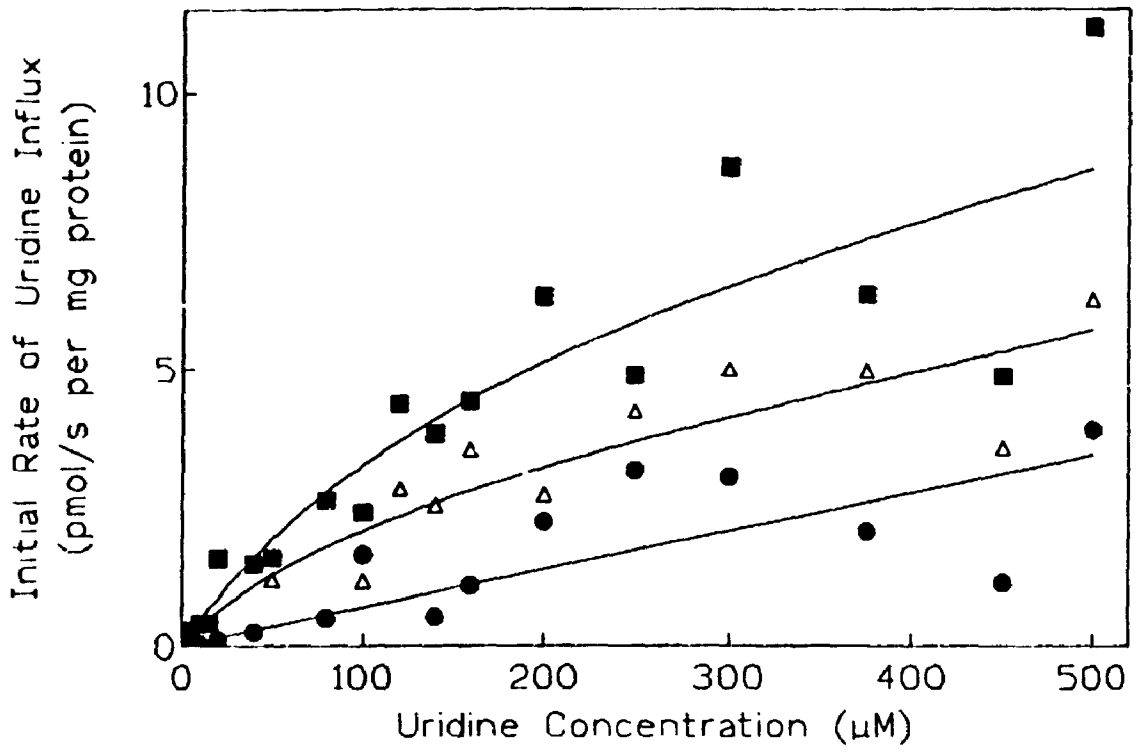


Figure 2.14: Relationship between substrate concentration and the initial rate of [^{3}H]uridine uptake by rabbit cortical synaptosomes. The initial rate of total uptake (■), and that in the presence of 1 μM NBMPR (▵) or 100 μM dilazep (non-mediated, ●) was examined at various uridine concentrations. Each of these initial rates were derived from at least three experiments conducted in duplicate, wherein the total uptake and that in the presence of 1 μM NBMPR or 100 μM dilazep was examined at 5 time points (see Figure 2.13). The results from these experiments were averaged and an initial rate of uptake was found for each uridine concentration. F-tests determined that the line of best fit was linear for non-mediated uptake and curvilinear for total uptake and that in the presence of NBMPR ($P<0.01$). Kinetic constants were determined by non-linear least-squares fit to the averaged data and are shown in Table 2.7.

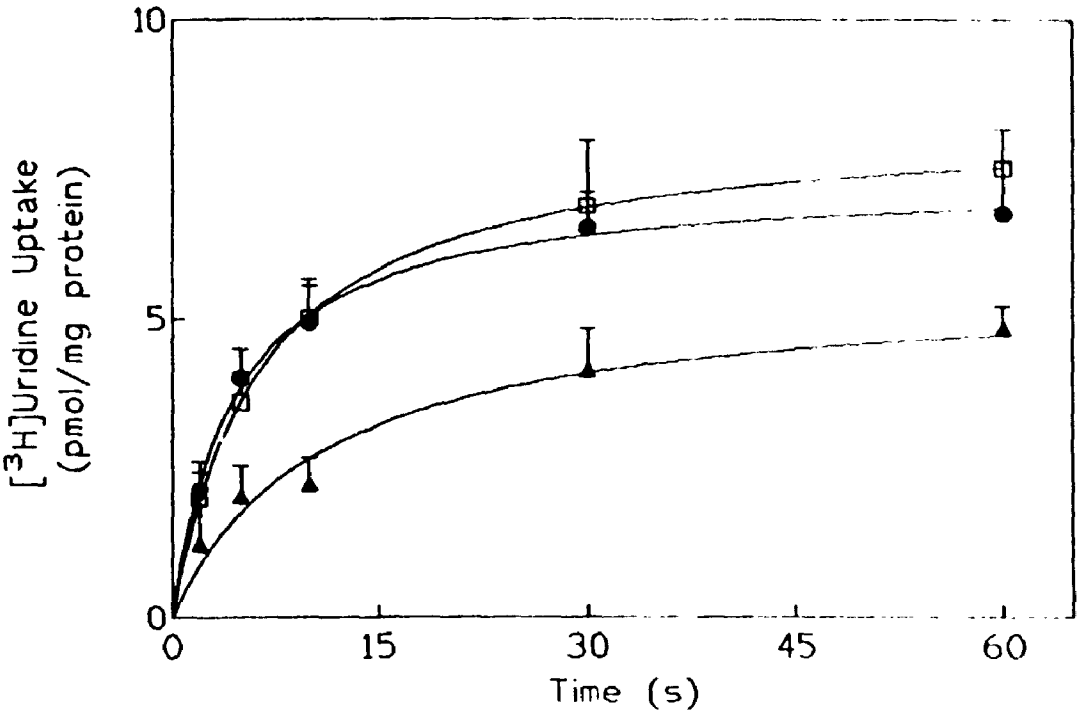
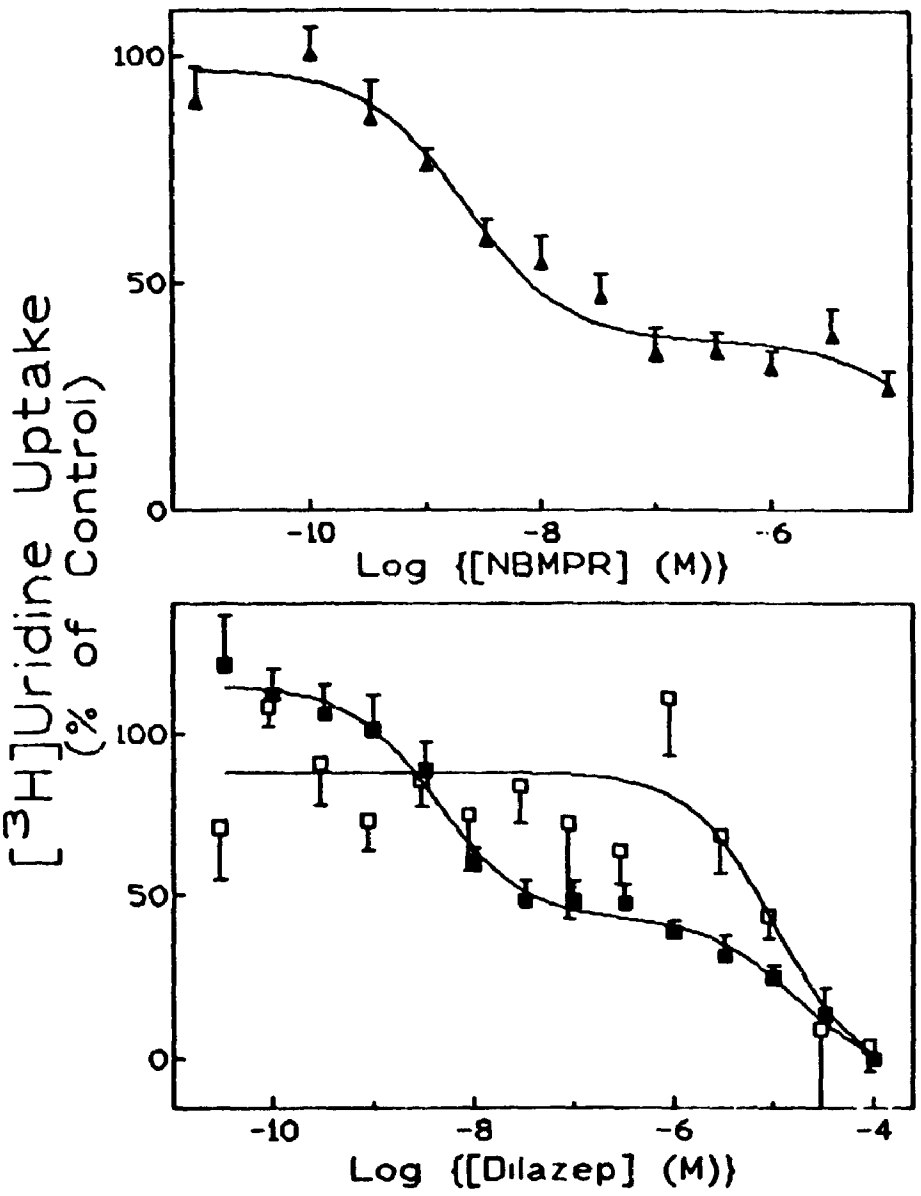


Figure 2.15: Examination of the sodium dependence of uridine (10 μM, final concentration) uptake by rabbit cortical synaptosomes. Synaptosomes were either incubated in $+Na^+$ medium and then transferred to $+Na^+$ medium containing [^{3}H]uridine (●, no gradient), incubated in $-Na^+$ medium and then transferred to $+Na^+$ medium containing [^{3}H]uridine (□, inward Na^+ gradient), or incubated in $-Na^+$ medium with 330 nM NBMPR and then transferred to $+Na^+$ medium containing [^{3}H]uridine with 330 nM NBMPR (▲, inward Na^+ gradient + NBMPR). Na^+ was replaced iso-osmotically with lithium in each case. Each point represents the mean ± SEM from four experiments performed in duplicate.

Figure 2.16: Inhibition of transporter-mediated [^{3}H]uridine uptake into rabbit cortical synaptosomes by NBMPR and dilazep. Synaptosomes were preincubated with various concentrations of NBMPR (▲) or dilazep (■,□) and the mediated influx of [^{3}H]uridine (final concentration, 10 μM) during a 30 s incubation was determined. The transporter mediated uptake in the presence of dilazep was found in the absence (closed symbols) or presence (open symbols) of 333 nM NBMPR. Control uptake was the amount of transporter-mediated accumulation of [^{3}H]uridine observed in the absence of inhibitors (closed symbols) or in the presence of 333 nM NBMPR alone (open symbols). Each point represents the mean ± SEM from four experiments performed in duplicate.


100
50
0
-10
-8
-6
Log {[NBMPR] (M)}
[3H]Uridine Uptake
(% of Control)
100
50
0
-10
-8
-6
-4
Log {[Dilazep] (M)}

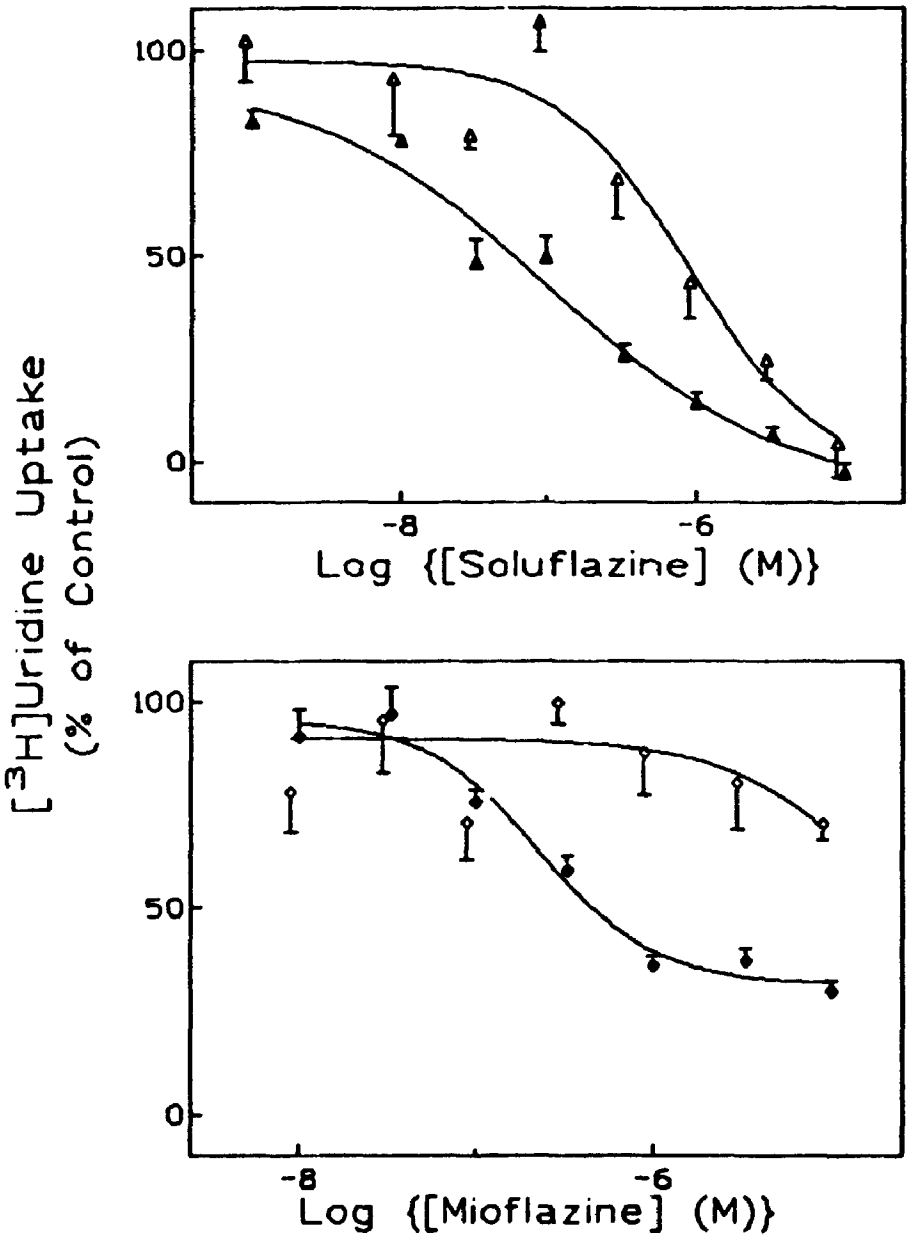


Figure 2.17: Inhibition of transporter-mediated [^{3}H]uridine uptake into rabbit cortical synaptosomes by soluflazine and mioflazine. Synaptosomes were preincubated with various concentrations of soluflazine (▲,△) or mioflazine (◆,◇) in the absence (closed symbols) or presence (open symbols) of 333 nM NBMPR. All other conditions are as described for Figure 2.16.

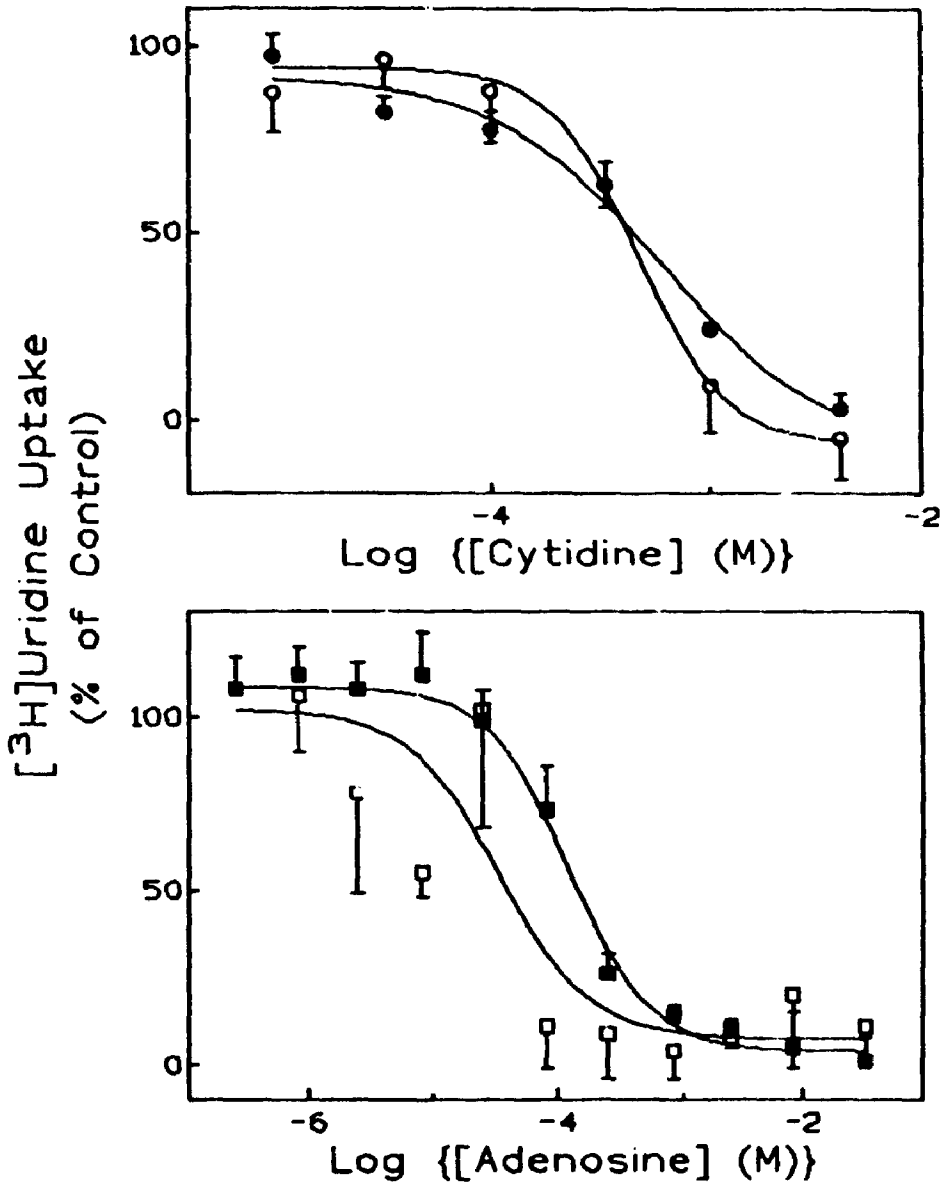


Figure 2.18: Inhibition of transporter-mediated [^{3}H]uridine uptake into rabbit cortical synaptosomes by cytidine and adenosine. The capacity of various concentrations of cytidine (●,○) or adenosine (■,□), in the absence (closed symbols) or presence (open symbols) of 333 nM NBMPR, to inhibit 10 μM [^{3}H]uridine uptake into synaptosomes was assessed. All other conditions are as described for Figure 2.16.

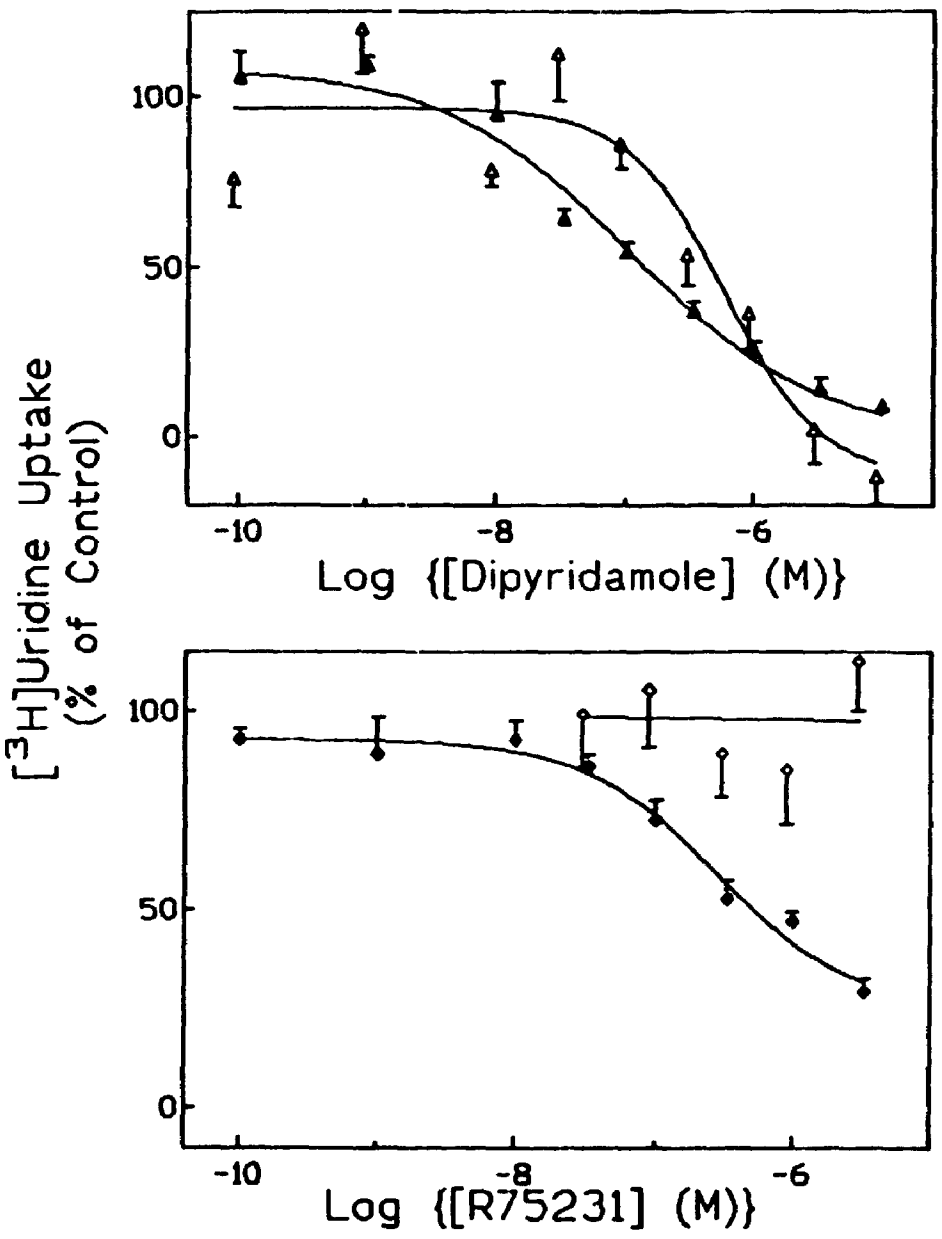


Figure 2.19: Inhibition of transporter-mediated [^{3}H]uridine uptake into rabbit cortical synaptosomes by DPR and R75231. Synaptosomes were preincubated with various concentrations of DPR (▲,△) or R75231 (♦,◇) in the absence (closed symbols) or presence (open symbols) of 333 nM NBMPR. All other conditions are as described for Figure 2.16.

