SYNTHESIS AND BIOLOGICAL ACTIVITY OF CONFORMATIONALLY
CONSTRAINED NUCLEOSIDES AND NUCLEOTIDES

by

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A thesis submitted in conformity with the requirements
for the degree of Masters of Science
Graduate Department of Chemistry
University of Toronto

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ABSTRACT

Synthesis and Biological Activity of Conformationally Constrained Nucleosides and Nucleotides

Degree of Master of Science, 1998
by Girolamo Tusa
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This thesis outlines the synthesis of a variety of conformationally constrained nucleosides and nucleotide analogues. The conformationally “locked” analogues closely resemble specific conformers of natural nucleosides and nucleotides and were used to probe the conformational specificity of particular physiological processes in PC12 cells; nucleoside transport (NT) activity and P2-purinoceptor response. 8,2′-thioanhydroadenosine (3) and the corresponding 5′- triphosphate derivative are “locked” in the anti-conformation about the glycosidic bond and in the S-type sugar pucker. Both characteristics are known to be highly favoured conformations of adenosine and ATP in solution state, thus it is hypothesised that these endogenous compounds are recognized by NT proteins and P2-purinoceptors in these conformations. 8,2′-thioanhydroadenosine (3) had no effect on NT activity and the triphosphate derivative did not elicit any P2-purinoceptor responses. 2,2′-anhydrouridine (6) and the corresponding 5′- triphosphate analogue are “locked” in the syn-conformation about the glycosidic bond and in the S-type sugar pucker. Similarly this analogue did not inhibit NT processes, although the 5′- triphosphate analogue was an agonist for the P2\(\times\)2-purinoceptor. 8-bromoadenosine (1) and 8-mercaptoadenosine (8) represent molecules with modifications on the adenine base moiety; at concentrations of 300 \(\mu\)M these were found to inhibit the NT mediated uptake of \(^{3}H\)-adenosine by 42±2 and 58±2\% of control, respectively. Adenosine-2′,3′-carbonate (9) adopts a constrained sugar pucker “intermediate” between the S and N-type sugars. This analogue was found to be a potent inhibitor of the NT process (IC\(_{50}\) ~ 10 \(\mu\)M).
DEDICATION

This thesis is dedicated to my parents who with their optimism, unrelenting support and continuous patience have inspired me during the difficult times.
ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my supervisor Dr. Juta Reed. This research would not have been possible without her extensive support, enthusiasm and encouragement throughout these past two years. Furthermore I thank her for devoting time to help screen the nucleoside and nucleotide analogues and hasten the completion of this work. I would also like to extend my gratitude to my volunteer workers Bogdan Paun, Cathy Tusa and Mae Chiang for their diligent work in both synthesizing intermediates and screening analogues. I would also like to thank Dr. Sandy Raha for the selection and background research for the synthesis of 8,2'-thioanhydroadenosine (3), Dr. K. K. Ogilvie for sending the authentic sample of 8,2'-thioanhydroadenosine (3), Dr. Scott Taylor for his guidance using the Varian Gemini-200 FT-NMR and Dr. Alex Young for running the FAB mass spectra of the nucleotide analogues.

My thanks also go out to all the chemistry graduate students, faculty and staff, especially those at Erindale. Not only have I gained new friendships, they have been supportive throughout my research, and will provide me with years of enjoyable memories. Finally, I wish to thank my family for their tolerance and understanding, particularly during times of stress.
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1. INTRODUCTION

Nucleosides and nucleotides are biologically ubiquitous substances that participate in nearly all biochemical processes. The roles of nucleosides and nucleotides in all organisms can be classified accordingly:

1. They comprise the monomeric units of nucleic acids (DNA and RNA).
2. Nucleoside triphosphates (NTPs), mainly adenosine triphosphate (ATP), are the “high-energy” molecules, whose utilization drives most energy-requiring processes.
3. Many metabolic pathways are regulated or mediated by nucleosides and nucleotides.
4. Adenine nucleotides are components of the coenzymes such as NAD⁺, NADP⁺, FMN, FAD, and coenzyme A.
5. Extracellular nucleosides and nucleotides can act as signaling molecules in many cells.

1.1 Chemical structure of nucleosides and nucleotides

Nucleotides are composed of three units, a sugar, a phosphate and a base. The sugar is a pentose (C₅) and the nitrogenous base is either a purine or a pyrimidine. Nucleosides lack the phosphate ester moiety. In ribonucleotides the pentose is the D-ribose residue while in deoxyribonucleotides, which occur in DNA, the sugar is 2'-deoxy-D-ribose. The phosphate group may be bonded to the C(3') or C(5') of the ribose to form a 3'-nucleotide or 5'-nucleotide, respectively. In all naturally occurring nucleosides and nucleotides, the N-glycosidic bond linking the nitrogenous base to the pentose C(1') atom has the β configuration, extending from the same side of the furanose ring as the C(4')-C(5') bond. The purine components of nucleic acids are adenine (A) and guanine (G) residues; the pyrimidine residues are cytosine (C), uracil (U), and thymine (T). The structures and numbering system for some common purines and pyrimidines are shown below (Fig. 1). The “primed” numbers refer to the atoms of the ribose residue and “unprimed” numbers refer to the nitrogenous base.
Figure 1. Chemical structure of common nucleosides

1.2 Metabolism of nucleosides and nucleotides

1.2.1 De novo synthesis

Nearly all cells can synthesize nucleosides/nucleotides de novo and from the degradation products of nucleic acids (salvage pathways). A full description of the steps in the de novo synthesis of nucleosides is beyond the scope of this thesis, although it is important to state that the process requires a myriad of cellular enzymes and requires considerable energy.

All purines are derived from a common intermediate, inosine monophosphate (IMP), which is synthesized from α-D-ribose-5-phosphate and the amino acids glycine, glutamine and aspartate (Voet and Voet, 1995). Adenosine monophosphate (AMP) and guanosine monophosphate (GMP) are then produced from this intermediate via separate pathways and further phosphorylated.
The de novo synthesis of pyrimidines is a much simpler process and involves the common template molecule orotic acid (uracil-6-carboxylic acid). Orotic acid, synthesized from the amino acids glutamate and aspartate, is coupled to 5-phosphoribosyl-α-pyrophosphate (PRPP) to form orotidine monophosphate (OMP) which is decarboxylated to uridine monophosphate (UMP) (Voet and Voet, 1995).

The formation of cytosine is accomplished at the nucleotide triphosphate level, where UTP is converted to cytidine triphosphate (CTP), by CTP synthetase, whereas thymine is formed at the thymidine deoxyribose monophosphate (dTMP) level from dUMP by the actions of thymidylate synthase.

Phosphorylation of nucleosides to their corresponding nucleoside monophosphates (NMPs) are accomplished by nucleoside kinases. NMPs are converted to nucleoside diphosphates (NDPs) by the actions of NMP kinases which utilize ATP as a phosphate donor. Both nucleoside kinases and NMP kinases are base-specific as to their choice of substrate. Subsequent phosphorylation of NDPS to nucleoside triphosphates (NTPs) is accomplished by NDP kinase, which catalyzes the exchange reaction between an NDP and an NTP (Voet and Voet, 1995). NDP kinase is nonspecific and does not discriminate between bases on either substrates or whether the sugar residue is ribose or deoxyribose. This thesis is concerned only with the enzymes involved in the phosphorylation of adenosine; these are adenosine kinase (AK), adenylate kinase and NDP kinase (scheme 1).