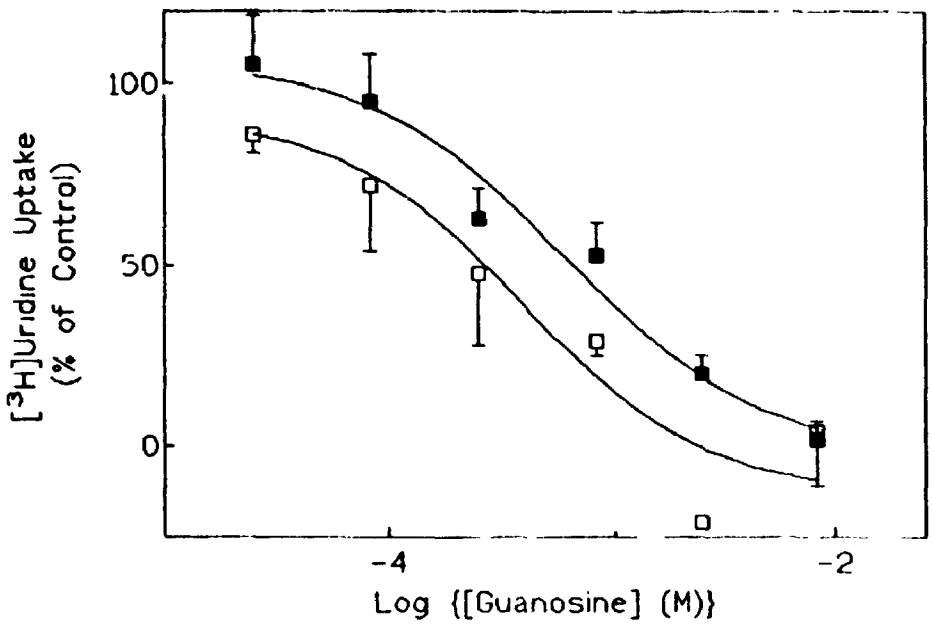


Figure 2.20: Inhibition of transporter-mediated [^{3}H]uridine uptake into rabbit cortical synaptosomes by guanosine. The capacity of various concentrations guanosine (■,□), in the absence (closed symbols) or presence (open symbols) of 333 nM NBMPR, to inhibit 10 μM [^{3}H]uridine uptake was assessed. All other conditions are as described for Figure 2.16.

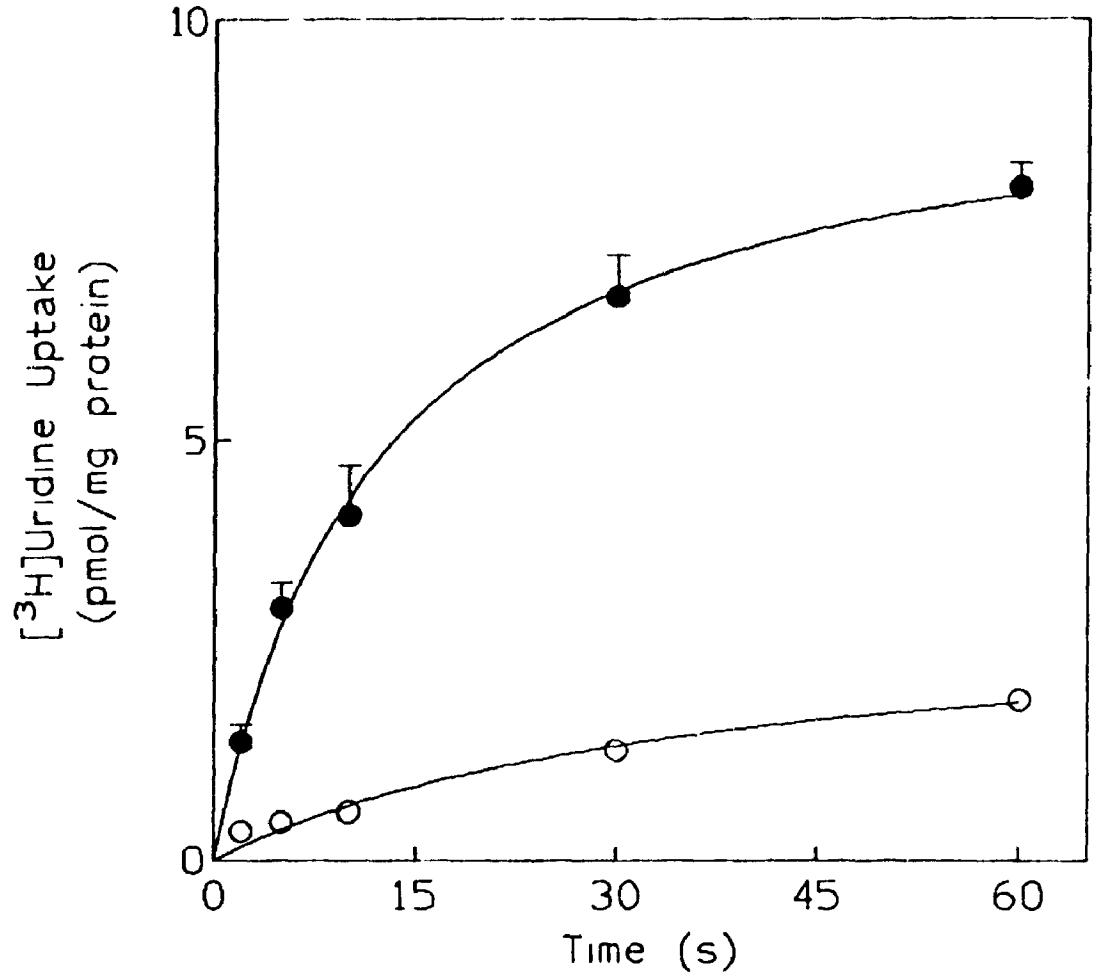


Figure 2.21: Uptake of [^{3}H]uridine by guinea pig cortical synaptosomes. Synaptosomal accumulation of 10 μM [^{3}H]uridine was measured in the absence (total uptake, ●) and presence (non-mediated uptake, ○) of 100 μM dilazep. Each point represents the mean ± SEM from four experiments conducted in duplicate.

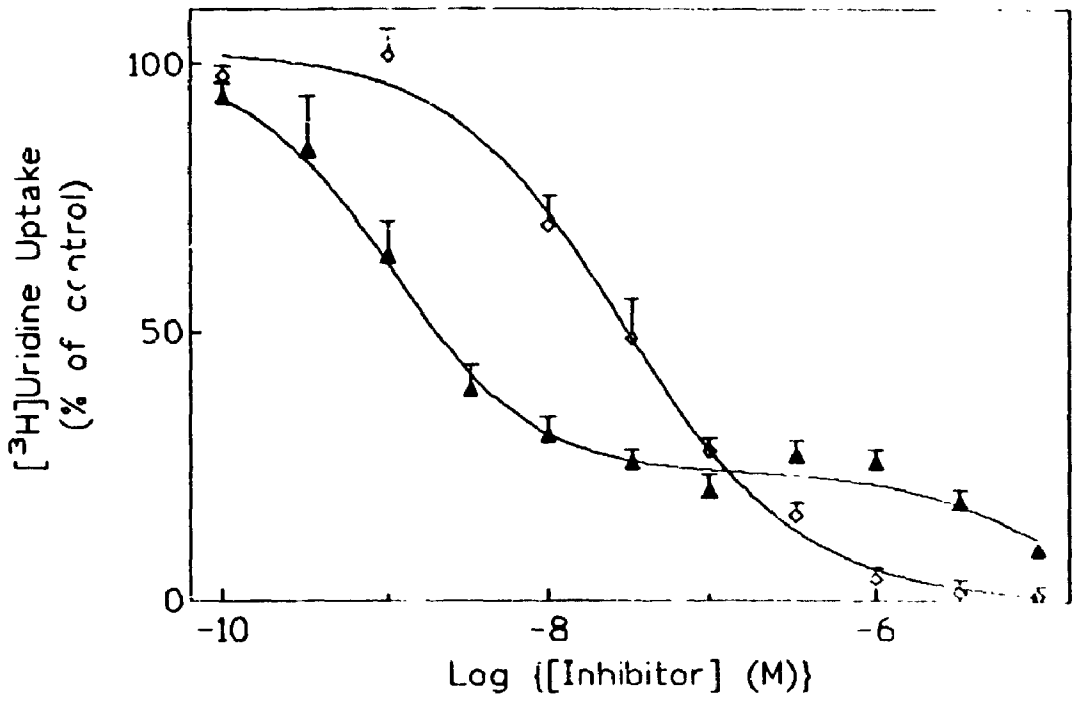


Figure 2.22: Inhibition of transporter-mediated [^{3}H]uridine uptake into guinea pig cortical synaptosomes by NBMPR and DPR. Synaptosomes were preincubated with various concentrations of NBMPR (▲) or DPR (◇) and the mediated influx of [^{3}H]uridine (final concentration 10 μM) during a 30 s incubation was determined. Control uptake was the amount of transporter-mediated accumulation of [^{3}H]uridine observed in the absence of inhibitors. Each point represents the mean ± SEM from four experiments conducted in duplicate.

Table 2.1 Comparison of [^{3}H]DPR binding constants in rabbit and guinea pig cortical membranes. Binding parameters were determined by mass law analysis of specific binding data similar to that shown in Figure 2.4. In rabbit, K_D and B_{max} values for NBMPR-resistant [^{3}H]DPR binding could not be determined in an independent experiment due to the small quantity of this component. However, a B_{max} for NBMPR-resistant [^{3}H]DPR binding was estimated at 20 fmol/mg (total [^{3}H]DPR binding B_{max} -NBMPR-sensitive [^{3}H]DPR binding B_{max}). Values shown are the means ± SEM from at least four experiments done in duplicate.

	NBMPR-Resistant		NBMPR-Sensitive	
Species	K_D (nM)	B_{max} (fmol/mg)	K_D (nM)	B_{max} (fmol/mg)
Rabbit	N.D.	20[a]	1.4±0.2	150±20
Guinea Pig[b]	60±20	840±40	2.8±0.5	110±30

[a] calculated from: total [^{3}H]DPR binding B_{max} (170 fmol/mg) - NBMPR-sensitive [^{3}H]DPR binding B_{max} (150 fmol/mg)

[b] A computer-generated curve split of the total specific [^{3}H]DPR binding (DPR-sensitive) into two components resulted in the following K_D and B_{max} values (high-affinity site, K_D = 1.37±0.06 nM and B_{max} = 100±11 fmol/mg; low affinity site K_D = 96±6 nM and B_{max} = 920±120 fmol/mg) which are similar to the values for NBMPR-sensitive and -resistant [^{3}H]DPR binding given in Table 2.1.

Table 2.2 Comparison of the inhibition of [^{3}H]NBMPR binding and NBMPR-sensitive [^{3}H]DPR binding to rabbit cortical membranes by various substrates and inhibitors. Compounds were tested over a range of 8-11 concentrations (Figures 2.5, 2.6) to determine their capacity to inhibit the specific binding of [^{3}H]NBMPR (0.4 nM) or the NBMPR-sensitive binding of [^{3}H]DPR (0.5 nM). NBMPR-sensitive [^{3}H]DPR binding was defined as that which could be inhibited by 1 μM NBMPR (or 10 μM dilazep, both yielded similar results). Values shown are the means ± SEM from three-five experiments done in duplicate.

Inhibitor	[^{3}H]DPR Binding		[^{3}H]NBMPR Binding	
	K_i	n_H	K_i	n_H
NBTGR (nM)	0.8±0.2	0.8±0.1	0.6±0.1	0.92±0.06
DILAZEP (nM)	0.10±0.04[a]	-	0.09±0.03	1.4±0.1
DPR (nM)	0.9±0.1[a]	0.95±0.04	0.95±0.08	1.04±0.02
R75231 (nM)	0.7±0.2	1.48±0.08	0.44±0.02	1.46±0.09
NBMPR (nM)	0.56±0.03	0.95±0.03	-	-
DIAZEPAM (μM)	3.5±0.5	0.82±0.08	-	-
ADENOSINE (μM)	70±5	1.02±0.05	-	-
URIDINE (μM)	270±20	0.9±0.1	-	-

[a] IC_{50} value.

Table 2.3 Inhibition of the NBMPR-resistant [^{3}H]DPR binding component in guinea pig cortical membranes by various agents. Assays were conducted in the presence of 1 μM NBMPR to inhibit the NBMPR-sensitive [^{3}H]DPR binding. Control binding of [^{3}H]DPR was defined as the DPR (10 μM)-sensitive binding of [^{3}H]DPR (1 nM) which was resistant to inhibition by 1 μM NBMPR. Final assay concentrations of inhibitors are shown in parentheses. Values shown are the means ± SEM from three experiments done in duplicate.

Inhibitor	% Control Binding
Adenosine (10 mM)	73±1
Uridine (10 mM)	103±7
Thymidine (10 mM)	78±5
Adenine (10 mM)	74±9
Soluflazine (10 μM)	73±1
Mioflazine (10 μM)	56±7
Dilazep (10 μM)	79±11
Nifedipine (10 μM)	60±2
ω-Conotoxin (500 nM)	114±3
DPCPX (10 μM)	59±2
D-Glucose (333 mM)	75±4

Table 2.4 Comparison of [^{3}H]NBMPR binding constants in rabbit and guinea pig cortical membranes. Binding parameters were determined by mass law analysis of specific binding data as shown in Figure 2.8. Values shown are the means ± SEM from at least four experiments done in duplicate.

	K_D (nM)	B_{max} (fmol/mg)	n_H
Rabbit	0.30±0.01	130±30	1.05±±0.02
Guinea Pig	0.097±0.007	174±4	0.96±0.03

Table 2.5 LDH activity in the synaptosomal fractions isolated from rabbit cerebral cortex. The synaptosomal fractions were isolated, as described in section 2.2.2, and the total and extravesicular LDH activity was determined. Intravesicular LDH activity was found by subtracting the extravesicular LDH activity from the total. The experiment was conducted twice and the average result is shown.

Synaptosomal Fraction	Total	Extra-vesicular	Intra-vesicular	Intravesicular
	(absorbance/min/μg protein)			(% of total)
P_2	0.109	0.060	0.049	45%
Myelin	0.123	0.068	0.055	45%
P_v	0.59	0.071	0.52	88%
Mitochondria	0.075	0.074	0.001	1%

Table 2.6 Comparison of [^{3}H]DPR binding constants in P_2 and P_v synaptosomal fractions of guinea pig cortex. Binding parameters were determined by mass law analysis of specific binding data. NBMPR-sensitive [^{3}H]DPR binding was defined as the specific binding that was sensitive to inhibition by 1 μM NBMPR. NBMPR-resistant binding of [^{3}H]DPR was defined as the binding that could be inhibited by 10 μM DPR but not by 1 μM NBMPR. No significant differences were found in the K_D or B_{max} values of the two fractions. Values shown are the means ± SEM from four experiments done in duplicate.

	NBMPR-resistant		NBMPR-Sensitive	
Fraction	K_D (nM)	B_{max} (fmol/mg)	K_D (nM)	B_{max} (fmol/mg)
P_2	60±20	840±40	2.8±0.5	110±30
P_v	81±15	1060±280	2.5±1.2	140±60

Table 2.7 Comparison of nucleoside transport and [^{3}H]NBMPR binding characteristics of mammalian cortical synaptosomes. Kinetic constants for [^{3}H]nucleoside transport via *es* and *ei* transporters, and B_{max} values ([^{3}H]NBMPR binding) for rat, guinea pig, and rabbit cortical synaptosomes are shown. [^{3}H]Uridine transport and [^{3}H]NBMPR binding constants for rabbit were determined as described in the text. All other values were taken from the indicated source. Substrate translocation rates were calculated based on the V_{max} for NBMPR-sensitive [^{3}H]uridine transport and the B_{max} for [^{3}H]NBMPR binding, assuming that one [^{3}H]NBMPR binding site represents one NBMPR-sensitive nucleoside transporter (Plagemann et al., 1988).

Species	[^{3}H]Nucleoside Transport					[^{3}H]NBMPR
	NBMPR-Sensitive			NBMPR-Resistant		
	K_m (μM)	V_{max} (pmol/s/mg)	Translocation (molecules/s)	K_m (μM)	V_{max} (pmol/s/mg)	B_{max} (fmol/mg)
Rat[a]	300	12	21	214	16	580
Guinea Pig[b]	13	2.9	5	73	6.9	620
Rabbit[c]	320	4.9	38	94	2.7	130

[a]from Lee and Jarvis, 1988a using uridine as the substrate
[b]from Lee and Jarvis, 1988b using adenosine as the substrate
[c]using uridine as the substrate

Table 2.8 Inhibition of total transporter-mediated, and NBMPR-resistant, [^{3}H]uridine uptake in rabbit cortical synaptosomes. Compounds were tested over a range of concentrations to determine their capacities to inhibit the transporter-mediated uptake of 10 nM [^{3}H]uridine (Figures 2.16 to 2.20). The ratio represents the relative affinity of the inhibitor for the ei transporter (Total IC_{50}/NBMPR-resistant IC_{50}). Values shown are the means ± SEM from at least four experiments.

Inhibitor or Substrate	Total Influx IC_{50}	NBMPR-Resistant Influx IC_{50}	Ratio[a]
Dipyridamole	110±40 nM	640±180 nM[c]	0.17
R75231	310±50 nM	>3 μM[bc]	<0.1
Soluflazine	52±6 nM	850±290 nM[c]	0.08±0.03
Mioflazine	740±180 nM	20±7 μM[bc]	0.08±0.06
Adenosine	100±20 μM	60±20 μM	1.6
Guanosine	450±90 μM	370±160 μM	1.2
Cytidine	390±50 μM	360±70 μM	1.3±0.3

[a] SEM for ratios are shown only if data were paired such that the ratio could be calculated for each individual experiment.

[b] Extrapolated IC_{50}. Mioflazine (9 μM) and R75231 (3 μM) produced 30±4% and 0% inhibition of NBMPR-resistant uptake, respectively.

[c] Significantly different from the IC_{50} value obtained for inhibition of total mediated uptake of [^{3}H]uridine (Student's t-test, paired where appropriate, $P<0.05$).

CHAPTER 3: INTERACTION OF R75231 WITH THE NUCLEOSIDE TRANSPORTER

3.1 Introduction

The radiolabelled inhibitors [^{3}H]NBMPR (Cass et al., 1974; Jarvis and Young, 1980; Hammond, 1991), [^{3}H]DPR (Marangos et al., 1985; Marangos and Deckert, 1987; Woffendin and Plagemann, 1987; Jones and Hammond, 1992), and [^{3}H]dilazep (Gati and Paterson, 1989) have all been used as specific high-affinity probes for proteins associated with nucleoside transport. There is growing evidence that inhibitor binding to the transporter is not a simple 1:1 relationship but involves a complex model, possibly involving multiple inhibitor binding sites and allosteric interactions. In this manner, inhibitor binding to one site can effect the binding of inhibitors to a different binding site(s). The evidence for a complex model includes pseudo-Hill coefficients of less than and greater than unity for inhibition of [^{3}H]NBMPR binding by DPR or lidoflazine analogues, respectively (Wu and Phillis, 1982*b*; Hammond and Clanachan, 1985; IJzerman et al., 1989; Jones and Hammond, 1992). Various agents have also been shown to modify the rate of dissociation of [^{3}H]NBMPR from its binding sites (Jarvis et al., 1983; Koren et al., 1983; Wohlhueter et al., 1983; Hammond, 1991). As well, saturable and reversible [^{3}H]dilazep binding to two distinct sites, as demonstrated by kinetic data, including a 50-fold difference in K_D values, was observed in S49 mouse lymphoma cells (Gati and Paterson, 1989). Both of these sites may be located on a single nucleoside transporter protein, as neither binding component was detected in AE_1 cells, a nucleoside transport deficient S49 mutant (Gati and Paterson, 1989). Due to the growing interest in nucleoside transport inhibitors as cardioprotective (Haga et al., 1986; Van Belle et al., 1989,

1991, 1992; Geiger and Fyda, 1991; Masuda et al., 1991, 1992; Chang-Chun et al., 1992; de Haan et al., 1993; Wainwright et al., 1993; Beukers et al., 1994; Bohm et al., 1994; Yang et al., 1994) and neuroprotective agents (Deckert et al., 1988; Dragunow, 1988; Daval et al., 1991; Geiger and Fyda, 1991; Marangos, 1991; Marangos and Miller, 1991), it is critical that their interactions with the transporter be understood thoroughly.

The present study used the newly developed mioflazine derivative from the Janssen Research Foundation, R75231 (2-(aminocarbonyl)-4-amino-2,6-dichlorophenyl)-4-[5,5-bis(4-fluorophenyl)-pentyl]-1-piperazineacetamide·2HCl), as a probe to study inhibitor interactions at the nucleoside transporter. R75231, a substituted piperazine, has unique and interesting binding properties, giving it great potential both as a probe for the nucleoside transporter, and clinically. Studies, *in vitro*, have shown that R75231 binds to the transporter extremely tightly (Masuda et al., 1991; Van Belle and Janssen, 1991), perhaps irreversibly (IJzerman et al., 1992). Compatible with these observations is the finding that R75231 has long-lasting inhibitory effects *in vivo* (Baer et al., 1991; Van Belle et al., 1991). This, and the finding that R75231, and congeners, inhibit [^{3}H]NBMPR binding with pseudo-Hill coefficients greater than one (IJzerman et al., 1989; Jones and Hammond, 1992), suggest that R75231 binds to the nucleoside transporter in a different manner than does [^{3}H]NBMPR. Because of this extremely tight binding, specificity for the nucleoside transporter, long duration of action, and very good absorption when given orally to rabbits (Baer et al., 1991; Van Belle and Janssen, 1991; Van Belle et al., 1991; IJzerman et al., 1992), the clinical potential of R75231 may be greater than that of any of the other nucleoside transport inhibitors tested, so far. However, it should be noted that the tight binding of R75231 could also prove to be a deleterious characteristic for clinical use.

Specifically, if the negative chronotropic effects of adenosine were to produce a complete conduction block in the heart, the tight binding of R75231 might make this condition extremely difficult to reverse. Currently, R75231 is being examined for its cardioprotective effects which include: inhibition of platelet aggregation, antiarrhythmic effects, arterial dilation, cardiopreservation for transplantation, protection from ischemia and from stress due to a high concentration of catecholamines (Masuda et al., 1991, 1992; Van Belle and Janssen, 1991; Mollhoff et al., 1992; de Haan et al., 1993; Van Belle et al., 1993; Wainwright et al., 1993; Beukers et al., 1994; Bohm et al., 1994).

In the present study, the nature of R75231 binding to nucleoside transporters was examined. The interaction of R75231 with nucleoside transporters in rabbit cortical synaptosomes was examined based on its capacity to inhibit the binding of [^{3}H]NBMPR and the uptake of [^{3}H]uridine. In addition, binding assays with the recently synthesized [^{3}H]R75231 were performed using human erythrocyte ghost membranes, as they possess a larger number of nucleoside transporters than do rabbit cortical synaptosomes.

3.1.1 Hypothesis

R75231 binds to the *es* nucleoside transporter in an irreversible manner.

3.2 Methods

3.2.1 Materials

[^{3}H]NBMPR, 26-36 Ci/mmol, was purchased from Moravek Biochemicals, Inc. (Brea, CA) and [^{3}H]uridine, 60 Ci/mmol, was purchased from ICN Canada. The R75231 and mioflazine were generously provided by Dr. H. Van Belle, Janssen Research Foundation (Beerse, Belgium). [^{3}H]R75231 (5 Ci/mmol) was obtained from Dr. A.P. IJzerman, Center for Bio-Pharmaceutical Sciences (Leiden, The Netherlands), and dilazep was a gift

from Asta Werke (Frankfurt, Germany). NBTGR, DPR, NBMPR, CHAPS, trypsin (EC 3.4.21.4, 10,200 units/mg), adenosine and uridine were supplied by Sigma (St. Louis, MO). All other compounds were of reagent grade.

3.2.2 Membrane Preparation

Cortical membranes (P_2 fraction) and a partially purified synaptosomal preparation (P_v fraction) were prepared as described previously (see section 2.2.2). All [^{3}H]NBMPR binding assays were done using the P_2 fraction. [^{3}H]Uridine uptake studies utilized the partially purified P_v preparation, as this preparation had the greatest concentration of intact synaptosomes (see section 2.3.3). Binding characteristics for the P_2 and P_v fractions were not significantly different (see section 2.3.3).

Human erythrocyte ghosts were prepared as described by Dodge et al. (1963), except that membranes were resuspended ultimately in 5 mM sodium phosphate (pH 8.0 at 22 °C) and stored at -70 °C.

3.2.3 [^{3}H]NBMPR Binding

[^{3}H]NBMPR binding was performed at room temperature (≈22°C) in 50 mM Tris (pH 7.1). Incubations (final volume of 1 ml) were initiated by adding an aliquot of the P_2 fraction (approx. 0.5 mg protein), or erythrocyte ghost membranes (approx. 3 μg protein), to a glass tube containing the appropriate concentration of radioligand (± inhibitors). In some cases, as indicated in Results, membranes were incubated with inhibitors for 30 min prior to the [^{3}H]NBMPR binding assays. Incubations were terminated after 45 min, unless otherwise noted, by dilution with 5 ml of ice-cold 10 mM Tris (pH 7.1). This was followed by rapid filtration through Whatman GF/B filters, which were then washed once with 5 ml of ice-cold 10 mM Tris (pH 7.1). Non-specific binding of [^{3}H]NBMPR was determined in the presence of 10 μM dilazep. In cases where membranes were washed after incubation with

inhibitor, each wash consisted of a centrifugation (40,000 g, 10 min), followed by resuspension in 10 ml of buffer (unless otherwise noted), and incubation at room temperature for 30 min.

3.2.4 [^{3}H]R75231 Binding

[^{3}H]R75231 was found to be >95% pure when tested by thin layer chromatography as described in IJzerman et al. (1992). In preliminary experiments with synaptosomal membranes, specific binding of [^{3}H]R75231 was not detected; this was likely due to the relatively low number of nucleoside transporters in this preparation and the high nonspecific binding observed with [^{3}H]R75231. Therefore, human erythrocyte ghosts, a more homogeneous membrane preparation with a large number of nucleoside transporters, were used for all [^{3}H]R75231 binding studies. Incubations (final volume of 1 ml) were conducted at room temperature in 50 mM Tris (pH 7.1), containing 0.01% CHAPS (w/v) to reduce non-specific binding (see section 2.3.1.1). The incubations were initiated by adding an aliquot of membrane suspension (approx. 3 µg protein) to a glass tube containing the appropriate concentration of radioligand (± inhibitors). Incubations were terminated after 2 h, unless otherwise noted, by dilution with 5 ml of ice-cold 10 mM Tris (pH 7.1). This was followed by rapid filtration through Whatman GF/B filters and four washes with 5 ml of ice-cold 10 mM Tris (pH 7.1). The filters were presoaked in 0.1% bovine serum albumin for 2 h at room temperature to reduce the non-specific binding of [^{3}H]R75231 to the filters. Non-specific binding of [^{3}H]R75231 to the erythrocyte membranes was determined by preincubating membranes with 10 µM NBMPR and 10 µM dilazep for 30 minutes prior to the assay and then conducting the assay in the presence of these inhibitors. [^{3}H]R75231 concentrations were determined from aliquots of incubation mixtures removed immediately prior to filtration.

To assess the reversibility of [^{3}H]R75231 binding (0.3

nM), ligand dissociation was initiated, after a 2 h association period, by the addition of 1 ml of buffer containing displacing agent(s) or by 100-fold dilution in incubation buffer. The effects of relatively weak inhibitors on the dissociation of [^{3}H]R75231 (e.g. adenosine, diazepam) were determined in the presence of a supramaximal inhibitory concentration of NBMPR (5 μM); this ensured a complete block of [^{3}H]R75231 reassociation and allowed the study of potential allosteric interactions as described by Hammond (1991). For some dissociation agents, similar experiments were conducted in the presence of 0.005% saponin to eliminate the possibility of intravesicular [^{3}H]R75231 trapping.

3.2.5 [^{3}H]Uridine Uptake

Assays were conducted as described in section 2.2.4, with the following modifications. Synaptosomes were incubated with inhibitors for 30 min prior to exposure to 10 μM [^{3}H]uridine. In cases where synaptosomes were washed after exposure to inhibitors, each wash consisted of a centrifugation (16,000 g, 2 min), resuspension in 1 ml buffer, and a 15 min incubation. Following the 5 washes (which in binding studies was shown to be sufficient to eliminate the inhibitory effect of NBMPR, see Figure 3.1), the synaptosomes were resuspended (approx. 200 μg protein/10 μl) in buffer or 100 μM dilazep (to quantify non-mediated uptake) and the uptake assay was conducted.

3.2.6 Data Analysis

Protein concentrations were determined as described by Lowry et al. (1951) using bovine serum albumin as the standard. Dimethyl sulfoxide, used as a solvent in some cases, did not have a significant effect on binding ([^{3}H]NBMPR or [^{3}H]R75231) or [^{3}H]uridine uptake at the concentrations used; up to 0.1 and 0.33% respectively. K_D, B_{max}, and $t_{1/2}$ values were derived by nonlinear regression analysis (GraphPAD Inplot, version 4.03) of non-transformed experimental data. Dissociation rate constants (k_{-1}) were found by linear

regression using the relationship $\ln(B/B_0)=k_{-1}t$, where B_0 is the bound ligand at time 0, and B is the bound ligand at specific times (t) after initiation of ligand dissociation. Statistics were completed as described in section 2.2.6.

3.3 Results

3.3.1 [^{3}H]NBMPR Binding

The interaction of R75231 with the nucleoside transporter was first analyzed by examining its capacity to interfere with the binding of [^{3}H]NBMPR. R75231 is a potent inhibitor of [^{3}H]NBMPR binding in rabbit cortical synaptosomes, K_i=0.44±0.02 nM, (see section 2.3.2). The reversibility of R75231 binding was examined by incubating synaptosomes with 3.75 nM R75231 or 3.75 nM NBTGR for 30 min and then washing the membranes repeatedly, with aliquots being removed after each wash for assay of [^{3}H]NBMPR binding (Figure 3.1). Even after 5 washes there was no significant change in the degree of R75231 inhibition of [^{3}H]NBMPR binding (Student's t-test), whereas NBTGR inhibition of [^{3}H]NBMPR binding was eliminated after the second wash. In a set of similar experiments, synaptosomes were washed once with 1 μM NBTGR or 10 mM adenosine in an attempt to displace the R75231. This was followed by 5 washes with buffer. These washes were effective in eliminating all of the inhibitory activity of NBTGR and adenosine (i.e. not significantly different from the binding observed in the absence of inhibitor; ANOVA). However, the majority of R75231 inhibition of [^{3}H]NBMPR binding still remained after the washes. The washing with adenosine or NBTGR resulted in a decrease of R75231 inhibition of [^{3}H]NBMPR binding of 9% (from 90.9 ± 0.4 to 81.8 ± 0.4%), and 5% (from 88.6 ± 1.3 to 83.7 ± 0.4%), respectively (ANOVA; Bonferroni, n=3).

To better understand the nature of R75231 interaction with the [^{3}H]NBMPR binding site (competitive or non-competitive), the binding of a range of concentrations of [^{3}H]NBMPR was examined using synaptosomes pre-incubated (30 min) with 0 nM (control), 1.5 nM, or 3.75 nM R75231 (Figure

3.2). R75231 caused a significant (repeated measures ANOVA, $P<0.01$), concentration dependent increase in apparent K_D and a decrease in apparent B_{max} values (Table 3.1). A similar shift in kinetic constants remained after 5 washes, although the differences in B_{max} were no longer statistically significant (Table 3.1).

3.3.2 [^{3}H]Uridine Uptake

R75231 selectively inhibited NBMPR-sensitive [^{3}H]uridine uptake in rabbit cortical synaptosomes without affecting NBMPR-resistant [^{3}H]uridine uptake, even at concentrations up to 10 μM (see section 2.3.3.1.2). Since R75231 was observed to inhibit [^{3}H]NBMPR binding even after repeated washing, the ability of R75231 to inhibit transporter-mediated [^{3}H]uridine uptake after repeated washing of synaptosomes preincubated with R75231 was investigated. As shown in Figure 3.3, the inhibition of transporter-mediated [^{3}H]uridine uptake by 100 nM R75231 (54±5% before washing) was not decreased significantly (30±10%), by the wash procedure (ANOVA; Bonferroni). In parallel experiments, a comparable inhibition produced by 10 nM NBMPR (43±2% before washing) was decreased significantly (5%±9) (ANOVA; Bonferroni) by the same wash prctocol (n=4).

3.3.3 [^{3}H]R75231 Binding

3.3.3.1 Binding Kinetics

The time course for specific binding of 0.3 nM [^{3}H]R75231 to human erythrocyte ghost membranes (Figure 3.4) fit a single component model (correlation coefficient = 0.99) and was rapid, with a half-time of 1.6±0.4 min. Because of the unusual binding characteristics of [^{3}H]R75231, the association kinetics of [^{3}H]R75231 were also examined in the presence of 3 nM NBMPR or 10 nM DPR (Figure 3.4). From this it was determined that [^{3}H]R75231 binding in the presence of inhibitors reached equilibrium within 90 min.

In all cases, dissociation of [^{3}H]R75231 from the

membranes conformed to a single component (correlation coefficient for the data graphed according to the equation $\ln(B/B_0)=k_{-1}t$ was 0.99, regardless of method of dissociation). However, the rate of dissociation of [^{3}H]R75231 was relatively slow and depended on the compound used to prevent its reassociation (Figures 3.5, Table 3.2). The addition of 5 μM NBMPR, 5 μM DPR, or 5 μM diazepam (+ NBMPR), each induced a complete dissociation of site-bound [^{3}H]R75231 (±10%) with rates (≈0.01 min^{-1}, see Table 3.2) not significantly different from those observed using a protocol involving a 100-fold dilution in the absence of displacer (0.007±0.002 min^{-1}, Student-Newman-Keuls). However, the dissociation of [^{3}H]R75231 obtained with 100-fold dilution was incomplete (≈75%). In comparison, the dissociation rates observed using 5 μM R75231 or 5 μM mioflazine (+ NBMPR) as the displacing agents were significantly slower (≈0.0025 min^{-1}; see Table 3.2, Student-Newman-Keuls) than those obtained using 5 μM NBMPR alone, and the use of 6 mM adenosine (+ NBMPR) resulted in a faster rate of dissociation (0.020±0.005 min^{-1}).

In contrast to any of the other compounds used to prevent [^{3}H]R75231 reassociation, 5 μM R75231 or 5 μM mioflazine (+ NBMPR) resulted in an initial transient increase in [^{3}H]R75231 binding to the membranes (Figure 3.5). In order to eliminate experimental artifact as an explanation, the possibility that these displacers were causing an increased free [^{3}H]R75231 concentration, by displacing [^{3}H]R75231 bound to the glass tubes, was examined. In control experiments with no membranes, neither R75231 nor NBMPR caused an increase in free [^{3}H]R75231.

Different dissociation rates observed with different displacing agents could be a result of these agents altering the permeability of membrane vesicles which had trapped [^{3}H]R75231. Therefore, dissociation experiments were repeated for some inhibitors in the presence of 0.005% saponin to eliminate the possibility of intravesicular [^{3}H]R75231 trapping. The effectiveness of saponin in permeabilizing the

vesicles was demonstrated in control experiments with trypsin which cleaves the nucleoside transport protein on a site on the cytoplasmic side of the membrane (Plagemann et al., 1988). In the presence of trypsin (10 μg/ml) and 0.005% saponin, 84±5% of the [^{3}H]R75231 binding was eliminated compared to only 27±14% in the presence of trypsin alone. Therefore, the saponin was effectively permeabilizing the cells under these experimental conditions. The dissociation profile obtained in the presence of saponin (Figure 3.6) was similar to that obtained in its absence. The dissociation rates in the presence of 5 μM R75231, 5 μM NBMPR, or 6 mM adenosine (+ NBMPR) were 0.0023±0.0001, 0.0052±0.0004, 0.011±0.001 min^{-1} respectively, which are not significantly different from those obtained in the absence of saponin (ANOVA; Bonferroni).

3.3.3.2 Equilibrium Binding

Mass law analysis of [^{3}H]R75231 binding to erythrocyte ghost membranes resulted in K_D and B_{max} values of 0.35 nM and 44.1 pmol/mg protein, respectively (Figure 3.7). This B_{max} value is not significantly different from that found using [^{3}H]NBMPR (Figure 3.8, 41.3±4.1 pmol/mg protein, Student's t-test).

Results from studies on the inhibition of [^{3}H]R75231 (0.3 nM) binding to erythrocyte ghost membranes by a variety of transport inhibitors and substrates can be seen in Figure 3.9, with the corresponding IC_{50} values compiled in Table 3.3. The order of potency of these agents for inhibition of [^{3}H]R75231 binding (dilazep>mioflazine>DPR>adenosine>uridine) is the same as that found for inhibition of [^{3}H]NBMPR binding (Figure 3.10, Table 3.3). However, the IC_{50} values for inhibition of [^{3}H]R75231 binding obtained in the present study were all 15 to 25-fold higher than those obtained for inhibition of [^{3}H]NBMPR binding. Since both the inhibition of [^{3}H]R75231 and [^{3}H]NBMPR binding experiments were done at radioligand concentrations approximately equal to their respective K_D values, these differences are probably due to the unique,

tight-binding characteristics of [^{3}H]R75231. Finally, R75231 inhibition of [^{3}H]NBMPR gave a pseudo-Hill coefficient significantly greater than unity (Student's t-test).

3.4 Discussion

R75231 has been shown to be a potent inhibitor of nucleoside transport in a variety of systems (Van Belle and Janssen, 1991; Van Belle et al., 1991; Baer et al., 1991; Masuda et al., 1991, 1992; IJzerman et al., 1992; Wainwright et al., 1993; Beukers et al., 1994; Bohm et al., 1994). As a result, the potential of R75231 and other related compounds, such as lidoflazine, mioflazine, and draflazine, to become clinically useful drugs, is being investigated (see section 1.2.6). The mode of action of R75231 is believed to be blockade of nucleoside transport which results in increased adenosine interaction with its receptors; however, a recent study has proposed that some of R75231 protective characteristics may be due to calcium channel blockade (Grover and Sleph, 1994). This proposal cannot be discounted; however, it should be noted that the R75231 concentration used in this study was ≈10 fold higher than that used in other studies due to the relative insensitivity of rat nucleoside transporters to inhibition by flazines (Masuda et al., 1991, 1992; Van Belle and Janssen, 1991; Mollhoff et al., 1992; de Haan et al., 1993; Van Belle et al., 1993; Wainwright et al., 1993; Beukers et al., 1994; Bohm et al., 1994; Grover and Sleph, 1994). This high concentration and species differences may explain the different results observed. The property of R75231 as a calcium channel blocker remains to be tested.

R75231 is of specific interest because of its unique tight binding properties to the nucleoside transporter and its long-lasting effects *in vivo*. Van Belle and Janssen (1991) demonstrated this tight binding when they showed that R75231-mediated inhibition of inosine transport in erythrocytes was not reversed by washing the erythrocytes. Similarly, the present study showed that in synaptosomal membranes

preincubated with R75231, repeated washing with buffer, even with the initial wash containing a known displacer such as adenosine or NBTGR, had little or no effect on the degree of inhibition of [^{3}H]NBMPR binding by R75231. Likewise, the inhibitory effect of R75231 on transporter-mediated [^{3}H]uridine uptake by synaptosomes was resistant to wash-out, whereas NBTGR was removed completely using the same experimental protocol. The complex interactions of R75231 with the NBMPR binding site on the nucleoside transporter were also reflected in the "mixed" type kinetics observed for R75231 inhibition of [^{3}H]NBMPR binding to rabbit cortical synaptosomes (dose dependent shift in apparent K_D and B_{max}, Table 3.1). This "mixed" inhibition, neither exclusively competitive or non-competitive, suggests that R75231 may bind to the NBMPR-binding site as well as another site on the nucleoside transporter. Once again, repeated washing of the synaptosomes did not eliminate the inhibitory effect of R75231 on [^{3}H]NBMPR binding (Table 3.1), demonstrating the extremely tight binding characteristics of this inhibitor.

Further detailed investigation of these interactions has been made possible by the availability of [^{3}H]R75231. IJzerman et al. (1992) reported that [^{3}H]R75231 dissociated from its binding sites in calf lung membranes extremely slowly ($t_{1/2}$>24 h), leading these authors to suggest that R75231 bound "pseudo-irreversibly". However, the results of the present study show that the nature of [^{3}H]R75231 binding to the nucleoside transporter, like that of other transport inhibitors (see section 1.2.3.1), may be dependent upon the species or tissue used. [^{3}H]R75231 bound to human erythrocyte ghost membranes could be displaced entirely by the transport inhibitors NBMPR and DPR. Furthermore, 100-fold dilution with buffer also led to the dissociation of the majority (74±6%) of the bound [^{3}H]R75231 within 5 h. Therefore, the site specific binding of [^{3}H]R75231 to human erythrocyte ghost membranes is reversible, albeit slowly when compared to other nucleoside transport probes such as [^{3}H]NBMPR. This slow dissociation

was not due to intravesicular trapping of [^{3}H]R75231 as similar dissociation profiles and rates were observed in the presence of a membrane permeabilizing concentration of saponin. The initial increase of [^{3}H]R75231 binding when R75231 was used as a dissociating agent was also present, but smaller, in the presence of saponin. This may be due to the membrane disrupting effects of saponin.