The formation of deoxyribonucleotides is accomplished by the actions of ribonucleotide reductase which reduces NDPS to dNDPS. This enzyme is nonspecific, and also does not discriminate among the bases.

Scheme 1. Synthesis of adenosine nucleotides
1.2.2 Salvage pathways

In contrast to the *de novo* synthesis, nucleosides can also be obtained through the salvage pathways. Most cells have active turnover of many of their nucleic acids which through degradative processes result in the release of adenine, guanine, and hypoxanthine (base of inosine). For the most part, these purines are salvaged by two different enzymes; adenine phosphoribosyltransferase (APRT) which mediates AMP formation through the transfer of adenine to PRPP with release of PPi and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) which catalyzes the analogous reactions with hypoxanthine and guanine (Voet and Voet, 1995). Salvage pathways for pyrimidines do not exist, possibly due to the fact that their *de novo* synthesis is not as energetically unfavoured as for the purines.

The final source of intracellular nucleosides, requires the process of nucleoside transport. Nucleosides can be obtained from ingested material, either in the form of nucleic acids or in their native form. Nucleic acids are usually degraded by a variety of group-specific nucleotidases and nonspecific phosphatases. Both nucleosides and the free bases can be directly transported into cells through the actions of nucleoside and nucleobase transport proteins. A complete introduction of nucleoside and nucleobase transport will be discussed later.

1.2.3 Catabolism

The major pathways of purine nucleotide and deoxynucleotide catabolism are summarized (scheme 2). Inosine, xanthosine and guanosine are hydrolyzed by the actions of one enzyme, purine nucleotide phosphorylase (PNP) to release the corresponding base. Adenosine and deoxyadenosine are not degraded by the actions of PNP. Rather, adenine nucleosides and nucleotides are deaminated first by adenosine deaminase (AD) and AMP deaminase, to their corresponding inosine derivatives. Inosine is then degraded by PNP, to yield the free base hypoxanthine, which is converted to xanthine. Similarly, guanosine is degraded to guanine, which is deaminated by guanine deaminase to yield xanthine. This common intermediate is converted to the waste product uric acid which is then excreted.
Similarly, pyrimidine nucleosides and nucleotides are catabolized by a variety of base-specific deaminases and phosphorylases.

\[
\begin{align*}
\text{AMP} & \xrightarrow{\text{AMP deaminase}} \text{IMP} \\
\text{adenosine} & \xrightarrow{\text{adenosine deaminase}} \text{inosine} \\
\text{hypoxanthine} & \xrightarrow{\text{xanthine oxidase}} \text{xanthine}
\end{align*}
\]

Scheme 2. Pathways of purine nucleotide and deoxynucleotide catabolism

1.3 Nucleoside and nucleobase transport

One of the major salvage pathways for nucleosides in the cell involves nucleoside and nucleobase transport. Since physiological nucleosides and nucleobases are hydrophilic, specialized transport systems are required for their movement into or out of cells (Griffith and Jarvis, 1996). The transport of nucleosides and nucleobases is mediated by a diverse group of proteins. Some of these proteins are non-specific and transport both nucleosides and their free bases, although for the most part they are specific and transport either one or the other.
The nucleobase transporters mediate translocation of nucleobases either by a facilitated diffusion or Na\textsuperscript{+}-dependent mechanisms (Griffith and Jarvis, 1996). Since this thesis will be concerned with the nucleoside transport processes, nucleobase transporters will not be mentioned further.

The nucleoside transport (NT) processes can be divided into two distinct classes, equilibrative or concentrative. The equilibrative NT processes, which exhibit the typical features associated with facilitated diffusion, are driven by the concentration gradient of the nucleoside being transported and function both in uptake and release of nucleosides in cells (Cass, 1995). The concentrative NT processes are systems that are driven by transmembrane Na\textsuperscript{+} gradients and, in isolated cells and vesicles, are inwardly directed Na\textsuperscript{+}-nucleoside cotransporters or symporters (Cass, 1995). Equilibrative NT processes are widely distributed among mammalian cells and tissues and may be ubiquitous, whereas concentrative NT processes are limited to specialized cell types, including intestine, kidney, spleen, lymphocytes, macrophages, and choroid plexus (Cass, 1995).

1.3.1 Classification of nucleoside transporters

The total number of NT processes present in mammalian cells and tissues is uncertain. To date, seven subclasses are evident from the functional and pharmacological characteristics of permeant fluxes (Cass, 1995). The characteristics that have been used to identify NT processes are: (i) dependance on Na\textsuperscript{+} gradients, (ii) sensitivity to inhibition by nitrobenzylthioguanosine (NBTI) also called nitrobenzylmercaptopurine (NBMPR), one of several potent inhibitors of NT processes, and (iii) preference for purine or pyrimidine nucleosides as permeants (Cass, 1995).

The NT classification scheme involves the use of trivial names that are related to the functional characteristics of the various NT processes. Letters are used to designate the transport mechanism (e = equilibrative, c = concentrative), the sensitivity to inhibition by NBTI (s = sensitive, i = insensitive), and the diagnostic nucleoside used to establish permeant selectivity (f = formycin B or purine selective; t = thymidine or pyrimidine selective) (Cass, 1995). When a third concentrative NT process was discovered that accepted a variety of
purine and pyrimidine nucleosides as permeants, the classification was expanded to include a third designation (b = broad) (Belt et al., 1993). The classification of Belt et al. (1993) recognizes five subclasses of NT processes: equilibrative NBTI-sensitive (es) transport; equilibrative NBTI-insensitive (ei) transport; concentrative NBTI-insensitive purine-selective (cif) transport; concentrative NBTI-insensitive pyrimidine-selective (cit) transport and concentrative NBTI-insensitive broadly selective (cib) transport (Belt et al., 1993). The recent observation of NBTI-sensitive concentrative transport of the nucleoside chemotherapeutic, fludarabine in freshly isolated human leukemic cells suggests that there may also be concentrative NBTI-sensitive (cs) transport (Paterson et al., 1993).

<table>
<thead>
<tr>
<th>Trivial(^c)</th>
<th>Equilibrative(^a)</th>
<th>Concentrative(^b)</th>
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<tbody>
<tr>
<td></td>
<td>es</td>
<td>ei</td>
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<tr>
<td>Na(^{+})-dependent</td>
<td>-</td>
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<tr>
<td>Na(^{+})-nucleoside stoichiometry</td>
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<td>Inhibited by</td>
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<td>NBMPR</td>
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<td>Dilazep</td>
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<td>Thymidine</td>
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ND, not determined.