Not all displacers resulted in the same rate of [^{3}H]R75231 dissociation. Specifically, adenosine enhanced the rate of dissociation, while the addition of R75231 or mioflazine slowed the rate of dissociation significantly and actually resulted in a transient increase in [^{3}H]R75231 binding to the membranes (Figure 3.5a). Other examples of complex binding interactions with the nucleoside transporter have been observed using [^{3}H]NBMPR. As was the case with [^{3}H]R75231 dissociation (Table 3.2), substrates such as adenosine increased the rate of [^{3}H]NBMPR dissociation (Jarvis et al., 1983; Koren et al., 1983; Hammond and Clanachan, 1985; Hammond, 1991). DPR, which had no significant effect on [^{3}H]R75231 dissociation (Table 2.3), slowed the rate of [^{3}H]NBMPR dissociation (Jarvis et al., 1983; Wohlhueter et al., 1983; Hammond and Clanachan, 1985; Shi and Young, 1986; Hammond, 1991). R75231 slowed the rate of [^{3}H]R75231 dissociation (Table 2.3), but R75231 and the related compound R57974 had no effect on [^{3}H]NBMPR dissociation (Ogbunude and Baer, 1989; Hammond, 1991).

The ability of nucleoside transport inhibitors and substrates to alter the rate of [^{3}H]R75231 (and [^{3}H]NBMPR) dissociation can be explained by the presence of multiple interacting inhibitor binding sites on the nucleoside transporter, as proposed by Hammond, 1991. In this model, the first site is the [^{3}H]NBMPR binding site to which many other inhibitors and substrates also bind as evidenced by competition studies (Hammond and Clanachan, 1984; Jarvis, 1986; Jones and Hammond, 1992). The second site binds certain transport inhibitors, such as R75231 and mioflazine, as well

as some nucleoside substrates, and interactions with this site affect the affinity of the first site for its ligands. This model is supported by results from several other studies where: a) two [^{3}H]dilazep binding components were observed to be associated with the nucleoside transporter in S49 mouse lymphoma cells, with only the high affinity component being inhibited by NBMPR (Gati and Paterson, 1989); b) approximately twice as many [^{3}H]R75231 binding sites were found on calf lung membrane as [^{3}H]NBMPR binding sites (IJzerman et al., 1992); and c) transport inhibitors and substrates were demonstrated to modify the rate of dissociation of site-bound [^{3}H]NBMPR (Koren et al., 1983; Jarvis et al., 1983; Wohlhueter et al., 1983; Hammond and Clanachan, 1985; Hammond, 1991).

Relatively high concentrations of R75231 may interact with the second, allosteric, site on the transporter leading to an increase in the affinity of the first site for R75231, in effect, "locking" R75231 onto this binding site (positive cooperativity). This hypothesis is supported by the following observations: a) approximately twice as many [^{3}H]R75231 binding sites were found on calf lung membrane as [^{3}H]NBMPR binding sites (IJzerman et al., 1992); b) [^{3}H]R75231 displacement by mioflazine (+NBMPR) or R75231 was significantly slower than that produced by the other transport inhibitors tested (Figure 3.5). In fact, both R75231 and mioflazine resulted in an initial increase in [^{3}H]R75231 binding prior to inducing dissociation; presumably, as a result of interactions with the "allosteric" site which increased the affinity of the first site for [^{3}H]R75231; c) IJzerman et al. (1992) could not displace more than 35% of the pre-bound [^{3}H]R75231 with inhibitors; however, complete inhibition was observed if [^{3}H]R75231 was not given prior access to the binding site; and d) pseudo-Hill coefficients greater than unity have been found for R75231 (and related compounds) inhibition of [^{3}H]NBMPR binding in this study and others (IJzerman et al., 1989; Jones and Hammond, 1992). IJzerman et al. (1989) postulated that these high pseudo-Hill

coefficients may be due to each R75231 molecule displacing 2 or more bound [^{3}H]NBMPR molecules, but these results can also be interpreted as positive cooperativity.

It should be noted that although approximately twice as many [^{3}H]R75231 binding sites were found on calf lung membrane as [^{3}H]NBMPR binding sites (IJzerman et al., 1992), this study found the two radioligands, at the concentrations used, to label approximately the same number of sites in human erythrocyte ghost membranes. This discrepancy may be due to species differences, with the second [^{3}H]R75231 binding site in human erythrocyte ghost membranes having a lower affinity for [^{3}H]R75231 than that in calf lung membranes. Unfortunately, it was not possible to confirm the proposed second [^{3}H]R75231 binding site in human erythrocyte ghost membranes using saturation binding experiments because of the high background binding present when high concentrations of [^{3}H]R75231 were used (Figure 3.7). The higher affinity for the second [^{3}H]R75231 binding site in calf lung membranes may also be connected to the apparent "irreversible" binding seen in this preparation (IJzerman et al., 1992), as opposed to the reversible binding observed in human erythrocyte ghost membranes.

[^{3}H]R75231 is a racemate, with the (-)-isomer of R75231 being a more potent inhibitor of [^{3}H]NBMPR binding (25-30 fold) than the (+)-isomer (IJzerman et al., 1992). In addition, the pseudo-Hill coefficient for (-)-R75231 inhibition of [^{3}H]NBMPR binding was 2.0, whereas that for (+)-R75231 was unity (IJzerman et al., 1992). Therefore, (-)-R75231 is likely responsible for the unique binding characteristics of [^{3}H]R75231. As a result, (-)-R75231, or draflazine, is being focused on for clinical application (Beukers et al., 1994; Bohm et al., 1994).

In conclusion, [^{3}H]R75231 binds tightly, but reversibly, to components of the human erythrocyte nucleoside transporter. Data obtained from [^{3}H]R75231 dissociation studies and analysis of inhibition of [^{3}H]NBMPR binding by R75231 suggest

the presence of multiple interacting binding sites on the nucleoside transport complex for R75231 and congeners. The observed kinetics of ligand interactions with the transporter will, therefore, be dependent on the relative affinities of the compounds for these interacting binding sites.

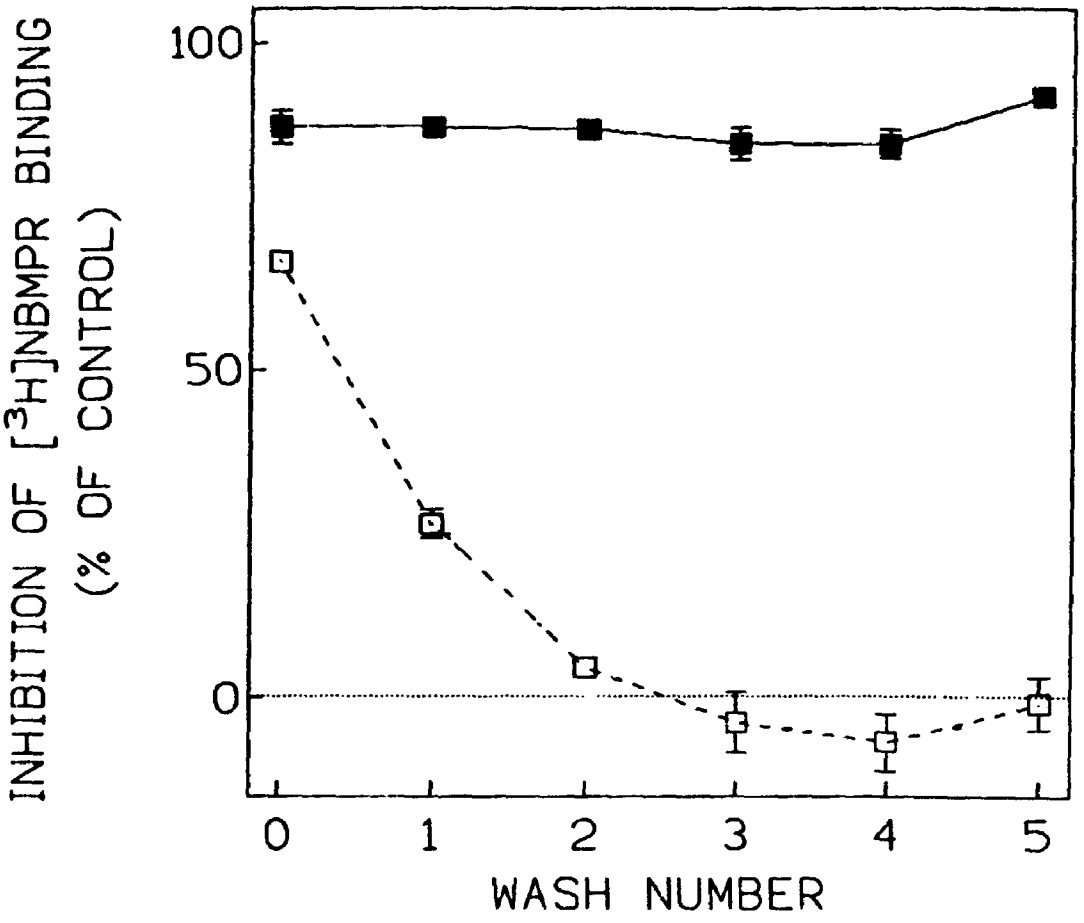


Figure 3.1: The effect of repeated washes on 3.75 nM R75231 (■) and 3.75 nM NBTGR (□) inhibition of 0.4 nM [^{3}H]NBMPR binding in rabbit cortical synaptosomes. Inhibition is expressed as a percentage of the maximum inhibition of [^{3}H]NBMPR binding as determined in the presence of 10 μM dilazep (control). Each point represents the mean ± S.E.M. from three experiments conducted in duplicate.

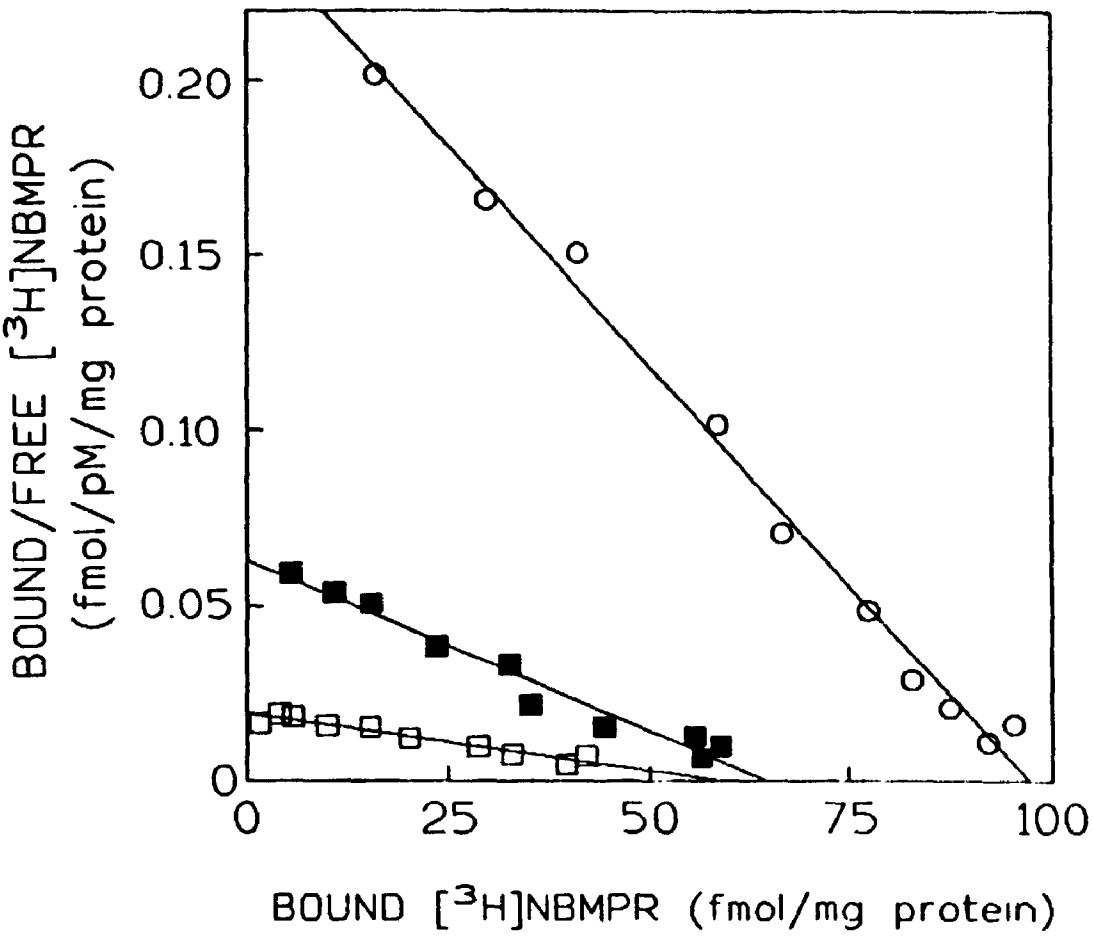


Figure 3.2: Mass law analysis of R75231 inhibition of [^{3}H]NBMPR binding in rabbit cortical synaptosomes. The specific binding of 10 concentrations (0.1-10 nM) of [^{3}H]NBMPR was measured in the presence of 0 nM (○), 1.5 nM (■), or 3.75 nM (□) R75231. This is a representative Scatchard plot from five experiments. Average K_D and B_{max} values derived from these experiments are shown in Table 3.1.

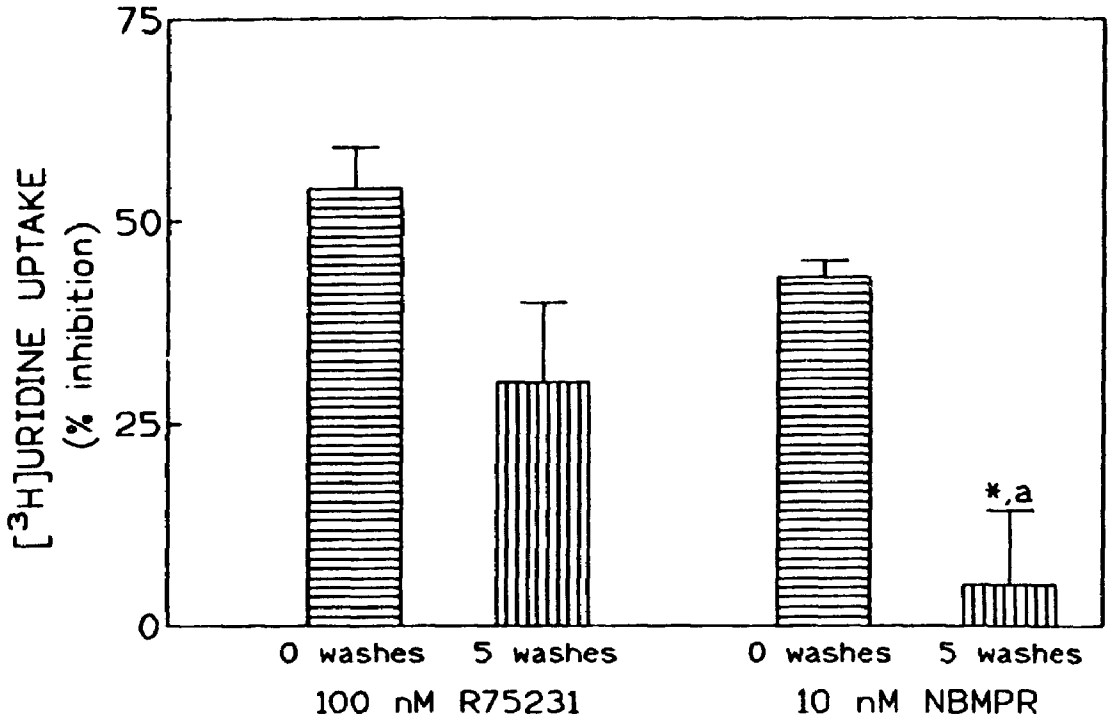


Figure 3.3: The effect of repeated washes on 100 nM R75231 and 10 nM NBTGR inhibition of mediated 10 μM [^{3}H]uridine uptake in rabbit cortical synaptosomes. Inhibition is expressed as a percentage of the maximum inhibition of transporter-mediated [^{3}H]uridine uptake as determined in the presence of 100 μM dilazep (control). Bars with horizontal lines and vertical lines represent inhibition before washing (0 washes) and after 5 washes, respectively. Each bar represents the mean ± S.E.M. from four experiments conducted in triplicate.

* = significant difference between 0 wash and 5 wash results (ANOVA; Bonferroni, $P<0.05$).

a = inhibition not significantly different from 0

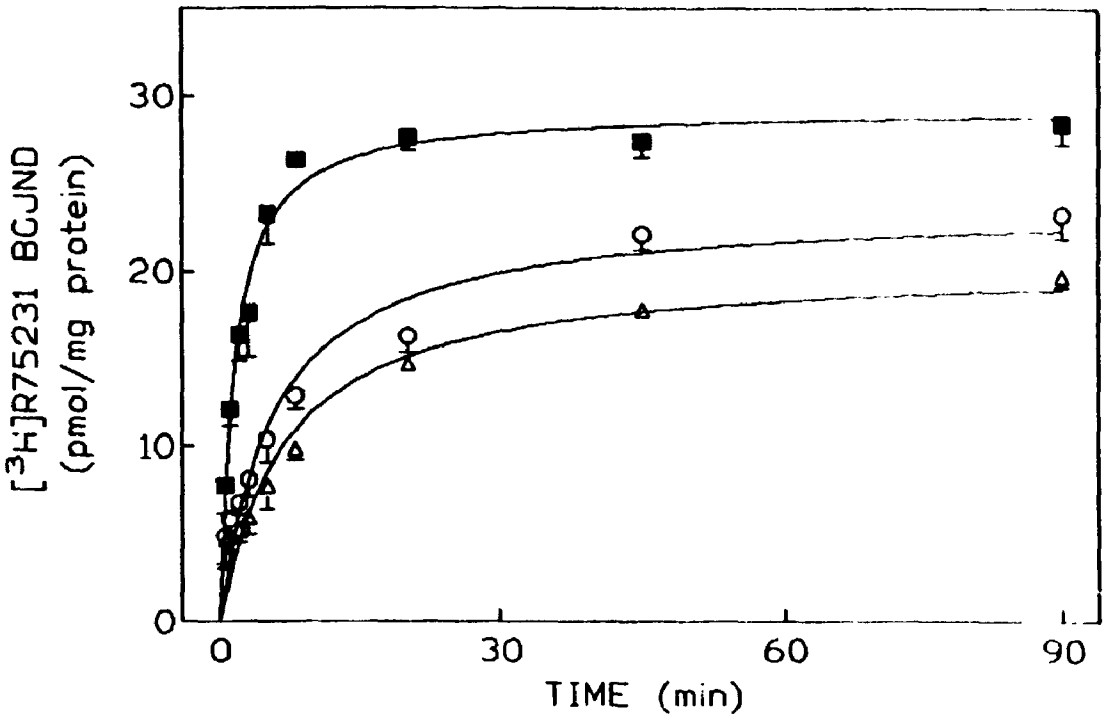
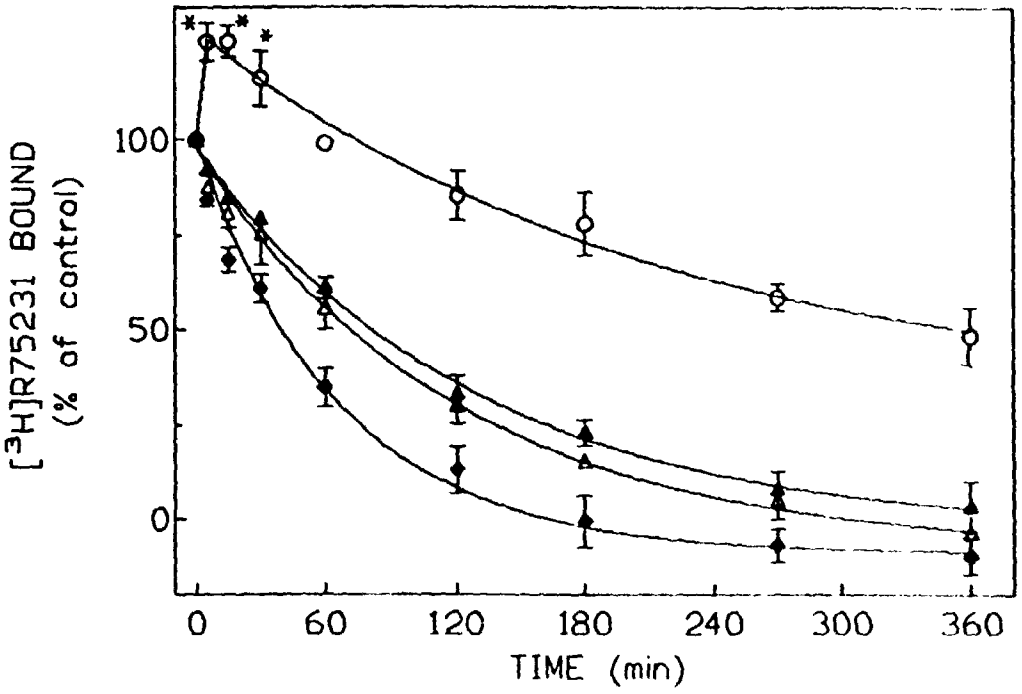
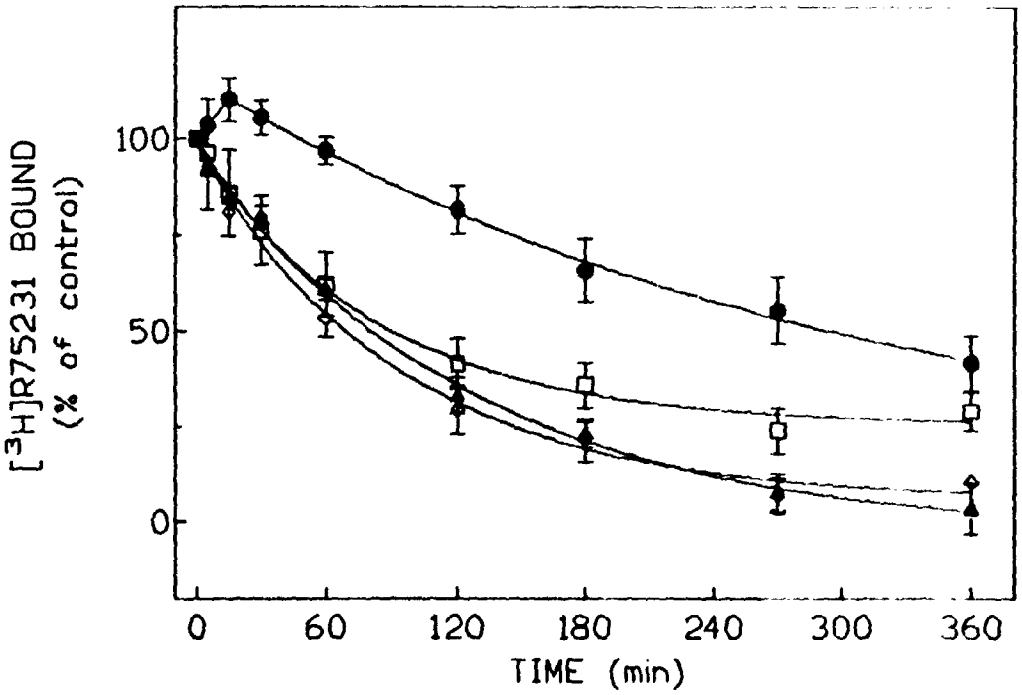


Figure 3.4: Time course of 0.3 nM [^{3}H]R75231 binding in human erythrocyte ghost membranes. Specific binding was observed in the absence of inhibitors (■), and in the presence of 3 nM NBMPR (▲) or 10 nM DPR (○). Each point represents the mean ± S.E.M. from three experiments.