**Table 1.** Classification and functional properties of nucleoside transporters (from Cass, 1995)
Figure 2. Functional NT processes of mammalian cells (from Cass, 1995)

The functional characteristics of the seven NT subclasses are outlined (Table 1). The NT studies in this thesis involve only the equilibrative transport systems (es and ei), and the specifics of concentrative transport systems will not be discussed. The two equilibrative processes of mammalian cells exhibit the classic features of facilitated diffusion including exchange diffusion and countertransport. The equilibrative NT processes are remarkably broad in their permeant selectivities and transport all of the native nucleosides as well as a diverse group of structural analogues with various substituents in the base or sugar moieties (Cass, 1995).

1.3.2 Nitrobenzylthioinosine (NBTI) a potent inhibitor of equilibrative NT

NBTI, a tight-binding and highly specific inhibitor has proven to be an invaluable chemical reagent for distinguishing between the two subclasses of equilibrative NT processes,
primarily due to the large differences ($10^4$) in sensitivity to inhibition by NBTI. (Cass, 1995). The es NT processes are inhibited by NBTI by low concentrations ($\leq 1 \text{ nM}$) as a result of a noncovalent interaction of NBTI with a high-affinity ($K_d \sim 0.1 \text{ nM}$) binding site located on the extracellular face of the plasma membrane (Agbanyo et al., 1988). In contrast, the ei NT processes are less sensitive to NBTI and are inhibited only at higher concentrations ($> 10 \mu\text{M}$) (Belt et al., 1983). Since NBTI competitively inhibits the transport of nucleosides, it is assumed to bind in the active-site “pore” created by the protein thereby preventing translocation of the permeant. NBTI itself is not transported and the binding was shown to be reversible (Jarvis et al., 1983). This high affinity of NBTI for the es transporter is thought to be stabilized by the interaction of the nitrobenzene ring with an adjacent hydrophobic domain on the outward-facing conformation of the transporter (Jarvis et al., 1983). In accordance, nucleosides are competitive inhibitors of NBTI binding with apparent $K_i$ values for different nucleosides corresponding closely to their apparent $K_m$ values for their influx (Jarvis et al., 1983).

*Figure 3.* Structure of NBTI (nitrobenzylthioinosine)
1.3.3 Non-nucleoside inhibitors of equilibrative NT

A vast number of compounds with structures that appear unrelated to nucleosides have been and continue to be discovered that inhibit the equilibrative NT processes. Some of the most potent include the vasodilators, dipyridamole (DPR) and dilazep (Fig. 4)

![Dipyridamole (DPR) and Dilazep](image)

*Figure 4. Structures of dipyridamole (DPR) and dilazep*

Both es and ei NT processes are inhibited by low concentrations (0.1-100 nM) of dipyridamole and dilazep, although there are differences among cell types and species (Plagemann et al., 1988). Dipyridamole like NBFI, behaves as a competitive inhibitor of nucleoside influx (Jarvis, 1986). Such results suggest that dipyridamole inhibits es nucleoside transport in a similar manner to that of NBFI and moreover that both ligands interact at common or overlapping sites on the transporter. The exact nature of inhibition of both dipyridamole and dilazep has yet to be determined, and it is unclear if these hydrophobic molecules are interacting with the NT protein itself or altering the nature of the lipid membrane. Some coworkers have suggested dipyridamole destabilizes the protein-lipid interface, consequently altering the protein conformation (Plagemann et al., 1988).
1.3.4 Relationships between nucleoside transport (NT) and metabolism

Cellular uptake of nucleosides involves several processes. Depending on the nucleoside, it involves permeation across the plasma membrane and metabolism by either anabolic (ie. nucleoside kinases) or catabolic enzymes (ie. nucleoside deaminases, nucleoside phosphorylases). The transport of nucleosides across the cell membrane is usually measured by determining the rate of uptake, ie. incorporation of radioactive nucleosides from the extracellular medium into the intracellular space. Overall, this is a two step process, consisting of facilitated translocation across the membrane followed by conversion to impermeant nucleotides via nucleoside kinases (Thampy and Barnes, 1983). The process can be further complicated by the catabolism of the radioactive analog by deamination or glycosidic bond cleavage. Originally it was assumed that transport was the rate-determining step for the overall uptake process. This assumption was proven invalid, since it has been established that nucleoside transport is the more rapid process, reaching near equilibrium in less than 1 min (Thampy and Barnes, 1983). Thereafter, the incorporation of radiolabel into cells reflects an accumulation of nucleotides which persists for many minutes (Thampy and Barnes, 1983). Thus in cells with high metabolic activity, it is nucleoside metabolism rather than transport which is reflected in the measured kinetic parameters. This problem can be alleviated by using cell lines deficient in certain metabolic enzymes, using inhibitors specific for these enzymes, or measuring the initial uptake process ie. within the first 20s after addition of the radioactive nucleoside (Plagemann et al., 1988).

1.4 The Special role of adenosine and adenine nucleotides in mammalian cells

Adenosine and adenine nucleotides are local signaling molecules that are released into the extracellular space in response to various stimuli. The ability of adenosine and adenine nucleotides to regulate cellular function has long been recognized (Burnstock, 1972). Such varied physiological processes as lipolysis, neurotransmitter release, platelet aggregation, coronary vasodilation, cardiac contractility, and renal vasoconstriction are regulated by adenosine or adenine nucleotides through the interaction with a heterogeneous group of cell surface receptors called purinoceptors.
1.4.1 Overview of $P_1$ and $P_2$-purinoceptors

Purinoceptors, are classified as $P_1$ or $P_2$-purinoceptors, depending on their preference, respectively, for adenosine or adenine nucleotides (Burnstock, 1997). Functional studies with various agonists and antagonists have revealed a bewildering array of $P_1$ and $P_2$-purinoceptor subtypes. The $P_1$-purinoceptors which are selective for adenosine, usually act through activation or inhibition of adenylate cyclase, thus regulating the levels of intracellular cyclic AMP (cAMP) (Burnstock, 1997).

The $P_2$-purinoceptors are generally selective for ATP, although some $P_2$-purinoceptors subtypes can be activated by uridine nucleotides (UTP) and ADP. The hypothesis that ATP acts as an extracellular signalling molecule has only recently been proposed. The $P_2$-purinoceptors are broadly classified into two large groups, G-protein coupled receptors (ie. $P_{2Y1}$, $P_{2Y2}$, etc.) and ligand-gated cation channels (ie. $P_{2X1}$, $P_{2X2}$, etc.) (Burnstock, 1997). Classification of receptor types or subtypes within these two groups has traditionally involved establishment of a “rank-order of potency” that characterizes the ability of naturally occurring or synthetic ligands (either agonists or antagonists) to interact with particular cell specific receptors. Although highly selective antagonists for these receptors have yet to be identified or synthesized, various nucleotides and nucleotide analogues have been shown to mimic, with different potencies, the ability of ATP to elicit diverse physiological responses.