Figure 3.5: Time course of dissociation of 0.3 nM [^{3}H]R75231 binding in human erythrocyte ghost membranes. Dissociation was initiated, after a 2 h association period, by the addition of: *Panel (a)*, 5 μM R75231 (○), 5 μM NBMPR (▲), 5 μM diazepam + 5 μM NBMPR (△), or 6 mM adenosine + 5 μM NBMPR (◆); *Panel (b)*, 5 μM mioflazine + 5 μM NBMPR (●), 5 μM DPR (◇), or 100-fold dilution (□). Results are expressed as a percentage of the maximum binding (control) obtained prior to the addition of displacers. Each point represents the mean ± S.E.M. from at least three experiments.

* = significant increase in binding over control (Student-Newman-Keuls, $P<0.05$).


[3H]R75231 BOUND
(% of control)
100
50
0
0
60
120
180
240
300
360
TIME (min)


[3H]R75231 BOUND
(% of control)
100
50
0
0
60
120
180
240
300
360
TIME (min)

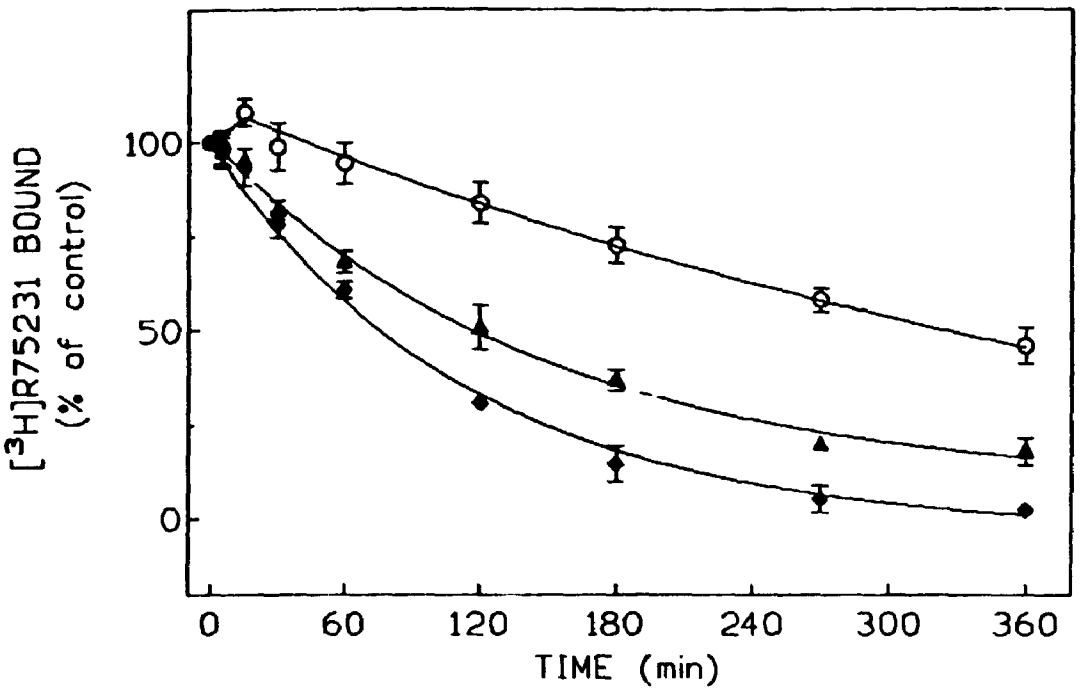


Figure 3.6: Time course of dissociation of 0.3 nM [^{3}H]R75231 binding in the presence of 0.005% saponin in human erythrocyte ghost membranes. Dissociation was initiated, after a 2 h association period, by the addition of 5 μM R75231 (○), 5 μM NBMPR (▲), 6 mM adenosine + 5 μM NBMPR (◆). Results are expressed as a percentage of the maximum binding (control) obtained prior to the addition of displacers. Each point represents the mean ± S.E.M. from four experiments.

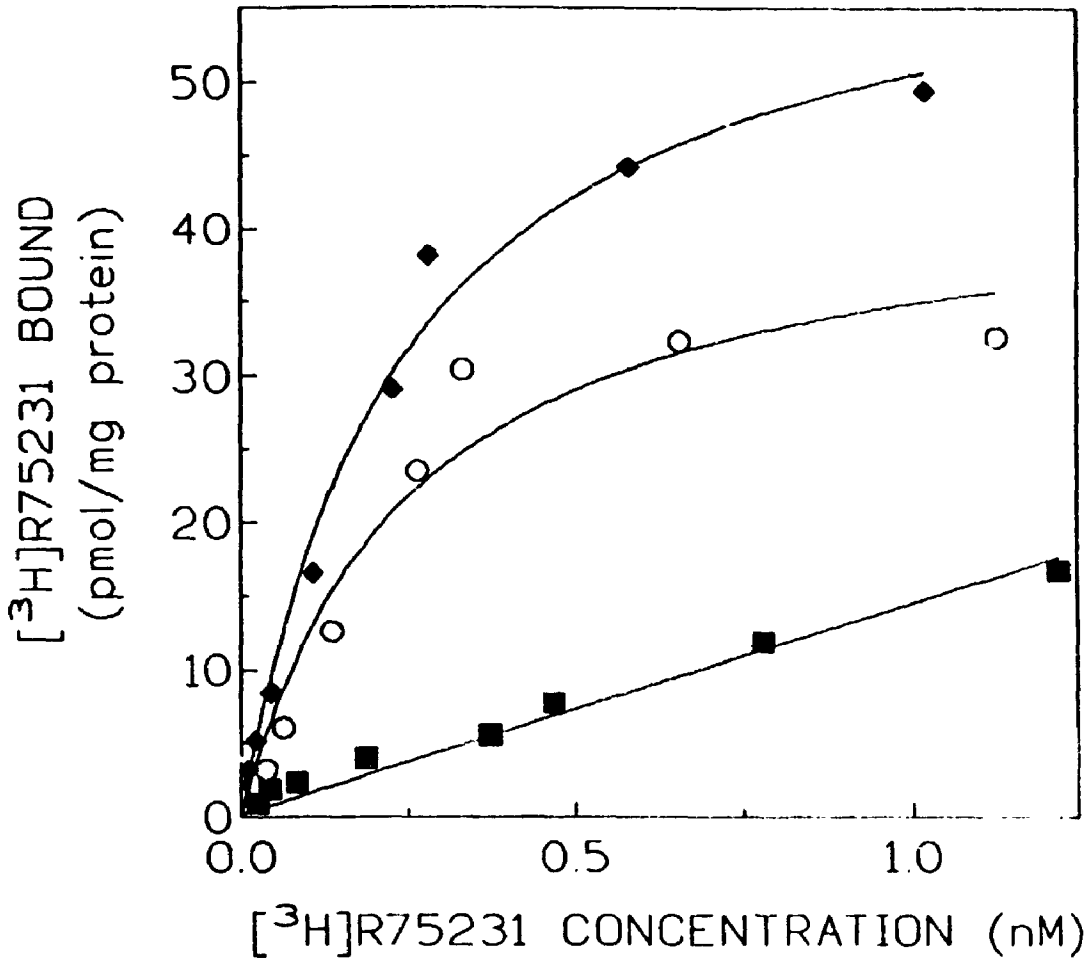


Figure 3.7: Mass law analysis of [^{3}H]R75231 binding to human erythrocyte ghost membranes. The binding of a range of concentrations of [^{3}H]R75231 were tested in the absence (total, ◆) and presence (non-specific, ■) of 10 μM NBMPR/10 μM dilazep. Specific binding (O) was calculated as the difference between the total and nonspecific binding components. These are representative data from four experiments conducted in duplicate. Average (±S.E.M.) K_D = 0.35 ± 0.07 nM and B_{max} = 44.1 ± 6.2 pmol/mg protein.

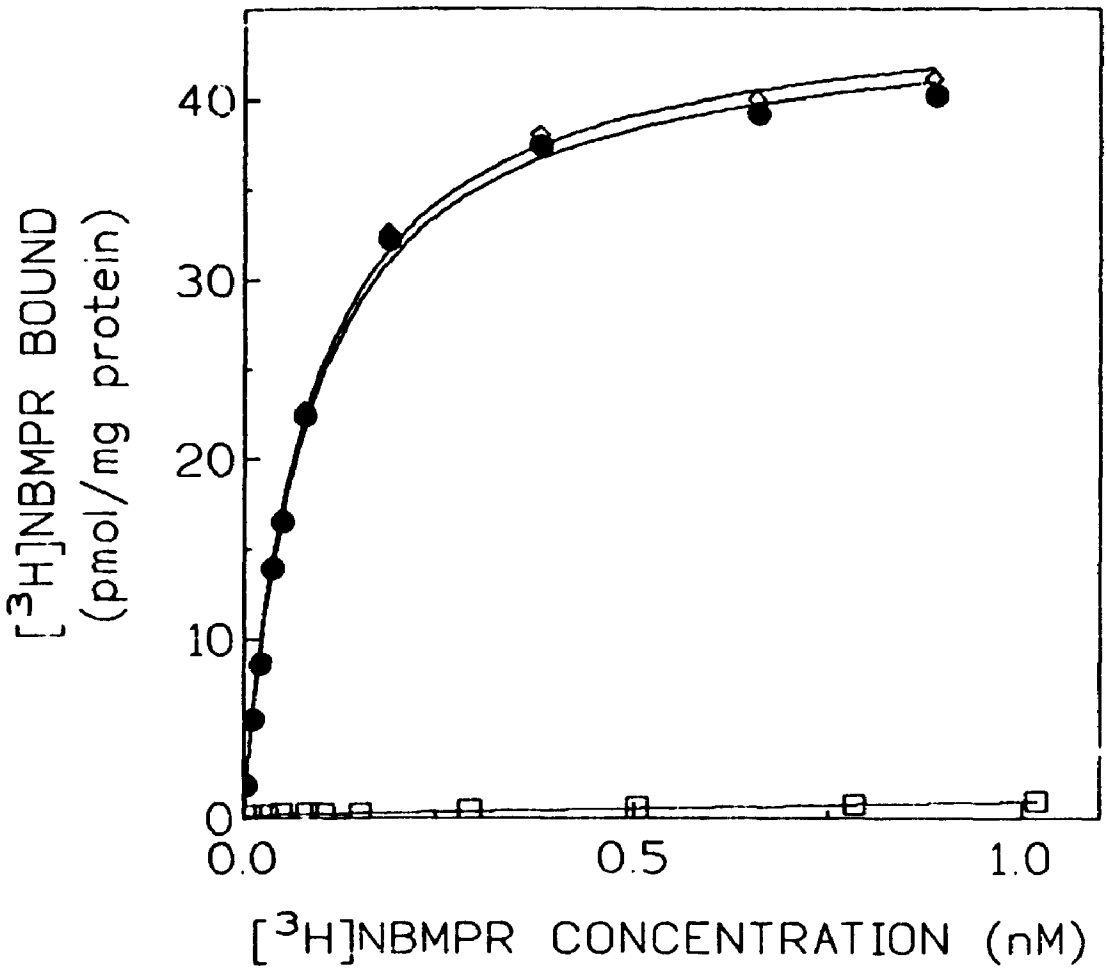


Figure 3.8: Mass law analysis of [^{3}H]NBMPR binding to human erythrocyte ghost membranes. The binding of a range of concentrations of [^{3}H]NBMPR were tested in the absence (total, ◇) and presence (non-specific, □) of 10 μM dilazep. Specific binding (●) was calculated as the difference between the total and nonspecific binding components. These are representative data from four experiments conducted in duplicate. Average (±S.E.M.) K_D = 0.09 ± 0.005 nM and B_{max} = 41.3 ± 4.1 pmol/mg protein.

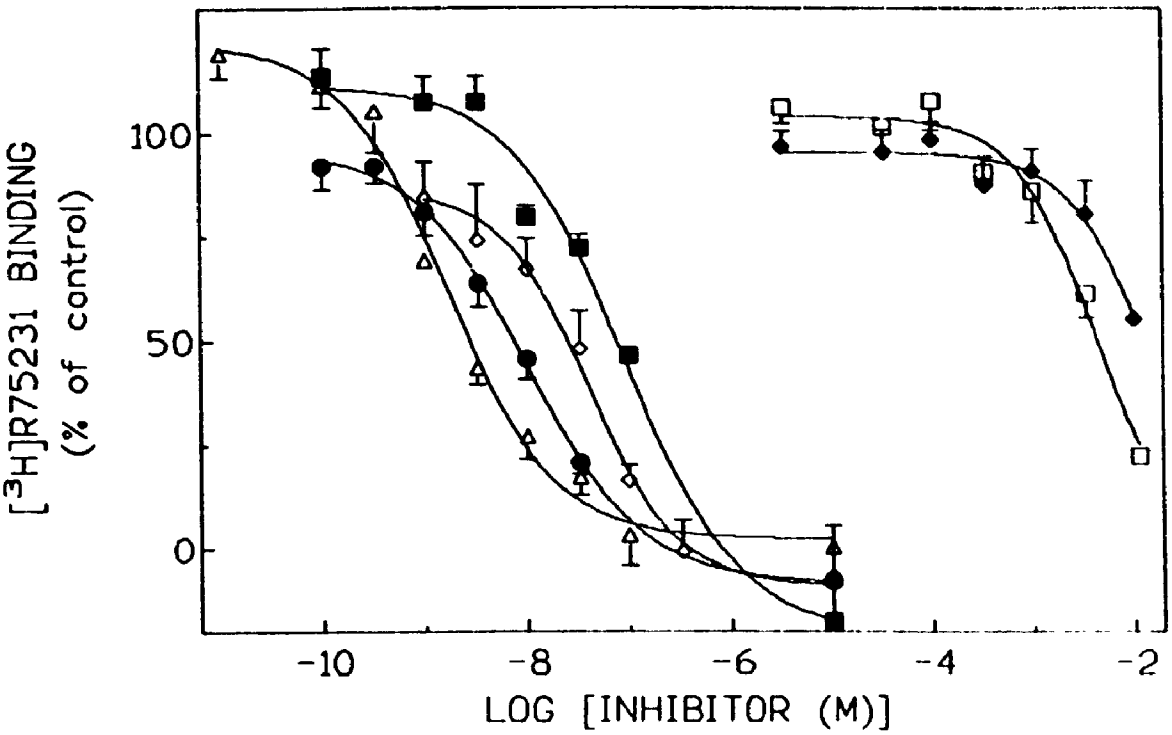


Figure 3.9: Inhibition of 0.3 nM [^{3}H]R75231 binding to human erythrocyte membranes in the presence of a range of concentrations of dilazep (▲), NBMPR (●), mioflazine (◇), DPR (■), uridine (▲), or adenosine (□). Control binding was defined as the specific binding observed in the absence of inhibitor. Membranes were exposed to inhibitor and [^{3}H]R75231 concurrently and incubated for 5 h to ensure that binding equilibrium was attained. Each point represents the mean ± S.E.M. from three experiments conducted in duplicate. The corresponding IC_{50} values and pseudo-Hill coefficients are shown in Table 3.3.

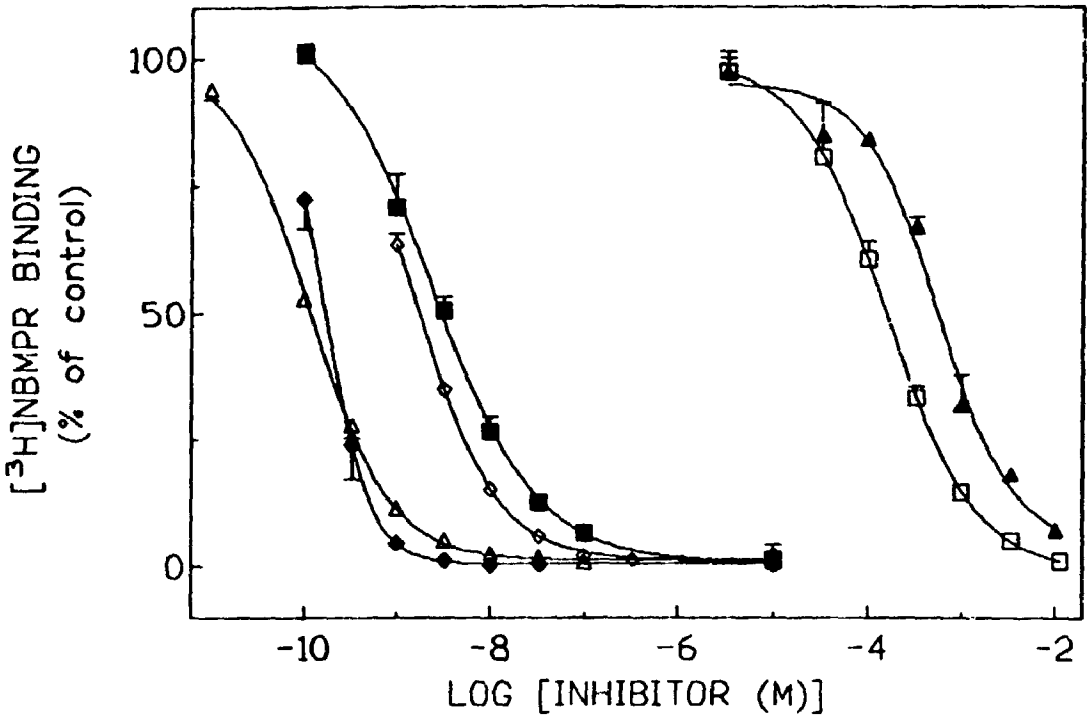


Figure 3.10: Inhibition of 0.08 nM [^{3}H]NBMPR binding to human erythrocyte membranes in the presence of a range of concentrations of dilazep (△), R75231 (◆), mioflazine (◇), DPR (■), uridine (▲), or adenosine (□). Control binding was defined as the specific binding observed in the absence of inhibitor. Membranes were exposed to inhibitor and [^{3}H]NBMPR concurrently and incubated for 5 h. Each point represents the mean ± S.E.M. from three experiments conducted in duplicate. The corresponding IC_{50} values and pseudo-Hill coefficients are shown in Table 3.3.

Table 3.1: Binding of [^{3}H]NBMPR to rabbit cortical synaptosomes preincubated with 0 nM (control), 1.5 nM, or 3.75 nM R75231. Binding constants (K_D, B_{max}) were derived from nonlinear regression analyses of site-specific binding data (0.1 nM to 10 nM [^{3}H]NBMPR), both before (UNWASHED) and after (WASHED) washing the preparations 5 times. Each value is expressed as the mean ± S.E.M. from five experiments.

	UNWASHED		WASHED	
	K_D (nM)	B_{max} (fmol/mg)	K_D (nM)	B_{max} (fmol/mg)
CONTROL	0.41±0.03	129±14	0.42±0.03	140±20
1.5 nM R75231	1.7±0.2[a]	106±13[a]	1.9±0.4[a]	126±26
3.75 nM R75231	4.0±0.5[a]	93±17[a]	2.6±0.5[a]	104±16

[a] significantly different from the corresponding 0 wash CONTROL, repeated measures ANOVA, $P<0.01$

Table 3.2: Effects of nucleoside transport inhibitors and substrates on 0.3 nM [^{3}H]R75231 dissociation from human erythrocyte membranes. The final concentration of displacer was 5 μM for all compounds tested except adenosine which was 6 mM (also see Figure 3.5). The rate of [^{3}H]R75231 dissociation found using R75231 or mioflazine (+NBMPR) as the displacer was calculated starting at the point of maximum [^{3}H]R75231 binding (see Figure 3.5). Each point represents the mean ± S.E.M. from at least three experiments conducted in duplicate.

	[^{3}H]R75231
	k_{-1} (min^{-1})
R75231	0.0026±0.0004
NBMPR	0.009±0.002
mioflazine[b]	0.0024±0.0007
DPR	0.011±0.002
adenosine[b]	0.020±0.005
diazepam[b]	0.010±0.002

[b] 5 μM NBMPR was included with the displacer to ensure full dissociation

Table 3.3: Effects of nucleoside transport inhibitors and substrates on 0.3 nM [^{3}H]R75231 and 0.08 nM [^{3}H]NBMPR binding to human erythrocyte membranes. Membranes were exposed to inhibitor and radioligand concurrently and incubated for 5 h (also see Figure 3.9, 3.10). Each point represents the mean ± S.E.M. from at least three experiments conducted in duplicate.