1.5 Nucleosides as chemotherapeutic agents

Nucleoside-based drugs have important clinical applications in the treatment of various cancers and viral diseases. The most important of these are used for treating leukemias, lymphomas, and cancers of the gastrointestinal tract, or a variety of viral diseases, including human immunodeficiency virus (HIV) and herpesvirus infections. A large number of nucleoside analogues with anti-cancer or anti-viral activity are currently in various stages of clinical development. Some of the more famous nucleoside drugs are those that have been used to treat HIV, namely Zidovudine (azidothymidine, AZT), Didanosine (dideoxyinosine, ddIno), and Zalcitabine (dideoxycytidine, ddCyd). All three analogues are phosphorylated by the host cell kinases to their corresponding nucleoside triphosphates and these analogues then interact
with HIV-reverse transcriptase, terminating viral DNA synthesis (Kamali, 1993). Other nucleoside drugs that have gained widespread acceptance as potent anti-cancer agents include Cladribine (chlorodeoxyadenosine, Cl-dAdo), Cytarabine (arabinosylcytosine, araC), Gemcitabine (2',2'-difluorodeoxycytidine, dFdC), 2-Fludarabine (fluoroarabinosyadenine, F-araA), 5-Fluorodeoxyuridine (FdU) and Pentostatin (2'-deoxycoformycin, dCF). All such nucleosides are called prodrugs, since they require intracellular phosphorylation before they can inhibit DNA.

1.5.1 Importance of nucleoside transport (NT) in the design and activity of nucleoside chemotherapeutics

The design of nucleoside drugs which may have therapeutic or diagnostic value is greatly aided by an understanding of the mechanism by which the naturally occurring, endogenous compounds exert their effects in vivo. Since the activity of many of these compounds depends upon their entry into intracellular metabolic pathways, the ability to cross cell membranes is a key requisite for their effectiveness. Since the first part of this thesis will focus on the affects of various nucleoside analogs on the transport of adenosine, it is useful to examine the effects of structural modifications of the nucleoside on membrane transport. Competitive inhibitors of adenosine transport may offer clues into the structure-selectivity of the NT processes and suggest possible refinements that may increase the potency of existing nucleoside therapeutics. Furthermore, endogenous nucleosides and nucleobases can often reduce the effectiveness of inhibitors of the de novo synthesis of purines and pyrimidines, and consequently, inhibitors of NT taken in combination with nucleoside chemotherapeutics can often enhance the toxicity of these substances (Plagemann et al., 1988).

The central role of adenosine in the regulation of a variety of cellular functions, makes it an attractive target for chemotherapy. Potent inhibitors of NT processes would be invaluable tools for prolonging the affects of adenosine P1-purinoceptors activation by preventing the re-uptake of adenosine. Indeed, the coronary vasodilator effects of dipyridamole and dilazep result from elevations in extracellular adenosine following inhibiton of transport by these agents (Plagemann et al., 1988). Also elevated levels of extracellular
Figure 5- Structures of common therapeutic nucleosides
adenosine has been shown to lessen the damage caused after severe ischemia and NT inhibition may be a useful therapeutic approach in preventing cerebral damage following stroke (Rongen et al., 1995).

1.6 Three-dimensional conformations of nucleosides and nucleotides

In order to predict the structure of a viable nucleoside/nucleotide inhibitor one must first understand the three-dimensional conformations of the parent compound. Nucleosides and nucleotides can adopt a variety of possible conformations. There are three key features of all nucleosides/nucleotides which confer three-dimensional conformation. These are: (i) conformation of the glycosidic bond, (ii) the conformation of the C(4')-C(5') exocyclic bond and (iii) the sugar pucker (Sarma et al., 1974).

1.6.1 Conformations of the glycosidic bond

The "conformation of the glycosidic bond" refers to the fact that this bond can "freely" rotate about its axis; C(1')-N(9) for purines and the C(1')-N(1) bond for pyrimidines. For simplicity, the nomenclature of syn and anti has been designated for these conformations. The base is in the anti-conformation when its larger substituent is facing away from the plane of the sugar, and in the syn-conformation when the larger substituent is toward or on top of the sugar plane.

Since neither the syn nor anti-conformations of nucleosides are actually static, and a range of possible glycosidic bond angles should be considered, a more accurate description of the glycosidic conformation requires defining the dihedral angle $X_{CN}$. For purines, a $X_{CN}$ of 0° corresponds to a cis planar arrangement of the O(1')-C(1')-N(9)-C(8) atoms (ie. when O(1') of sugar and C(8) of base are eclipsed) (Paul et al., 1982). For the pyrimidines a $X_{CN}$ of 0° corresponds to a cis planar arrangement of the O(1')-C(1')-N(1)-C(6) atoms (ie, when the O(1') of sugar and C(6) of base are eclipsed) (Hatano et al., 1980). Therefore referring to Fig. 7, anti-conformations are defined by $X_{CN}$ angles between 0° and 75°, and 345° to 360°. $X_{CN}$ angles between 75° and 165° are termed the high anti region. The pure syn region occurs
between $X_{CN}$ angles of $165^\circ$ and $255^\circ$, and the high syn region occurs between $X_{CN}$ angles of $255^\circ$ and $345^\circ$ (Paul et al., 1982).

Figure 6. The syn and anti-conformations of adenosine and uridine.
Figure 7. Definition of glycosidic conformational ranges (from Paul et al., 1982).
1.6.2 Conformation of the C(4')-C(5') exocyclic bond

This bond angle, denoted $\Psi$, can be considered to exist in one of three possible states; (i) gauche-gauche (gg); (ii) gauche-trans (gt); and (iii) trans-gauche (tg) (Sarma et al., 1974). A gauche interaction occurs when two substituents are separated by 60° in a Newman projection. A trans interaction occurs when two substituents are 180° apart in a Newman projection. Both gauche and trans interactions are characterized by a local minimum in a plot of potential energy versus the dihedral angle between two particular substituents. Thus, such conformations are considered to be the most stable about a particular bond. Fig. 8, represents the Newman projections along the C(4')-C(5') bond axis. The three stable conformers are shown below.

![Newman projections showing the three staggered conformations about the exocyclic C(4')-C(5') bond](image)

**Figure 8.** Newman projections showing the three staggered conformations about the exocyclic C(4')-C(5') bond (from Sarma et al., 1974)

The gg, gt, and tg, conventions are used since in the plane of this projection, for this bond, three possible atoms can interact with greatest steric affect on the molecule. These atoms are O(5'), C(3'), and the bridging oxygen (O) of the sugar. The first interaction of the double convention characterizes the spatial arrangement of the O(5') with respect to bridging O, and the second interaction denotes the arrangement of the O(5') with respect to C(3') (Sarma et al., 1974).
1.6.3 Configurations of the sugar

The third portion of the "nucleotidyl unit" that must be considered is the sugar pucker. The conventions used to describe the conformation of the sugar were determined from analysis of crystal structures of nucleosides and nucleotides. It was shown that the furanose ring exists primarily in one of two possible conformations, S or N-type sugars (Davies and Danyluk, 1974). The N-type is when the sugar is puckered with the 2'-exo, 3'-endo configuration and the S-type is denoted by the 2'-endo, 3'-exo configuration (Davies and Danyluk, 1974). Atoms lying on the same side of the plane as C(5') are designated endo and those on the opposite side as exo.