	[^{3}H]R75231		[^{3}H]NBMPR	
	IC_{50}	n_H	IC_{50}	n_H
dilazep	1.7±0.3 nM	0.82±.08	0.11±0.01 nM	1.00±0.04
R75231	-	-	0.18±0.03 nM	1.91±0.06
NBMPR	8.2±1 nM	0.87±0.07	-	-
mioflazine	40±10 nM	0.98±0.05	1.7±0.2 nM	1.02±0.04
DPR	57±5 nM	0.78±0.01	3.1±0.6 nM	0.92±0.07
adenosine	4.0±0.3 mM	1.2±0.1	160±30 μM	1.0±0.2
uridine	>10 mM	-	520±70 μM	1.0±0.2

CHAPTER 4: CHANGES IN NUCLEOSIDE TRANSPORT CHARACTERISTICS AS A RESULT OF CELLULAR DIFFERENTIATION IN HUMAN NEUROBLASTOMA (LA-N-2) CELLS

4.1 Introduction

This study involved examining changes in nucleoside transport characteristics (number and types of transporters) as a result of cellular differentiation. Cellular differentiation of neuroblastoma cells includes the formation of neurites, increase in size of soma, inhibition of cell division, and increased activities of neural enzymes such as tyrosine hydroxylase, choline acetyltransferase and acetylcholinesterase (Prasad, 1975; Seeger et al., 1977; Sidell et al., 1983; Singh et al., 1990; Rylett et al., 1993). The rationale for this work is two-fold: 1/ Knowledge of the nucleoside transport characteristics of a neuroblastoma cell line may aid in the selection and development of treatment strategies involving cytotoxic nucleoside analogues; 2/ To evaluate the suitability of a human, neuronal, tumour cell line as a model for studying the mechanisms of cellular regulation of nucleoside transporter activity.

Neuroblastoma, a neural crest tumour, originates from the autonomic nervous system and is most commonly associated with mutations on the short arm of chromosome 1 (Siegal and Sato, 1986; Schwab, 1988; Sreedhar, 1991). It is one of the most common childhood cancers, accounting for 5-7% of all childhood malignancies (Siegal and Sato, 1986; Sreedhar, 1991) and approximately 15% of cancer deaths in childhood (Siegel and Sato, 1986). Neuroblastoma is also one of the most difficult tumours to treat with a 2-year survival rate of ≈25% (Siegel et al., 1986; Sreedhar, 1991). Therefore, alternative therapeutic strategies are being examined (Mirkin and Fink, 1988; Rosenthal, 1991). One of these strategies involves inducing terminal differentiation of the tumour (*in vivo*) with

low doses of cytotoxic nucleoside analogues (Prasad, 1975; Rosenthal, 1991). This strategy stems from the idea that malignant cells represent a blockade in cell maturation which may be reversible. This is supported by reports of spontaneous differentiation of malignant neuroblastoma tumours into a benign state, in 1-2% of all cases (Siegel et al., 1986; Mirkin and Fink, 1988; Prasad, 1991; Rao, 1991). Cytosine arabinoside and 5-azacytidine were able to induce differentiation in human leukaemic cells (Rosenthal, 1991). As well, cytosine arabinoside and 5-bromodeoxyuridine induced differentiation in mouse and human neuroblastoma cells (Prasad, 1975; Ponzoni et al., 1991; Lanciotti et al., 1992). Since these cytotoxic nucleoside analogues are substrates for the nucleoside transporter (Paterson et al., 1981; Zimmerman et al., 1989; Cass, 1994), delineation of the pattern of expression of transporter subtypes in undifferentiated and differentiated neuroblastoma cells may allow for a more precise clinical application of the pharmacological tools currently available, as well as aid in the development of alternative therapeutic strategies.

Treatment for neuroblastoma can involve a combination of surgery, radiation therapy, and chemotherapy (Siegel et al., 1986; Sreedhar, 1991). Cytotoxic compounds which are transported by nucleoside transporters are not currently part of the clinical treatment for neuroblastoma. However, due to their success for treatment of other cancers (Plunkett and Saunders, 1991; Perigaud et al, 1992; Cass, 1994), and the relative ineffectiveness of current treatments for neuroblastoma (Siegel et al., 1986; Sreedhar, 1991), they may soon be evaluated for use as single agents or in combination with other agents. As discussed in section 1.2.6, knowledge of the quantity and type of nucleoside transport present in tumour cells is extremely important for the selection of appropriate drug combinations to apply clinically.

There has been little research into the regulation of nucleoside transport; it is apparent, however, that growth and

differentiation are linked to changes in transporter numbers and/or activity (see section 1.2.5). Due to the importance of adenosine in the nervous system (see section 1.2.4), the suitability of a human tumour cell line of neuronal origins as a model to study the mechanisms of nucleoside transporter regulation, was evaluated.

LA-N-2 cells are a cholinergic, human neuroblastoma line derived from the primary tumour, clinical Stage IV neuroblastoma, of a 3-year old female (Seeger et al., 1977). LA-N-2 cells can undergo differentiation in response to transfer of the cells from a basal, serum-containing medium to an enriched serum-free medium (Rylett et al., 1993). Undifferentiated LA-N-2 cells grew in clumps with peripheral cells having short cytoplasmic processes. Cells grown in serum free medium changed, with the cells tending to grow individually, the cell bodies becoming more rounded, and the extending of neurite-like processes with lengths of $\geq$300 μM (Rylett et al., 1993). This morphological differentiation was also accompanied by biochemical changes. These included changes in the metabolism of choline as well as an approximately 7-fold increase in the number and activity of hemicholinium-sensitive cholinergic transporters (Rylett et al., 1993). Since differentiation in various cell types has resulted in modified numbers and activities of nucleoside transporter subtypes (see section 1.2.5), it is possible that differentiation of LA-N-2 cells, which resulted in increased choline transport (Rylett et al., 1993), could also induce changes in nucleoside transport characteristics. Therefore, this study characterized nucleoside transport in the human neuroblastoma LA-N-2 cell line before and after differentiation.

4.1.1 Hypothesis

Nucleoside transporter subtype expression and activity changes upon the differentiation of LA-N-2 cells.

4.2 Methods

4.2.1 Materials

The neuroblastoma cell line LA-N-2 was generously provided by Dr. R. J. Rylett, University of Western Ontario, Canada (originally obtained from Dr. R.C. Seeger of Children's Hospital, Los Angeles, CA, U.S.A.). Leibovitz's L-15 medium, Dulbecco's-Hams F12 (1:1) medium, and gentamicin were obtained from Gibco (Oakville, Ontario). Transferrin, progesterone, insulin, sodium selenite, putrescine, adenosine, uridine, uridine monophosphate, uridine diphosphate, uridine triphosphate, uracil, DPR, NBMPR, and nitrobenzylthioguanosine, were supplied by Sigma (St. Louis, MO). Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT) and formycin B from Calbiochem (La Jolla, CA). [^{3}H]NBMPR (30 Ci/mmol) and [^{3}H]formycin B (14 Ci/mmol) were from Moravek Biochemicals, Inc. (Brea, CA). [^{3}H]Uridine (35-50 Ci/mmol) was purchased from ICN Biochemicals (Costa Mesa, CA). ^3H_2O (1 mCi/g) and [*carboxyl*-^{14}C]-dextran-carboxyl (0.58 mCi/g) were purchased from Du Pont Canada (Markham, ON). Dilazep was provided by Asta Werke (Frankfurt, Germany). All other compounds were of reagent grade.

4.2.2 Cell Culture

Human neuroblastoma cells LA-N-2 (passage # 77-97) were grown in Corning flasks (25 cm^2) in Leibovitz's L-15 medium containing 10% fetal bovine serum and 50 μg/ml gentamicin in humidified air at 37°C. Cells were passaged weekly and medium replaced every three to four days. For assays, the cells were plated onto culture plates at a density of ≈ 50,000 cells/cm^2 and maintained for three days with L-15/serum. Cells were then divided into groups and were grown in either L-15/serum (undifferentiated cells) or serum-free defined medium (SF-N2) comprised of Dulbecco's-Hams F12 (1:1) containing transferrin (100 μg/ml), sodium selenite (30 nM), progesterone (20 nM), bovine insulin (5 μg/ml), putrescine hydrochloride (100 μM),

HEPES-NaOH, pH 7.4 (15 mM) and gentamicin (50 μg/ml) (differentiated cells). The medium was changed after two days and all assays were conducted four days after the cells were divided into groups.

4.2.3 [^{3}H]Nucleoside Uptake

Uptake assays were conducted on monolayer cultures in 35 mm culture dishes at room temperature, unless otherwise noted. Cultures were washed 3 times with 1.5 ml of incubation medium (130 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 3 mM glucose, and 4 mM HEPES, pH 7.4). For inhibition studies, substrates (adenosine, formycin B) and [^{3}H]formycin B were added simultaneously, whereas inhibitors (NBMPR, dilazep, DPR) were incubated for 20 min with the cells prior to initiation of the assay. Nucleoside uptake was initiated by the addition of 1 ml of either 10 μM [^{3}H]uridine (0.25 μCi/ml) or 25 μM [^{3}H]formycin B (0.5 μCi/ml), with appropriate inhibitors, in incubation medium. Assays were terminated by the addition of 4 ml of stop solution (25 μM DPR in ice-cold incubation medium) which was subsequently aspirated. Plates were then washed rapidly with two, 4 ml aliquots of stop solution. Non-mediated uptake was determined using plates preincubated with 10 μM NBMPR and 10 μM DPR. Uptake at time 0 s was calculated by adding stop solution immediately to an assay conducted with ice cold [^{3}H]uridine solution using plates preincubated with 10 μM NBMPR and 10 μM DPR. Cells were digested with 500 μl of 1 M NaOH and aliquots were taken for quantization of radioactivity (liquid scintillation spectrometry) and protein content.

The potential contribution of ion-dependent nucleoside transporters to the accumulation of [^{3}H]formycin B by LA-N-2 cells was assessed. Uptake of 25 μM [^{3}H]formycin B was measured as described above using the usual incubation media (130 mM NaCl), and "sodium-free" incubation media with iso-osmotic replacement of sodium chloride by lithium chloride or N-methylglucamine chloride. The effect of iso-osmotic

replacement of potassium chloride was examined similarly. It should be noted that the "sodium-free" and "potassium-free" buffer likely contained a low (<200 μM) concentration of the replaced ion due to contaminants in the other salts used to prepare the buffers. However, it is unlikely that the residual sodium or potassium would be sufficient to drive an ion-dependent transporter as it has been determined in other systems that the sodium-dependent and potassium-dependent transporters have a $K_{1/2}$ of about 10 mM (Jarvis, 1989; Jarvis et al., 1989; Williams et al., 1989; Dagnino et al., 1991*b*; Plagemann, 1991; Williams and Jarvis, 1991) and 300 mM (Lee et al., 1988), respectively.

Both cell volume and cell number estimates were found using plates (4 per cell type) which had undergone a "mock" assay (ie. initial washes in buffer, incubation in the absence of [^{3}H]substrate, and final washes in ice-cold buffer). The cells were then removed from the plates following a 2 min incubation in buffer containing trypsin (0.5 mg/ml, EC 3.4.21.4, 10 200 units/mg) and EDTA (0.5 mM). Intracellular and extracellular water volumes were determined by incubating the suspended cells for 3 min with a combination of [^{14}C]dextran-carboxyl (cell-impermeant) and ^3H_2O layered over a 200 μl cushion of silicone oil/mineral oil (21:4, v/v) in microcentrifuge tubes. At the end of the incubation, cells were pelleted (60 s, 12 000 g). The supernatant and oil were removed, the pellets were digested in 1 M NaOH, and the ^{3}H and ^{14}C were counted by liquid scintillation spectrometry. The number of intact cells was found using a Neubauer type counter and trypan blue exclusion (0.01%). The percentage of intact cells was 83±4 and 80±2 for differentiated and undifferentiated cells, respectively (n=10).

4.2.4 DNA Assay

Cell DNA content was found using a method described by Setaro and Morely (1976). In brief, cells were scraped from plates which had undergone "mock" assays. Cells were washed

twice in ice-cold 0.6 N trichloroacetic acid and once in potassium acetate/absolute alcohol (9.8 g/L) (760 g, 20 min, 4°C). The pellet was incubated in absolute alcohol for 15 min at 60°C, centrifuged (760 g, 20 min), and dried. DNA was extracted from the pellets in 1 N perchloric acid for 30 min at 75°C. Diaminobenzioc acid dihydrochloride (100 µl of 1.32 M) was added to 100 µl of samples and standards (Herring sperm DNA) and incubated at 60°C for 30 min. Perchloric acid (0.6 N, 2.3 ml) was added to each tube and the fluorescence was measured (420 nM excitation, 520 nM emission).

4.2.5 Nucleoside Metabolism

Uptake assays were conducted as above for [^{3}H]uridine (10 µM, 10 µCi/ml, 15 s incubation) or [^{3}H]formycin B (25 µM, 6 µCi/ml, 60 s incubation). After the stop solution had been aspirated, 50 µl of ice cold perchloric acid (7% w/v) was added and the precipitate was removed by centrifugation (12 000 g, 60 s). An aliquot of the acid extract was neutralized with $NaHCO_3$ and centrifuged (12 000 g, 60 s) to remove the precipitate. Samples were chromatographed as described previously for uridine (Jones and Hammond, 1992) or on polyethyleneimine cellulose paper using water for formycin B (Crawford et al., 1990). Radioactivity was extracted with water (1 ml) for 1 hour before the addition of scintillation fluid.

4.2.6 [^{3}H]NBMPR Binding

[^{3}H]NBMPR binding studies were conducted at room temperature, in Hank's Balanced Salt solution without Ca^{2+} and Mg^{2+} (138 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 0.3 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 6 mM glucose). LA-N-2 cells were removed from the plates by a 2 min incubation in 1 ml of buffer containing trypsin (0.5 mg/ml, EC 3.4.21.4, 10 200 units/mg) and EDTA (0.5 mM), after which trypsin inhibitor was added (1.5 mg of Type I-S). Incubations (final volume of 1 ml) were initiated by the addition of a 200 µl aliquot of LA-N-2 cells (≈ 60,000

cells) to a glass culture tube containing the appropriate concentration of [^{3}H]NBMPR (± inhibitors). Incubations were terminated after 30 min by dilution with 5 ml of ice-cold 10 mM Tris (pH 7.1) followed by rapid filtration through Whatman GF/B filters. Filters were washed once with 5 ml of ice-cold 10 mM Tris (pH 7.1). The radioactivity on the filters was counted by liquid scintillation spectrometry. Non-specific binding of [^{3}H]NBMPR was determined in the presence of 10 μM NBTGR.

4.2.7 Data Analysis

Protein concentrations were determined as described by Lowry et al. (1951) using bovine serum albumin as the standard. Dimethyl sulfoxide, used as a solvent in some cases, did not influence uptake at the concentrations used (up to 0.1%). K_i values were found using the relationship $K_i=IC_{50}/(1+[L]/K_D)$, where IC_{50} was interpolated from best fit sigmoid curves of percentage of inhibition versus log inhibitor concentration, [L] is the concentration of radioligand, and K_D is the equilibrium dissociation constant for the radioligand used. All other values reported were derived from computer-generated algebraic relationships obtained via nonlinear regression analysis (GraphPAD Inplot, version 4.03) of experimental data. Statistics were completed as described in section 2.2.6.

4.3 Results

4.3.1 Cell Differentiation

As described by Rylett et al. (1993), undifferentiated LA-N-2 cells can be induced to undergo morphological and biochemical differentiation by transfer to the SF-N2 medium of Bottenstein and Sato (1979). The cells in SF-N2 medium continue to differentiate for at least six days. Cells maintained beyond six days became only loosely adherent to the culture plate surface and began to degenerate (Rylett et al., 1993). For this reason, assays were conducted after four days

of differentiation.

[^{3}H]Nucleoside uptake results are expressed as pmol of nucleoside influx per intact cell. The accuracy of the cell counting for both differentiated and undifferentiated cells was confirmed by DNA assays. The DNA content per cell was not significantly different between the cell types (Table 4.1, Student's paired t-test), suggesting that the counting procedure was equally accurate for both undifferentiated and differentiated cells. In comparison, the protein per cell and cell volume was significantly higher in the differentiated cells (Table 4.1; Student's paired t-test). These changes are as expected for differentiated neuroblastoma cells (Prasad, 1975).

4.3.2 [^{3}H]Nucleoside Uptake

All nucleoside uptake assays were terminated by the addition of ice cold stop solution. This method was only valid if the stop solution terminated uptake immediately and prevented efflux of the substrate from the cells. Cells were incubated with 25 μM [^{3}H]formycin B for 60 s, after which 4 ml of ice cold stop solution was added to the plate. The stop solution was removed immediately (control) or after a defined interval to examine the efflux of uridine. This was followed by 2 more rapid washes in ice cold stop solution (as usual). This stop procedure was found to be effective in terminating [^{3}H]formycin B influx and subsequent efflux (Figure 4.1).

To assess potential changes in nucleoside transport characteristics due to differentiation of LA-N-2 cells, [^{3}H]nucleoside uptake assays were conducted using formycin B. Formycin B is a poorly metabolized nucleoside analogue which is a substrate for all known nucleoside transporters, except the N2 and N4 subtypes (Vijayalakshmi and Belt, 1988; Plagemann and Woffendin, 1989; Crawford et al., 1990; Crawford and Belt, 1991; Cass, 1994). As was the case in other cell types (Vijayalakshmi and Belt, 1988; Plagemann and Woffendin, 1989; Crawford et al., 1990; Crawford and Belt, 1991), LA-N-2

cells did not metabolize [^{3}H]formycin B significantly, whereas [^{3}H]uridine was metabolized extensively to nucleotides (Table 4.2, Students t-test).

Both undifferentiated and differentiated LA-N-2 cells accumulated [^{3}H]formycin B rapidly via a transporter-mediated process. The initial rate of transporter-mediated uptake of 25 μM [^{3}H]formycin B by the differentiated cells (3.2±0.4 pmol/10^6 cells/s) was significantly less (Student's paired t-test, $P<0.05$) than that by the undifferentiated cells (4.0±0.5 pmol/10^6 cell/s)(Figure 4.2). At equilibrium (extrapolated from the total uptake data), intracellular [^{3}H]formycin B was 36±4 and 40±6 μM for undifferentiated and differentiated cells, respectively. This intracellular [^{3}H]formycin B concentration is higher than expected (25 μM), if only a facilitated diffusion system is operating. However, no Na^+ or K^+-dependent transport of [^{3}H]formycin could be detected by iso-osmotic ion replacement with Li^+ or N-methylglucamonium$^+$ (Figure 4.3).

The sensitivity of undifferentiated and differentiated cells to nucleoside transport inhibitors and substrates was examined by comparing concentration-effect curves for their inhibition of transporter-mediated [^{3}H]formycin B uptake (Figures 4.4 and 4.5, Table 4.3). Of the agents tested, only the inhibition profile for NBMPR was significantly different in the two cell populations (Figure 4.4). In undifferentiated cells, NBMPR completely inhibited transporter-mediated [^{3}H]formycin B uptake in a monophasic manner with an IC_{50} value of 0.5±0.2 nM and a pseudo-Hill coefficient not significantly different from unity (Students's t-test). In contrast, differentiated cells fit a two-component model significantly better than a one-component model (F-test, $P<0.05$). Based on the two component model, the IC_{50} values for NBMPR inhibition were 0.9±0.2 nM and 7±2 μM for the NBMPR-sensitive and -resistant components, respectively. The NBMPR-insensitive component accounted for 17±5% of the total mediated uptake. The NBMPR-sensitive component of [^{3}H]formycin B uptake in the

differentiated cells had an IC_{50} for NBMPR (0.9±0.2 nM) that was not significantly different from that seen in the undifferentiated cells (0.5±0.2 nM, Students t-test).

The IC_{50} obtained for formycin B inhibition of transporter-mediated [^{3}H]formycin B uptake (≈280 μM, Table 4.3) provides an indication of its K_m for the nucleoside transporter, and is similar to that seen in other cell lines (Zimmerman et al., 1989; Plagemann and Woffendin, 1989; Vijayalakshmi et al., 1992). Likewise the potencies of DPR, dilazep and adenosine for inhibition of nucleoside transport in LA-N-2 cells are similar to those reported for other systems (Plagemann et al., 1988).

4.3.3 [^{3}H]NBMPR Binding

Specific binding of [^{3}H]NBMPR (0.4 nM) was measured in LA-N-2 cells that had been exposed to trypsin and trypsin inhibitor (as described in section 4.2.6) and those which had not been exposed to trypsin and trypsin inhibitor, so that the effect of removing cells from the plates with trypsin on the number of [^{3}H]NBMPR binding sites could be assessed (Table 4.4). The removal of the cells from the plates without trypsin was done mechanically (via rubber policeman) rather than enzymatically. There was no significant difference in specific [^{3}H]NBMPR binding as a result of being removed from the plates with trypsin (Student-Newman-Keuls, $P<0.05$). This is as expected due to the trypsin cleavage site on the transporter being located on the cytoplasmic side of the membrane (Plagemann, 1988).

Mass law analysis of the specific binding of [^{3}H]NBMPR to undifferentiated and differentiated cells (Figure 4.6) indicated a single class of binding sites for both cell populations (one-site model provided better fit than two site model; F-test, $P<0.05$). For undifferentiated and differentiated cells respectively, the K_D values of 0.14±0.02 and 0.14±0.03 nM, and B_{max} values of 220±30 and 230±30 fmol/10^6 cells, were not significantly different (Student's t-test).