\[ \text{S-type sugar (C-3'}\ exo,\ C-2'\ endo) \]
\[ \text{N-type sugar (C-2'}\ exo,\ C-3'\ endo) \]

B=purine or pyrimidine

Figure 9. Sugar pucker of nucleosides (S and N-type sugars)

1.6.4 The favoured conformation of nucleosides and nucleotides

Nucleoside/nucleotide structures in solution are actually a dynamical average of a number of rapidly interconverting conformers rather than a static state as implied in the previous analysis. X-ray studies on nucleosides and nucleotides have shown that the anti orientation for the base and the gauche-gauche (gg) conformation about the backbone C(4')-C(5') bond are strongly favored (Sundaralingam, 1969; Rubin et al., 1972). Both conformations can be attributed to the alleviation of steric hinderance in the molecule.
Solution state studies have been limited to NMR. Parallel with x-ray studies, $^{13}$C-NMR data has shown that the preferred conformation of adenosine in solution is anti (Nair and Young, 1987). $^{13}$C-NMR data reveal that there are diagnostic differences in chemical shifts in the carbohydrate portion of purine nucleosides in the syn compared with the anti conformations. In particular, the chemical shift of C(2') of the D-ribose moiety appears to be sensitive to changes in the glycosidic bond conformation. The C(2') experiences an upfield shift in the syn conformation in comparison with the anti conformation. The effect may be due to the proximity of the lone pair of electrons of N(3) to the D-ribose C(2')-H(2') bond in the syn conformation, and can be easily appreciated by examining the chemical shift difference between C(2') and C(3') (Nair and Young, 1987). For example, 8-bromoadenosine is known from x-ray crystallographic data to exist in the syn conformation in the solid state (Travale and Sobell, 1970). Its $^{13}$C-NMR spectrum shows that the chemical shift of C(2') is at 71.1 ppm, only 0.3 ppm downfield from that of C(3') (Nair and Young, 1987). In contrast, in the anti-preferring adenosine, the chemical shift of C(2') is 73.6 ppm, about 2.8 ppm downfield from that of C(3') (Nair and Young, 1987).

Assigning the most stable sugar pucker is not as straightforward, and varies greatly depending on the nucleoside or nucleotide under study. Normally a rapid interconversion between the two states (S and N) occurs, and structural data for the sugar pucker in given by a ratio (S:N), which defines a weighted average of the number of molecules in a particular population (Davies and Danyluk, 1974). In this study, which correlates both x-ray and $^1$H-NMR data, calculated equilibrium compositions of ~40% N : ~60% S-type configuration are reported for 5'-ribonucleotides, and ~30% N : ~70% S-type configuration are predicted for 5'-deoxynucleotides (Davies and Danyluk, 1974).

1.7 The rational design of nucleoside and nucleotide inhibitors

1.7.1 Nucleoside selectivities of nucleoside transport (NT) proteins

The selectivities of various NT processes provide a guide to important structural features that predict whether or not a nucleoside drug is likely to be an inhibitor. NT competition studies with radioactive adenosine can be complicated, since the putative
“inhibitor” can interact with the NT protein in one of two ways: (i) it can be a permeant or (ii) it may bind to the NT protein active site and not be transported into the cell. Both scenarios will predict inhibition of adenosine transport.

It has been shown previously that the equilibrative transport proteins are the most widespread, and that many cell lines possess both the es and ei transporters. In the study described in this thesis, the cell line used for the transport assays is the PC12 (pheochromocytoma) a tumor cell line isolated from the rat adrenal gland. Preliminary studies have shown that these cells possess equilibrative transport activity, although the NT process has not been characterized in detail (de Souza, 1995). No concentrative transport activity is evident since substitution of Na⁺ has no affect on nucleoside transport (de Souza, 1995).

Extensive studies of es transporters in other cells have shown that nucleosides with either purine or pyrimidine bases and a variety of different five-carbon sugars, including ribose, 2’-deoxyribose, and arabinose, compete with adenosine (or uridine) for entry and are probably permeants (Cass, 1995). The es transporter is highly stereoselective, with a strong preference for D- over the L-enantiomer (Gu and Geiger, 1992). Generally, transport is greatly decreased or eliminated by the presence of ionized residues, loss or substitution of the 3’-hydroxyl residue, or addition of bulky, hydrophobic substituents at the N(6) position of the purine moiety (Cass, 1995). The importance of the 3’-hydroxyl group in determining permeation suggests that it may be involved in hydrogen bonding of the nucleoside to the transporter protein (Cass, 1995).

A molecular model of the permeant binding site of the es transporter has recently been developed by the application of an algorithm for analysis of the three-dimensional structures and physiochemical properties of various nucleoside permeants (Viswanadhan et al., 1990). This study termed QSAR (quantitative structure-activity relationships) predicts that the permeant-binding site of the es transporter is sensitive to the size and hydrophobicity of the heterocyclic base moiety, prefers nucleosides in the anti-conformation, and has residues that hydrogen bond with the 5’-OH of the sugar moiety (Viswanadhan et al, 1990).
1.7.2 Structure-based synthesis of nucleoside transport (NT) inhibitors

The fact that the transporter favours nucleosides predominantly in the anti-conformation is not surprising, since this is the most stable conformer. A rational approach for obtaining structure-based inhibitors would be to probe this selectivity. Thus, in this research project a molecule 8,2′-thioanhydroadenosine (3), was synthesized and tested. This molecule is unique since it is “locked” permanently in the anti-conformation.

![Figure 10. Structure of 8,2′-thioanhydroadenosine (3)](image)

8-bromoadenosine (1) and 8-mercaptoadenosine (8) were also synthesized and tested. Both substitutions affect not only the hydrophobicity of the heterocyclic but also the conformation about the glycosidic bond. Finally, the selectivity and requirements for the “favoured” sugar pucker were also studied. This is of particular interest since such parameters have never before been examined. This aspect will be addressed once again with the synthesis of 8,2′-thioanhydroadenosine (3), since this molecule is not only “locked” in the anti-conformation, but the sugar is constrained to the S-type configuration (2′-endo, 3′ exo) which is favoured in the natural nucleosides.

![Figure 11. Sugar pucker of 8,2′-thioanhydroadenosine (3)](image)
The sugar pucker requirements were also studied further by synthesis and testing an analogue, adenosine-2',3'-carbonate (9). This constrained molecule cannot adopt either the S or N-type configurations and exists in some "intermediate" form of the two.

![Figure 12. Structure of adenosine-2',3'-carbonate (9)](image)

The pyrimidine/purine base selectivity of the nucleoside transporter in these cells was determined by utilizing another conformationally "locked" molecule 2,2'-anhydrouridine (6). This uridine analog is constrained in the syn-configuration about the glycosidic bond, and adopts the S-type conformation in the sugar.

![Figure 13. Structures of 2,2'-anhydrouridine (6)](image)
1.7.3 Agonist specificity of \(P_2\)-purinoceptors

The existence of multiple \(P_2\)-purinoceptors subtypes that are selectively expressed in different cell types has been largely determined by studying the efficacy of ATP analogs. These analogs represent substitutions on the base, sugar, and modifications of the polyphosphate tail. Substitutions on the base have involved modifications at the N(6), C(8) and C(2) positions (Burnstock et al., 1994). The sugar alterations generally involve 2'-deoxyribose, arabinose or the L-enantiomer. Modifications of the phosphate esters commonly involve replacement of the phosphorus bonded oxygen by methylene (-CH\(_2\)-), difluoromethylene (-CF\(_2\)-) or by dichloromethylene (-CC\(_1\)Cl\(_2\)-).

For the ligand-gated cation channel receptors (\(P_{2X}\) class) the various subclasses can be distinguished by some of these common modifications. For example, contraction of the guinea-pig urinary bladder is induced by ATP binding to the \(P_{2X1}\) -purinoceptors. For these \(P_{2X1}\) -purinoceptors, substitutions on the adenine base at N(6) abolishes activity, although modification at C(8) (as in 8-Bromo-ATP) or at C(2), do not improve potency relative to ATP (Cusack, 1991). Modifications in the polyphosphate tail, ie. \(\alpha\beta\)-methylene-ATP, show enhanced receptor potencies. Surprisingly the stereochemistry of the ribose can be altered since L-ATP binds with the same potency as the natural D-ATP. On the other hand the \(P_{2X7}\) -purinoceptors in rat mast cells are extremely sensitive to alterations of the ATP molecule. Substitutions on N(6) or C(8) of the adenine base leads to loss of activity, but C(2) substitution is allowed as 2-chloro-ATP, 2-methyl-S-ATP, and 2-ethyl-S-ATP are active (Cusack, 1991). The \(\beta\)-D-ribofuranose sugar is an absolute requirement and L-ATP is inactive (Cusack, 1991). For the \(P_{2X2}\)-purinoceptors found in a variety of cell types including PC12, adenine substitutions at C(2) display enhanced or similar potencies to that of ATP but substitutions on the phosphates yield analogs which are inactive (Burnstock, 1997). In summary, different \(P_{2X}\)-receptor proteins display markedly different agonist requirements for molecular recognition.

The structural specificity for the G-protein coupled receptors (\(P_{2Y}\)-class) is also quite broad. For the \(P_{2Y1}\)-purinoceptor, the adenine base is required for maximal activity, since ADP is equipotent with ATP, and GTP, CTP, and UTP are all inactive (Burnstock, 1997).
Substitutions on the adenine base at N(6) abolish activity, but those at C(8) are allowed, while C(2) substitutions enhances potency up to 200 times that of ATP (Cusack, 1991). Replacement of D-ribose by other sugars generates analogues less potent than ATP. For the P2Y2-purinoceptor the specificity for the adenine is flexible, since UTP is equipotent to ATP (Burnstock, 1997). Contrary to the P2Y1-purinoceptors substitutions at C(2) of adenine base reduce the agonist activity for P2Y2-purinoceptors (Cusack, 1991).

<table>
<thead>
<tr>
<th>P2-purinoceptor</th>
<th>Tissue</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y1</td>
<td>brain, placenta, endothelium, prostate and ovary</td>
<td>2-MeSATP ≥ ATP &gt; ADP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(UTP and α,β-meATP inactive)</td>
</tr>
<tr>
<td>P2Y2</td>
<td>lung, bone, pituitary and PC12 cells</td>
<td>ATP = UTP &gt; &gt; 2-MeSATP</td>
</tr>
<tr>
<td>P2X1</td>
<td>Vas deferens and urinary bladder</td>
<td>2-MeSATP ≥ ATP &gt; α,β-meATP</td>
</tr>
<tr>
<td>P2X2</td>
<td>PC12 cells, cochlea and DRG cells</td>
<td>2-MeSATP &gt; ATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(α,β-meATP inactive)</td>
</tr>
<tr>
<td>P2X7</td>
<td>Brain and macrophage</td>
<td>BzATP &gt; ATP &gt; 2MeATP &gt; ADP &gt; UTP</td>
</tr>
</tbody>
</table>

Table 2. Agonist “rank-order potency” profiles for some common P2-purinoceptors (Burnstock, 1997)

The second part of the thesis describes the synthesis of novel nucleoside triphosphate analogues, in hopes that they may target one or more specific P2-purinoceptor subtypes. The activities of the triphosphate analogues were assessed in whole cell experiments utilizing the PC12 (pheochromocytoma) cell line. PC12 cells resemble neurons found in the peripheral nervous system (PNS). An important aspect of these cells is that they secrete ATP which can
act as a feed-back neuromodulator by binding to specific P2-purinoceptors (Raha et al., 1993). PC12 cells express two distinct P2-purinoceptors in the plasma membrane P2Y2 and the P2X2 which can be experimentally distinguished (Raha et al., 1993; de Souza et al., 1995).

1.7.4 Design of structure-based agonists and antagonists of P2Y2 and P2X2-purinoceptors

The design and synthesis of specific P2-purinoceptor agonists are of great importance as cell specific pharmacological agents. To date a receptor-specific antagonist has not been identified. In this research, two nucleoside triphosphate analogs were synthesized and tested for P2Y2 and P2X2-purinoceptor activity in PC12 cells. Since it has been established that ATP in solution exists primarily in the anti-conformation about the glycosidic bond, the synthesis of an anti-"locked" analog of ATP, 8,2'-thioanhydroadenosine-5'-triphosphate (4) would be of considerable interest. Agonist activity of such a constrained molecule would clarify the P2-purinoceptor selectivity for the more "stable" conformer of ATP. Furthermore, analog requirements of sugar pucker for the P2-purinoceptors can be analyzed since this molecule is "locked" in the more abundant S-type sugar configuration. This is of special interest since such characteristics have never before been studied in P2-purinoceptors. The second triphosphate analog synthesized is the syn-"locked" analog of UTP, 2,2'-anhydrouridine-5'-triphosphate (7). UTP activates the P2Y2-purinoceptor with similar potency to that of ATP. Thus, this constrained nucleoside triphosphate will be used to determine the selectivity of the P2Y2-purinoceptor for the pyrimidine nucleotide unit. Once again both the requirements for the conformation about the glycosidic bond and the sugar pucker will be clarified (2,2'-anhydrouridine (6) adopts the favoured S-type configuration of the sugar moiety).

1.8 Physiological effects of ATP in PC12 Cells

The primary physiological response of activation P2Y2-purinoceptors in most cells including PC12 cells, is a marked transient increase in intracellular Ca\(^{2+}\) upon binding of ATP (Raha et al., 1993). This increase in intracellular Ca\(^{2+}\) results from G-protein linked activation of phospholipase C and release of Ca\(^{2+}\) from the endoplasmic reticulum (ER) stimulated by IP3 (inositol 1,4,5-triphosphate) formation (Raha et al., 1993). In PC12 cells P2Y2-purinoceptor
activation also results in a transient influx of Ca\textsuperscript{2+} from the extracellular environment mediated by membrane bound Ca\textsuperscript{2+}-channel proteins, although the exact nature of this apparent response is still unknown (Raha et al., 1993). Changes in intracellular [Ca\textsuperscript{2+}] is normally measured using a fluorescent dye (Fura-2). The fluorescence excitation of Fura-2 increases upon binding of Ca\textsuperscript{2+} (Gryniewicz et al., 1985).