Similarly, there was no difference between undifferentiated and differentiated cells for inhibition of 0.4 nM [^{3}H]NBMPR binding by dilazep or DPR (Figure 4.7, Table 4.5). All inhibition profiles had pseudo-Hill coefficients that were not significantly different from unity (ANOVA). Assuming that each [^{3}H]NBMPR binding site represents one functional nucleoside transporter (see section 1.2.3.1.2), there is an estimated 1.4 X 10^5 *es* transporters per cell. This value is within the range of 2 x 10^4 and 6 X 10^5 *es* transporters per cell reported for other cultured cell lines (Plagemann et al., 1988).

4.4 Discussion

This study is the first report to characterize the nucleoside transport system in a human neuroblastoma cell line. Undifferentiated LA-N-2 human neuroblastoma cells accumulate nucleosides via *es* nucleoside transporters. Differentiation of the LA-N-2 cells in serum-free media induced a change in nucleoside transport characteristics; specifically, the initial rate of transporter-mediated [^{3}H]formycin B accumulation by the cells was increased. This increase was not due to an increase in the number of *es* transporters as there was no significant change in the number of [^{3}H]NBMPR binding sites (which represent *es* transporters). This enhanced rate of transport (≈25%) was likely due to the functional expression of an *ei* transporter in the differentiated cells (17% of total transporter-mediated [^{3}H]formycin B uptake) that was not present in the undifferentiated cells.

The nucleoside transport characteristics of LA-N-2 cells were similar to those of other neuronal cells and cultured tumour cell lines. Both *es* (undifferentiated and differentiated cells) and *ei* transporters (differentiated cells) have been found in other neuronal preparations (see section 1.2.4). As well, both the K_D and B_{max} values for

[^{3}H]NBMPR binding were within the range of values observed in other neuronal preparations and cultured cell lines (Plagemann et al., 1988; Geiger and Nagy, 1990). Although NBMPR inhibition of transporter-mediated [^{3}H]formycin B uptake showed NBMPR-sensitive and -resistant uptake components (ie. a biphasic inhibition profile), dilazep did not. There have been reports of dilazep distinguishing between *es* and *ei* transporters, resulting in biphasic inhibition profiles for nucleoside uptake (Plagemann and Woffendin, 1987*b*; Geiger et al., 1988; Lee and Jarvis, 1988*a*,*b*; Hammond, 1991; section 2.3.3.1.2). However, these inhibition profiles for dilazep are often not as clearly biphasic as those of NBMPR (Plagemann and Woffendin, 1987*b*; Geiger et al., 1988; Lee and Jarvis, 1988*a*; Hammond, 1991; section 2.3.3.1.2). This is due to dilazep having a relatively similar affinity for *es* and *ei* transporters, as opposed to NBMPR which displays a >1000-fold difference in affinity for *es* and *ei* transporters. There are also instances where dilazep has demonstrated high affinity for both *ei* and *es* transporters (Cass, 1994) and where dilazep inhibition of mediated uptake could not be distinguished as biphasic in a system possessing both *es* and *ei* transporters (Plagemann et al., 1988; Johnston and Geiger, 1989). Therefore, although NBMPR could distinguish [^{3}H]formycin B uptake by LA-N-2 cells into two components (biphasic inhibition profile), a similar affinity of dilazep for *es* and *ei* nucleoside transporters and the low proportion of *ei* transport (<20%) in differentiated LA-N-2 cells may have resulted in the monophasic inhibition profile for dilazep inhibition of [^{3}H]formycin B uptake.

At equilibrium, the estimated intracellular concentration of [^{3}H]formycin B in both undifferentiated and differentiated cells (36 and 40 μM, respectively) was higher than the extracellular media concentration of 25 μM. This concentrative effect was not due to formycin B metabolism (Table 4.2), nor can it be attributed to ion-dependent nucleoside transport, as none was detected. A similar,

sodium-independent, concentrative accumulation of [^{3}H]formycin B has been observed previously (Plagemann and Woffendin, 1989) in mouse P388 leukaemia cells, and was speculated to be due to binding to intracellular components.

It is not known if the increase in NBMPR-resistant nucleoside transport in the differentiated cells was due to enhanced activity of existing membrane-located transporters, translocation from intracellular compartments, or increased transcription. However, increased choline uptake into serum-free differentiated LA-N-2 cells, due to an increased number of transporters, appeared to be under transcriptional control as the increase in uptake was largely blocked (>80%) by the RNA polymerase inhibitor, α-amanitin (Rylett et al., 1993). It should be noted, that the control of nucleoside transporter expression may be regulated by a different mechanism than that of choline transport. First, the increase in choline transport was much larger than the corresponding increase in nucleoside transport (600% vs. 25%) (Rylett et al., 1993). Second, the affected choline transporters are ion-dependent whereas the nucleoside transporters operate via facilitated diffusion. Third, the control of expression of cellular functions by differentiation of LA-N-2 cells appears to be regulated by multiple mechanisms, possibly involving different cell signalling pathways. This is suggested by the increase in the activity of choline acetyltransferase in response to differentiation of LA-N-2 cells by retinoic acid (Singh et al., 1990); whereas, choline transport was increased in response to differentiation by serum-free medium (Rylett et al., 1993).

This present report is the first to demonstrate cellular differentiation resulting in an increase in *ei* nucleoside transporter activity. Changes in nucleoside transport characteristics, including the number of transporters, the presence of transporter subtypes, and transporter activity, have been linked to cellular differentiation (see section 1.2.5). Differentiation of HL-60 human promyelocytic cells by

N,N-dimethylformamide (Chen et al., 1986), DMSO (Lee et al., 1990; Sokaloski et al., 1991), or phorbol 12-myristate 13-acetate (PMA) (Lee et al., 1991; Sokaloski et al., 1991; Lee, 1994), resulted in a decrease in *es* mediated transport. Cells differentiated by DMSO and PMA also showed an increase in sodium-dependent nucleoside transport (Lee et al., 1990, 1991; Sokaloski et al., 1991). An increase in sodium-dependent transport was seen in IEC-6 rat intestinal epithelial cells differentiated by low serum content (Jakobs et al., 1990). Transformation of rat-2 fibroblasts, by protein-tyrosine kinase, resulted in an increase in *es* mediated transport activity without an increase in the number of [^{3}H]NBMPR binding sites (Meckling-Gill and Cass, 1992). Treatment of S1 macrophages with colony-stimulating factor 1 resulted in an increased ratio of *es* to *ei* mediated transport with a corresponding increase in the number of [^{3}H]NBMPR binding sites (Meckling-Gill et al., 1993). From these studies it is apparent that cells are capable of regulating both the quantity of nucleoside transporters and the relative expression of transporter subtypes. In addition, these changes in nucleoside transport, as a result of differentiation, vary considerably depending on cell type and agent used to induce differentiation.

The intracellular mechanisms involved in nucleoside transport regulation are not clear. However, second messengers have been implicated in the regulation of nucleoside transport. These include protein kinase A (Sen et al., 1990; Miras-Portugal et al, 1991*b*; Nagy et al., 1991) and protein kinase C (Lee, 1994; Lee et al., 1991; Miras-Portugal et al., 1991*a*, 1991*b*; Sen et al., 1993). There is also evidence for cell membrane located nucleoside transporter movement to and from cytoplasmic pools of transporters (Blostein and Grafova, 1987; Liang and Johnstone, 1992; Torres et al., 1992), and for hormonal-mediated (triiodo-L-thyronine) increased transporter synthesis (Fideu and Miras-Portugal, 1992).

One of the objectives of this study was to assess the suitability of this cell line for examining nucleoside transporter regulation. Differentiation of the LA-N-2 cells did result in a change in nucleoside transport characteristics in the form of an increase in NBMPR-resistant transport. However, a larger change might be necessary to study, effectively, the mechanisms of transporter regulation. The degree of morphological differentiation, observed under serum-free conditions, was not as extensive as that seen by others using different conditions; consequently, a more complete differentiation, using inducing agents such as DMSO, retinoic acid, phorbol esters or nerve growth factors, may result in a larger change in nucleoside transport characteristics (Sidell et al., 1983; Singh et al, 1990; Prasad, 1975; Mirkin and Fink, 1988; Rosenthal, 1991; R.J. Rylett, Depts. of Physiology and Pharmacology, University of Western Ontario, personal communication). Also of interest are low doses of cytotoxic nucleoside analogues such as cytosine arabinoside, 5-bromodeoxyuridine, and 5-azacytidine which may be used as part of an alternative therapeutic strategy (Mirkin and Fink, 1988; Rosenthal, 1991) involving induction of terminal differentiation of the tumour (see section 4.1).

In conclusion, differentiation of LA-N-2 cells in serum-free media results in the enhanced functional xpression of an *ei* nucleoside transporter. Further study of nucleoside transport in human neuroblastoma LA-N-2 cells is important both as a potential model for examining nucleoside transporter regulation and for the possible clinical applications. This should involve the examination of other differentiating agents, including low doses of cytotoxic nucleoside analogues, for their capacity to induce differentiation in this cell line.

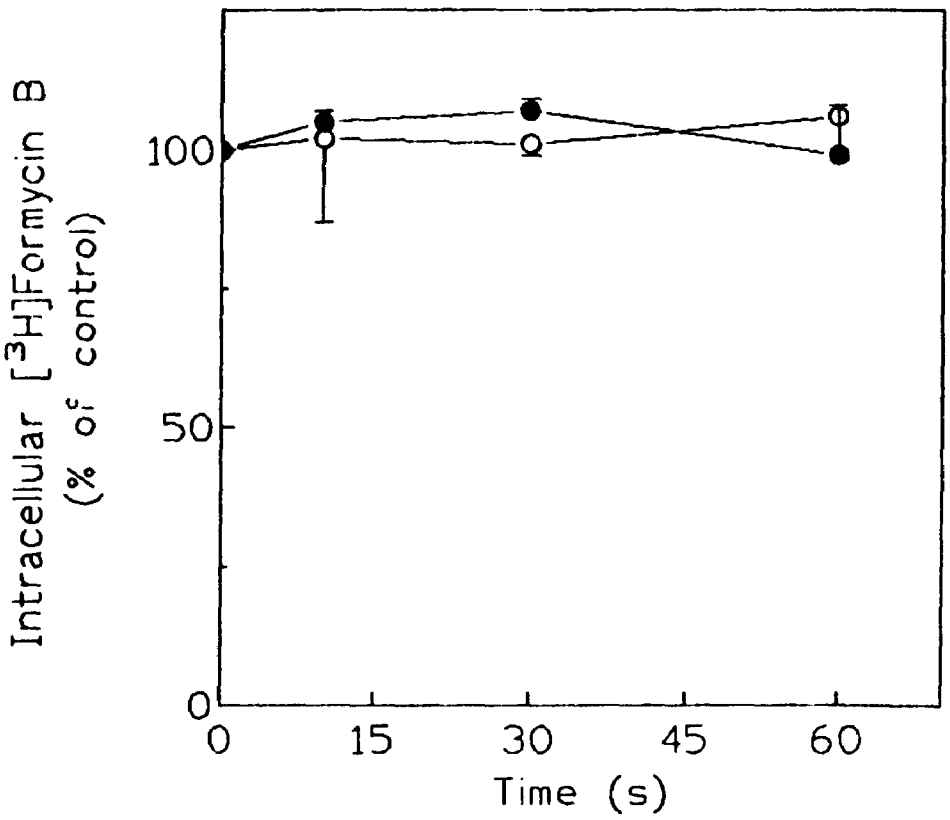


Figure 4.1: The effectiveness of the stop solution was assessed in undifferentiated and differentiated LA-N-2 cells. Undifferentiated (●) and differentiated (○) cells were incubated with 25 μM [^{3}H]formycin B for 60 s, after which 4 ml of ice cold stop solution was added to the plate. The stop solution was removed immediately (control) or after 10, 30, or 60 s. This was followed by 2 more rapid washes in ice cold stop solution (as usual). Each point represents the mean ± SEM from three experiments conducted in at least duplicate.

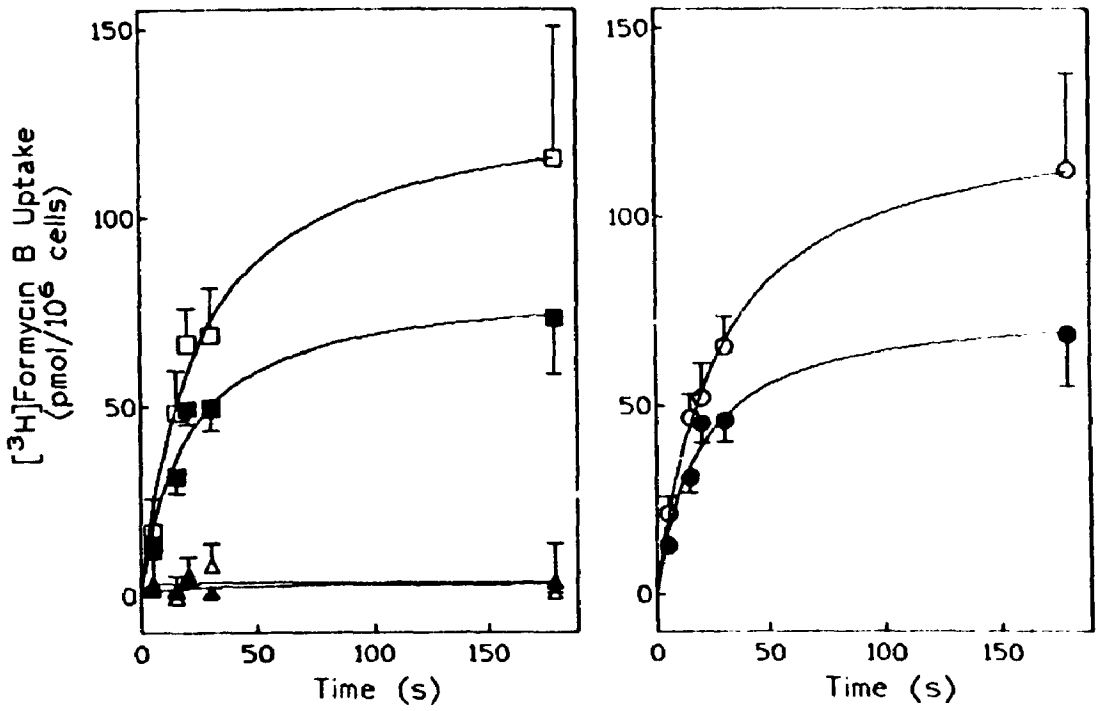


Figure 4.2: Uptake of [^{3}H]formycin B by undifferentiated and differentiated LA-N-2 cells. Accumulation of 25 μM [^{3}H]formycin B was measured in the absence (total; □, ■) and presence of 10 μM NBMPR and 10 μM DPR (non-mediated; △, ▲) for both undifferentiated (closed symbols) and differentiated cells (open symbols). Transporter-mediated uptake (○, ●) was defined as the difference between the total and non-mediated uptake components. Each point represents the mean±SEM from four experiments conducted in duplicate.

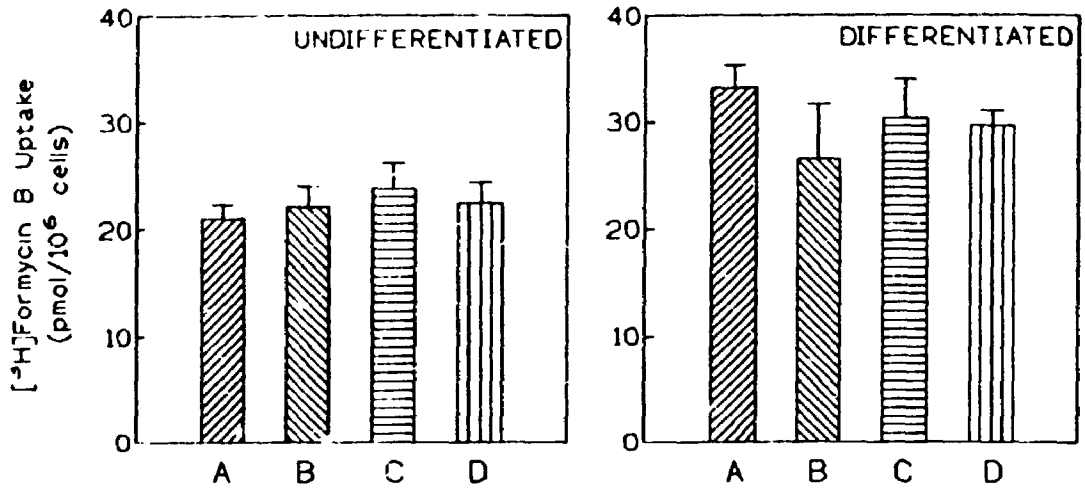


Figure 4.3 Ion dependence of [^{3}H]formycin B uptake by undifferentiated and differentiated LA-N-2 cells. Transporter-mediated accumulation of 25 μM [^{3}H]formycin B was measured after 30 s in normal incubation media (A; see section 4.2.3 for composition), or in incubation media with iso-osmotic replacement of Na^+ by Li^+ (B) or N-methylglucamine$^+$ (C), or iso-osmotic replacement of K^+ by N-methylglucamine$^+$ (D). None of these ion replacements had a significant effect on [^{3}H]formycin B uptake (ANOVA, $P<0.05$). Each point represents the mean±SEM from four experiments conducted in triplicate.

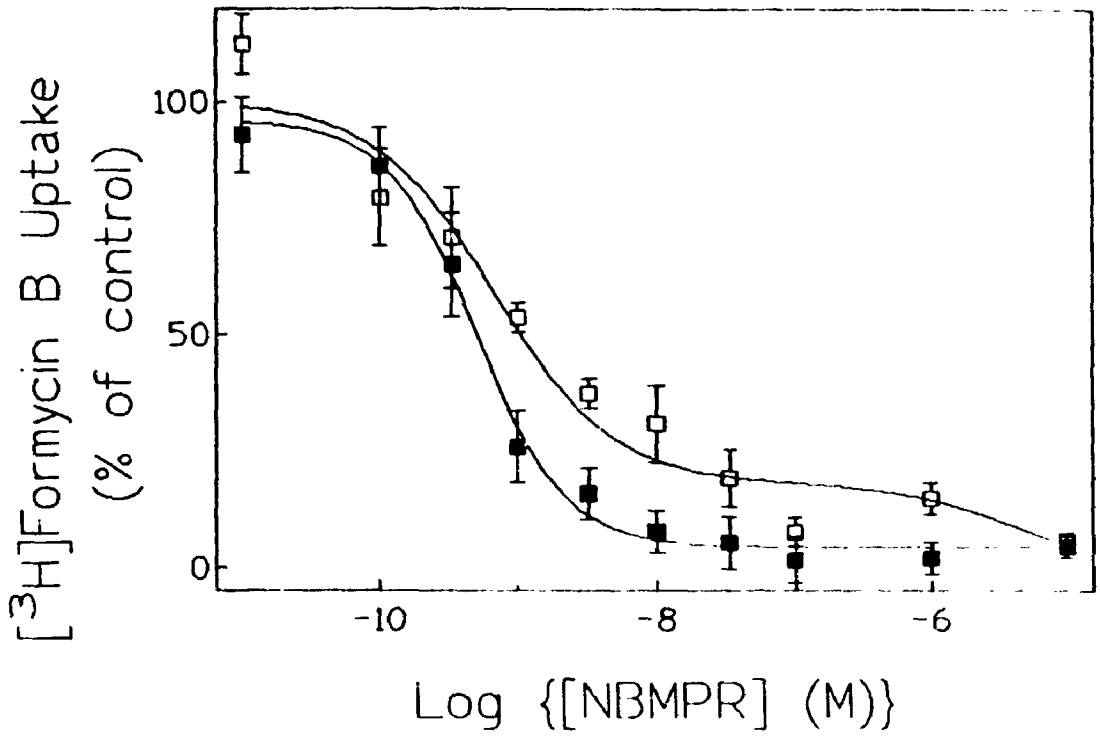


Figure 4.4: Inhibition of transporter-mediated [^{3}H]formycin B uptake by NBMPR. Both undifferentiated (■) and differentiated cells (□) were incubated with a range of concentrations of NBMPR and then the mediated influx of 25 μM [^{3}H]formycin B (15 s incubation) was determined. Control uptake was the amount of transporter-mediated accumulation of [^{3}H]formycin B observed in the absence of inhibitors. Each point represents the mean ± SEM from at least four experiments conducted in duplicate.

Figure 4.5: Inhibition of transporter-mediated [^{3}H]formycin B uptake by selected inhibitors and substrates. Undifferentiated (closed symbols) and differentiated (open symbols) LA-N-2 cells were incubated with a range of concentrations of DPR (◆, ◇), adenosine (▲, △), dilazep (●, ○), and formycin B (■,□), and the mediated influx of 25 μM [^{3}H]formycin B (15 s incubation) was determined. Control uptake was the amount of transporter-mediated accumulation of [^{3}H]formycin B observed in the absence of inhibitors. Each point represents the mean ± SEM from at least four experiments conducted in duplicate.