ATP activation of P\textsubscript{2X} purinoceptors in PC12 cells results in an influx of Na\textsuperscript{+} leading to membrane depolarization. Changes in membrane potential can be measured with the fluorescent potential-sensitive anionic dye, bisoxonol (Rink et al., 1980). In addition, activation of P\textsubscript{2X}-purinoceptor also results in a transient decrease in pH of the intracellular environment, which results from an indirect activation of the HCO\textsubscript{3}^- / Cl\textsuperscript{-} exchanger (Moore and Reed, 1997). The extrusion of HCO\textsubscript{3}^- displaces the CO\textsubscript{2} / HCO\textsubscript{3}^- equilibrium producing a transient burst of intracellular protons. Changes in intracellular pH is measured with the fluorescent dye BCECF, which displays differing spectral properties at various ionization states (Graber et al., 1986).

*Figure 14.* Physiological effects of ATP in PC12 cells
1.9 Synthetic schemes of nucleoside and nucleotide analogs

1.9.1 Synthesis of $8,2'\text{-thioanhydroadenosine-5'}$-triphosphate (4)

The retro-synthesis of $8,2'\text{-thioanhydroadenosine-5'}$-triphosphate (4) is shown (scheme 3). The starting material is the commercially available nucleoside adenosine. The key intermediate in the synthesis is $8\text{-bromoadenosine-2',3'}\text{-carbonate}$ (2) which reacts with thiourea to form the sulphur bridged nucleoside, subsequent phosphorylation of the $5'$-hydroxyl yields the desired product, compound (4).

\begin{center}
\includegraphics[width=\textwidth]{synthetic_schemes}
\end{center}

\textit{Scheme 3.} Retro-synthesis of $8,2'\text{-thioanhydroadenosine-5'}$-triphosphate (4)
1.9.2 Synthesis of 2,2'-anhydouridine-5'-triphosphate (7)

2,2'-anhydouridine-5'-triphosphate (7) is synthesized from uridine which reacts with diphenyl carbonate to form the cyclic-nucleoside 2,2'-anhydouridine (6). Phosphorylation of the 5'-hydroxyl of the cyclic-nucleoside yields the product (7).

Scheme 4. Retro-synthesis of 2,2'-anhydouridine-5'-triphosphate (7)

1.9.3 Synthesis of 8-Mercaptoadenosine (8)

The adenine analog, 8-mercaptoadenosine (8) was obtained by reacting 8-bromoadenosine (1) with thiourea.

Figure 15. Structure of 8-mercaptoadenosine (8)
1.9.4 *Synthesis of Adenosine-2',3'-carbonate (9)*

The synthesis of adenosine-2',3'-carbonate (9) utilizes diphenyl carbonate, to introduce the 2',3'-protecting group on adenosine.

*Figure 16. Structure of adenosine-2',3'-carbonate (9)*
II. EXPERIMENTAL PROCEDURES

2.1 Materials

The following materials were obtained from the indicated suppliers and were used without further purification: 1,8-bis-(dimethylamino)-naphthalene (proton sponge), anhydrous dimethylformamide (DMF), and DMSO-d$_6$ were purchased from Aldrich Chemical Co.; adenosine, uridine and ATP were from ICN Biochemicals Inc.; AMP, ADP, NBTI (nitrobenzylthioinosine) and DPR (dipyridamole) were obtained from Sigma Chemical Co.; EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine was purchased Research Biochemicals International (RBI). An authentic sample of 8,2'-thiohydrodadenosine (3) was the kind gift of Dr. K. K. Ogilvie.

Infrared spectra were run as KBr pellets on a Nicolet 5DXB FT-IR spectrometer. All 200 MHz $^1$H-NMR and $^{31}$P-NMR spectra of compounds were run on a Varian GEMINI-200 FT-NMR and analyzed with Felix for Windows (version 1.02, BIOSYM technologies, 1993) software. TLCs were carried out using Whatman Fluorescent-Silica (250 µM layer, uv 254 nm). Purification of organic compounds was accomplished by column chromatography with Silica-gel (70-230 mesh, 60 Å sphere, Caledon Chemical Co.). Cation exchange of sodium pyrophosphosphate was performed by Dowex-50W-X8 (20-50 mesh, ionic form H$^+$, J.T. Baker Chemical Co.). Tri-n-butylammonium pyrophosphate was stored over molecular sieves (4 Å beads, 4-8 mesh, Aldrich Chemical Co). Nucleoside triphosphates were purified by Sephadex-DEAE A-25 anion exchange resin (Pharmacia Fine Chemicals). Nucleotide containing fractions were analyzed by uv (254 nm) on a Gilford-250 UV spectrophotometer. Mass spectra of the nucleoside and nucleotides analogues were obtained on a VG 70-250S spectrometer under FAB (fast atom bombardment) conditions using 8-kV Xe atoms in a glycerol matrix. All nucleosides were analyzed on a Waters 501 HPLC system using a C-18 reverse-phase analytical column and eluted isocratically with 10% MeOH in a potassium phosphate buffer (4mM, pH 5.8) at a flow rate of 1.0 mL/min. Peaks were detected at 254 nm using a uv detector. Fluorescence measurements on PC12 cells in suspension were performed on an SLM model 4800 spectrofluorimeter.
2.2 Synthesis of nucleoside and nucleotide analogs

2.2.1 Preparation of 8,2'-thioanhydroadenosine-5'-triphosphate (4)

8-bromoadenosine (1)

In a procedure adapted from Ikehara and Kaneko (1970) adenosine (5.0 g, 19 mmole) was dissolved in sodium acetate buffer (0.5 M, pH 4, 150 mL) with stirring and slight heating. The resulting solution was cooled to room temperature and Br$_2$-water (200 mL of water + 2.08 mL of Br$_2$, 38 mmole) was added. After 3 h at room temperature the colour of the solution (dark-orange) was discharged by addition of 5M NaHSO$_3$ (light-yellow) and the pH of the solution was adjusted to 7 with 5 M NaOH (15 mL). After cooling for 10 h (4°C) the yellow crystalline precipitate was filtered by suction, washed with water (50 mL), then with acetone (50 mL) and dried and the first crop (4.09 g, 11.82 mmole) was obtained. The filtrate was evaporated to half its volume, cooled (4°C) overnight, and a second crop of 8-bromoadenosine (1) (1.22 g, 3.54 mmole) was obtained (total yield 81%, lit. (Ikehara and Kaneko, 1970) 75%): (mp decomposes at 223-230°C, lit. (Ikehara and Kaneko, 1970) mp >200°C); TLC (ethylacetate) R$_f$ = 0.20; IR (KBr) ν 3421, 3355, 1569, 1095, 870 cm$^{-1}$; $^1$H-NMR (200 MHz, DMSO-$d_6$) δ 3.57 (2x dd, 2H, 5’-H, 5''-H ribose), 4.00 (q, 1H, 4’-H, J$_H4.5,5''_H$ = 4.0 Hz), 4.21 (q, 1H, 3’-H, J$_H3.4$ = 2.4 Hz), 5.10 (q, 1H, 2’-H, J$_H2.3$ = 4.6 Hz), 5.20 (b, 1H, 2’-OH), 5.50 (m, 2H, 5’-OH, 3’-OH), 5.87 (d, 1H, 1’-H, J$_H1.2$ = 6.6 Hz), 7.62 (s, 2H, NH$_2$ adenine), 8.14 (s, 1H, 2-H adenine).