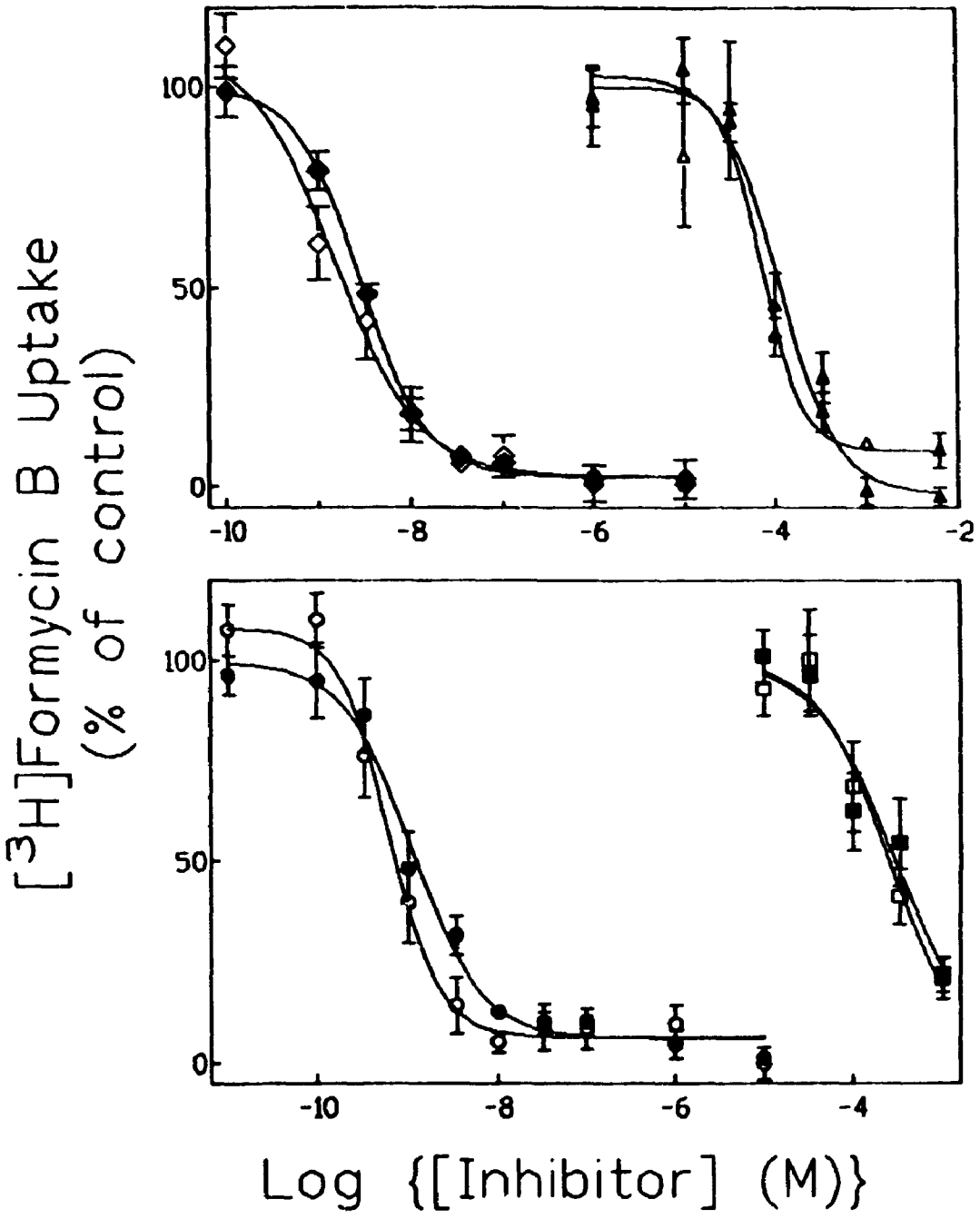
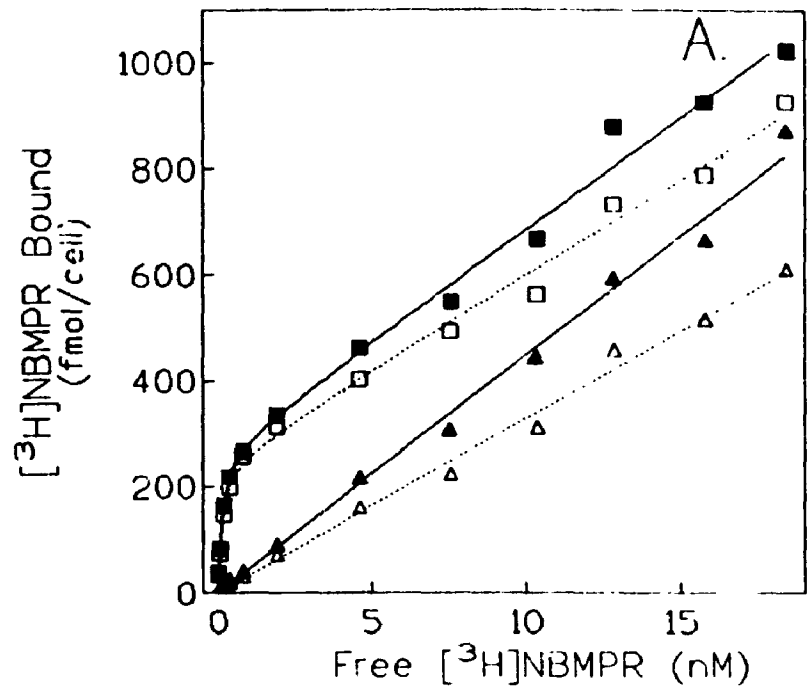
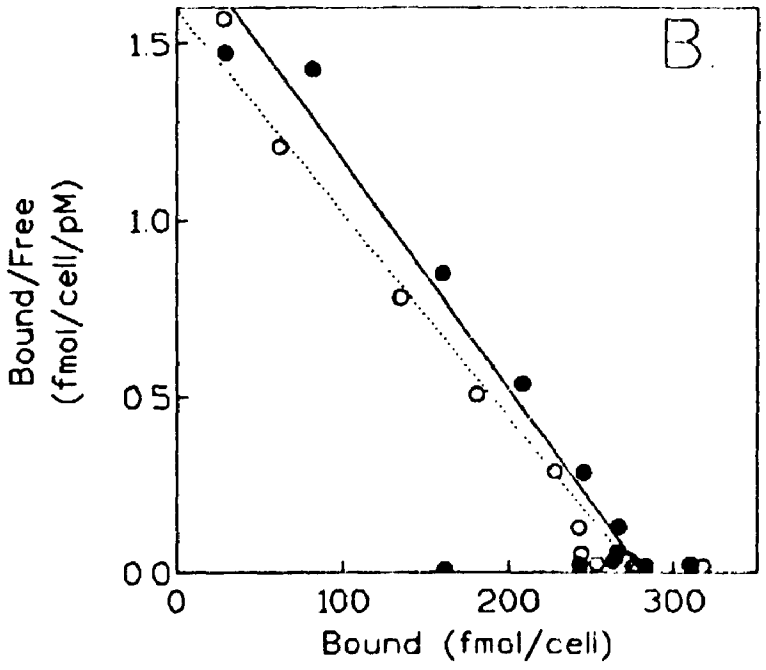

[3H]Formycin B Uptake
(% of control)
100
50
0
-10
-8
-6
-4
-2
100
50
0
-10
-8
-6
-4
Log {[Inhibitor] (M)}

Figure 4.6: Saturation analysis of [^{3}H]NBMPR binding to undifferentiated and differentiated LA-N-2 cells (A.) and the corresponding Scatchard transformations of the specific binding derived from these data (B.). Specific binding (●, ○) was defined as the total binding (■, □) minus the binding observed in the presence of 10 μM NBTGR (non-specific; ▲, △) for both undifferentiated (closed symbols) and differentiated (open symbols) cells. These are representative plots from six experiments.


A.
1000
800
600
400
200
0
0
5
10
15
[3H]NBMPR Bound (fmol/cell)
Free [3H]NBMPR (nM)


B.
1.5
1.0
0.5
0.0
0
100
200
300
Bound/Free (fmol/cell/pM)
Bound (fmol/cell)

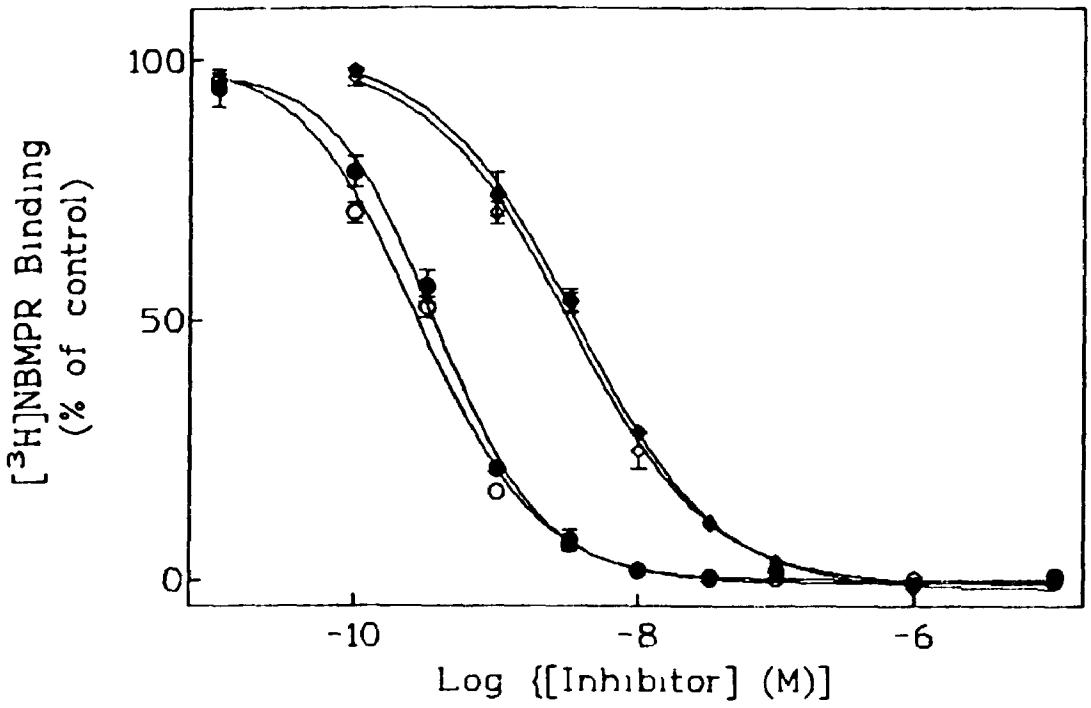


Figure 4.7: Inhibition of [^{3}H]NBMPR binding to undifferentiated and differentiated LA-N-2 cells by dilazep and DPR. Both undifferentiated (closed symbols) and differentiated (open symbols) cells were incubated with 0.4 nM [^{3}H]NBMPR in the presence and absence of a range of concentrations of dilazep (●, ○) and DPR (♦, ◇). Control binding was defined as specific binding in the absence of inhibitor. Each point represents the mean ± SEM from four experiments.

Table 4.1: Protein content, DNA content, and cell volume of undifferentiated and differentiated LA-N-2 cells. After undergoing a "mock" assay, the number of cells, protein content, DNA content and cell volume were determined using replicate plates from the same passage. Each value represents the mean ± SEM from 4 separate experiments.

Parameter (/10^6 cells)	Undifferentiated	Differentiated
DNA (μg)	19±5	18±3
Protein (μg)	200±40	290±45[a]
Cell volume (μl)	2.6±0.3	3.4±0.5[a]

[a] significantly different from the value obtained using undifferentiated cells (Student's paired t-test, $P<0.05$)

Table 4.2: Metabolism of formycin B and uridine by LA-N-2 cells. [^{3}H]Formycin B and [^{3}H]uridine (final concentration, 25 μM and 10 μM, respectively) were incubated with cells for 60 and 15 s, respectively. The products of metabolism were extracted and then identified by thin layer chromatography. Values are given as a percentage of the total radioactivity recovered. Values are the average of 2 experiments done in duplicate.

Substrate		% of Total Radioactivity Recovered		
		Nucleoside	Nucleotides	Nucleobase
Formycin B	undifferentiated	99	1	-
	differentiated	99	1	-
Uridine	undifferentiated	7	92	1
	differentiated	11	89	0

Table 4.3: Inhibition of transporter-mediated [^{3}H]formycin B uptake in undifferentiated and differentiated LA-N-2 cells. Compounds were tested over a range of concentrations to determine their capacity to inhibit the transporter-mediated accumulation of [^{3}H]formycin B (25 μM; Figures 4.4 and 4.5). Values shown are the means±SEM from at least 4 experiments.

INHIBITOR	[^{3}H]Formycin B Uptake (IC_{50})	
	undifferentiated	differentiated
NBMPR		
"sensitive" (nM)	0.5±0.2	0.9±0.2
"resistant" (μM)	_	7±2
DPR (nM)	2.9±0.2	2.6±0.9
Dilazep (nM)	1.2±0.3	0.9±0.3
Adenosine (μM)	110±20	80±10
Formycin B (μM)	280±60	270±80

Table 4.4: Binding of [^{3}H]NBMPR (0.4 nM) was conducted with undifferentiated and differentiated LA-N-2 cells that had been exposed to trypsin and trypsin inhibitor ("With Trypsin"), as described in section 4.2.6, and those which had not been exposed to trypsin and trypsin inhibitor ("No Trypsin"). "No Trypsin" cells were removed from the plates mechanically (via rubber policeman) rather than enzymatically. In each case, cells removed from the plates were diluted equally for use in the assay. Values given below are for specific binding of [^{3}H]NBMPR (fmol per assay tube of cells). There was no significant difference in [^{3}H]NBMPR binding as a result of method of cell removal from the plates (Student-Newman-Keuls, $P<0.05$). Each point represents the mean ± SEM from four experiments conducted in duplicate.

	[^{3}H]NBMPR Bound (fmol/assay tube)	
LA-N-2 cells	No Trypsin	With Trypsin
undifferentiated	12±3	10±3
differentiated	10±2	9±2

Table 4.5: Inhibition of [^{3}H]NBMPR binding in undifferentiated and differentiated LA-N-2 cells. Compounds were tested over a range of concentrations to determine their capacity to inhibit the specific binding of [^{3}H]NBMPR (0.4 nM) as shown in Figure 4.7. Values shown are the means±SEM from 4 experiments.

INHIBITOR	[^{3}H]NBMPR Binding (K_i, nM)	
	undifferentiated	differentiated
NBMPR	0.14±0.02*	0.14±0.03*
DPR	0.9±0.1	0.8±0.1
Dilazep	0.1±0.01	0.08±0.01

* K_D for [^{3}H]NBMPR binding

CHAPTER FIVE: CONCLUSIONS AND QUESTIONS FOR FURTHER STUDY

This thesis examined the heterogeneity of nucleoside transport in mammalian neuronal tissues. For greater clarity, the results were presented as three separate chapters. A brief description of where these results fit into the field of nucleoside transport, as well as a summary of results and questions for further study, follows.

A single system for mediated nucleoside transport was first characterized in the early 1970's. Since then at least seven functionally distinct nucleoside transporter subtypes have been described and, with the aid of molecular biology, it is likely that the discovery of other subtypes will follow. In order to gain a better understanding of nucleoside transport, characterization of each subtype is being pursued. Key to this are the many probes, such as [^{3}H]NBMPR, [^{3}H]DPR, and [^{3}H]R75231, which have been synthesized for the study of nucleoside transporters. These probes can be used to obtain information about nucleoside transporters including, the number of binding sites, interactions of inhibitors with binding sites, and interaction of binding sites with each other (Chapters 2, 3, and 4). However, the only transporter subtype that can be effectively examined by these probes is the *es* transporter (Chapters 2 and 3). Fortunately, the concentrative transporters can be examined selectively based on their substrate selectivity. This leaves the *ei* transporter for which no selective substrate or probe has yet been found (Chapter 2). As a result, detailed examination of this transporter has proven difficult (Chapter 2). In addition to the transporter heterogeneity, the study of nucleoside transporters has been complicated by species dependent differences in nucleoside transporter characteristics (number of transporters, transporter subtypes, kinetic characteristics, and inhibitor sensitivities)

(Chapters 2 and 3).

Regulation of nucleoside transporter numbers and subtypes is poorly understood. However, it is apparent that changes in transporter numbers and subtypes are linked to cellular differentiation (Chapter 4). Further study in this area may establish the factors that regulate nucleoside transporters under both physiological and pathological conditions.

Perhaps the most interesting area of future research involving nucleoside transport is in the clinical field. Current areas of research include using nucleoside substrates or inhibitors in cancer and viral (including AIDS) chemotherapy, for cardioprotection including the preservation of organs for transplant, and for neuroprotection under conditions such as stoke or seizure (Chapters 3 and 4). Because of these practical implications, it is likely that both basic and clinical research in the area of nucleoside transport will continue to increase.

The studies reported in this thesis have examined radioligand binding to, the functioning of, and the regulation of, nucleoside transporters in mammalian nervous systems. Several conclusions can be made based on these results.

The relationship between the nucleoside transport system and NBMPR-sensitive and -resistant [^{3}H]DPR binding was examined in rabbit and guinea pig cortical synaptosomes. As well, a pharmacological profile of the inhibitory effects of various compounds on nucleoside transport was conducted in rabbit cortical synaptosomes.

1. In agreement with previous studies, NBMPR-sensitive [^{3}H]DPR binding and [^{3}H]NBMPR binding involved the same, or overlapping, membrane sites associated with the es nucleoside transporter. Therefore, [^{3}H]DPR could be a useful probe for this transporter.

2. This study showed that CHAPS could be used to decrease [^{3}H]DPR binding to glass tubes and filters. This methodology also prevented the filter associated [^{3}H]DPR which could

potentially be misinterpreted as binding to specific tissue located sites.

3. This study proposed that the NBMPR-resistant [^{3}H]DPR binding site(s) involved membrane components distinct from those associated with functional, *ei* nucleoside transporters. As a result, past and future binding studies with [^{3}H]DPR must be interpreted with caution.

4. Nucleoside transport characteristics were examined in rabbit cortical synaptosomes. These results, when compared to studies involving other species agree with previous findings of species dependent differences in nucleoside transport characteristics. The effects produced by a particular transport blocker in the CNS are likely to be species dependent.

The interaction of the mioflazine derivative R75231 with the nucleoside transport system was investigated.

5. R75231 bound extremely tightly to nucleoside transporters of rabbit cortical synaptosomes. This tight binding, which has been observed in other systems, may be responsible for the long lasting inhibitory effects of R75231, *in vivo*.

6. This study was the first to detail many of the binding characteristics of R75231, which may be responsible for its tight binding and long-lasting effects, to the nucleoside transporter. For example: R75231 was a "mixed" type inhibitor of [^{3}H]NBMPR binding in rabbit cortical synaptosomes; [^{3}H]R75231 binding to the nucleoside transporter of human erythrocyte ghost membranes was reversible; the rate of [^{3}H]R75231 dissociation depended on the agent used to prevent its reassociation.

7. [^{3}H]R75231 binding characteristics support a nucleoside transporter model which has multiple, interacting binding sites. Specifically, this present study proposes that relatively high concentrations of R75231 may interact with the second, allosteric, site on the transporter leading to an increase in the affinity of the first site for R75231. This, in effect, "locks" R75231 onto this binding site (positive

cooperativity).

The nucleoside transport characteristics of undifferentiated and differentiated LA-N-2 human neuroblastoma cells were compared.

8. This study is the first report to characterize the nucleoside transport system in a human neuroblastoma cell line. Undifferentiated LA-N-2 human neuroblastoma cells accumulate nucleosides via *es* nucleoside transporters. The enhanced nucleoside transport in differentiated LA-N-2 cells appeared to be due to an increased expression of *ei* transporters. The mechanism which resulted in the change of transport characteristics is not yet known.

9. This present report is the first to demonstrate cellular differentiation resulting in an increase in *ei* nucleoside transporter activity. From this study and others, it is apparent that cells are capable of regulating both the number of nucleoside transporters and the relative expression of transporter subtypes. In addition, these changes in nucleoside transport, as a result of differentiation, vary considerably depending on cell type and agent used to induce differentiation.

The studies reported in this thesis have examined aspects of nucleoside transport in the mammalian nervous system. Still, many questions in these areas remain unanswered. Some potential avenues of further research include the following:

1. Does a strongly selective substrate/inhibitor for the *ei* transporter exist or could one be developed? Extensive examination of the structure-activity relationship of a variety of nucleosides and nucleoside derivatives may lead to the development of a substrate/inhibitor selective for the *ei* transporter.

2. Many of the characteristics of nucleoside transporters, are, at least in part, species dependent. Since nucleoside transport inhibitors may prove to be clinically useful in the CNS, the full characterization of the nucleoside transport

system of human CNS tissue could prove extremely beneficial. In order to examine the kinetics and pharmacology of all possible transporter subtypes, substrate influx studies should be performed using fresh brain tissue. Examination of heterogeneity of transport characteristics within the different areas of the brain may also prove to be useful.

3. There is considerable evidence for multiple, interacting, inhibitor binding sites on the *es* nucleoside transporter. There is also some evidence that suggests that individual nucleoside transporters may form complexes of 2 or more, which can result in allosteric regulation of adenosine transport (Casillas et al., 1993). Therefore, are the interacting inhibitor binding sites on the same individual transporter protein or are they actually on different transporter proteins within a complex? Analysis of cloned and sequenced *es* nucleoside transporters and information from subsequent mutation studies may clarify the number and location of inhibitor binding sites and possible transporter-transporter interactions.

4. Was the increase in *ei*-mediated nucleoside transport observed in the differentiated LA-N-2 neuroblastoma cells due to increased transcription or increased translation? This question could be addressed using inhibitors of protein synthesis (i.e. cycloheximide) and RNA polymerase inhibitors (i.e. α-amanitin). Increased *ei*-mediated nucleoside transport may also be due to enhanced activity of existing membrane-located transporters or translocation from intracellular compartments. Examination of this possibility would require a probe for the *ei* transporter, which is not yet available, so that the number of transporters could be estimated. The movement of transporters from intracellular sources could then be observed by separating cell membranes and intracellular vesicles through differential centrifugation or by using saponin to permeabilize the cell membranes and allow the labelling of intracellular transporters.

5. Differentiation of the LA-N-2 neuroblastoma cells, as a

result of growing in the absence of serum, did result in a change in nucleoside transport characteristics. However, a larger change might be necessary to study, effectively, the mechanisms of transporter regulation. Since the morphological differentiation observed in the absence of serum was not complete, would other differentiating agents, such as DMSO, retinoic acid, phorbol esters, or nerve growth factors, produce a more complete differentiation of LA-N-2 cells with a larger change in nucleoside transport characteristics?

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