8-bromoadenosine-2',3'-carbonate (2)

Using a procedure adapted from Ogilvie et al. (1972), 8-bromoadenosine (1) (3.92 g, 11.32 mmole), diphenyl carbonate (3.28 g, 15.2 mmole) and sodium bicarbonate (60 mg) were heated in DMF (12 mL) at 150°C (oil-bath) for 30 min. The mixture was cooled to room temperature and the resulting solution (orange) was evaporated to a total volume of 2 mL. The carbonate product had an R$_f$ = 0.53 by TLC (ethylacetate). The mixture (2 mL) was purified by silica gel gravity column chromatography with ethylacetate and 135 (3 mL) fractions were collected and analyzed by TLC. Fractions 100-135 were pooled and
evaporated to dryness under vacuum, yielding a chalky-white powder of pure 8-bromoadenosine-2',3'-carbonate (2) (2.47 g, 6.64 mmole) (yield 59%, lit. (Ogilvie et al., 1972) 70%) : (mp 164-168°C, lit. (Ogilvie et al., 1972) mp 159-162°C) ; IR (KBr) ν 3508, 3180, 3337, 1811 (carbonate) , 1661, 1611, 1462, 1298, 1162, 1062 cm⁻¹ (lit. (Ogilvie et al., 1972) 1802, 1470, 1130, 1081 cm⁻¹) ; ¹H-NMR (200 MHz, DMSO-d₆) δ 3.47 (t, 2H, H-5', H-5''), 4.40 (q, 1H, 4'-H, J₅',₅'' = 6 Hz), 5.15 (b, 1H, 5'-OH), 5.52 (dd, 1H, 3'-H, J₅',₄ = 4 Hz), 6.26 (dd, 1H, 2'-H, J₄,₃ = 8 Hz), 6.34 (d, 1H, 1'-H, J₁₄₂ = 0.8 Hz), 7.59 (s, 2H, NH₂ adenine), 8.15 (s, 1H, 2-H adenine).

a J₅',₅'' = (J₅',₄ + J₅',₃)/2

8,2'-thioanhydroadenosine (3)

Using a procedure adapted from Ogilvie et al. (1972), 6.45 mmole (2.40 g) of the cyclic carbonate (2) and 2 equiv. of thiourea (12.90 mmole, 1.01 g) were refluxed in freshly distilled n-butanol (20 mL) for 5 h (110°C). The solvent was evaporated to 2 mL and a small aliquot applied to a TLC plate and eluted with ethylacetate and ethanol (5:1), yielding two spots. The thio-product had an Rf = 0.32 compared to an authentic sample obtained from K. K. Ogilvie. The crude product (2 mL) was purified by flash silica gel chromatography eluted with ethylacetate and ethanol (5:1) and 160 (3 mL) fractions were collected and analyzed by TLC. Fractions 100-160 were pooled and evaporated to dryness under vacuum, yielding a white powder of pure 8,2'-thioanhydroadenosine (3) (1.16 g, 4.14 mmole) (yield 64%, lit. (Ogilvie et al., 1972) 66%) : (mp 141-153°C, lit. (Ogilvie et al., 1972) mp 140-150°C) ; HPLC >99% purity, retention time 13.8 min ; IR (KBr) ν 3352, 2995, 1669, 1596, 1463, 1071, 998 cm⁻¹ ; uv (H₂O) λmax =276 nm ; lit. (Ogilvie et al., 1972) same ; ¹H-NMR (200 MHz, D₂O) δ 3.57 (2x dd , 2H, 5'-H, 5''-H), 4.26 (ddd, 1H, 4'-H, J₄',₄ = 5.2 Hz), 4.56 (t, 1H, 3'-H, J₅',₄ = 3.2 Hz), 4.95 (dd, 1H, 2'-H, J₄,₃ = 2.6 Hz), 6.62 (d, 1H, 1'-H, J₁₄₂ = 6.6 Hz), 8.06 (s, 1H, 2-H adenine) ; ¹H-NMR (200 MHz, DMSO-d₆) δ 3.40 (unres. , 2H, 5'-H, 5''-H), 4.00 (q, 1H, 4'-H, J₄',₄ = 5.0 Hz), 4.40 (unres. , 1H, 3'-H), 4.86 (dd, 1H, 2'-H, J₄,₃ = 2.5 Hz), 5.50 (unres. , 1H, 5'-OH), 5.90 (unres. , 1H, 3'-OH), 6.52 (d, 1H, 1'-H, J₁₄₂ = 6.4 Hz), 7.13 (s, 2H, NH₂ adenine), 8.07 (s, 1H, 2-H adenine) ; FAB-MS m/z=282
The synthesis of the procedure nucleoside 5'-triphosphate was adapted from Zimmet et al., (1993) with modifications.

Preparation of tri-n-butylammonium pyrophosphate - 6.69 g of sodium pyrophosphate decahydrate (15 mmole) was dissolved in 150 mL of water and an excess of Dowex ion exchange resin (25 g, ~40 mequiv., 50X8, 20-50 mesh, H⁺ form) was added. The mixture was stirred gently for 20 min. A mixture of 60 mL of ethanol and 7.14 mL of tri-n-butylamine was placed in a flask in an ice-water bath, and the pyrophosphate solution was filtered by suction directly into this flask. The resin was washed with 200 mL of water, and the total filtrate (pH 3.2) was titrated to pH 7.0 by the dropwise addition of tri-n-butylamine. The solvent was evaporated under vacuum at 35°C, yielding a thick, nearly colourless syrup. This residue was treated twice with 90 mL of anhydrous ethanol and evaporated. This is to remove residual water. The residue was taken up in 50 mL of anhydrous DMF and evaporated once again. The resulting yellow oil, was taken up in an additional 10 mL of anhydrous DMF. DMF washes of the flash were added, finally yielding 30 mL of 0.5 M tri-n-butyl ammonium pyrophosphate. The preparation was stored cold (4°C) over 4 Å molecular sieves.

Preparation of triethylammonium bicarbonate (TEAB) buffer - A 1 M solution was prepared by vigorously stirring a mixture of 55 mL triethylamine and 400 mL of deionized water under a balloon of CO₂ until the pH of the resulting buffer was 7.5.

Synthesis of nucleoside triphosphate- 1.1 mmole (0.390 g) of dry nucleoside (dessicator stored over P₂O₅), 1.7 mmole (0.234 g, 1.5 equiv.) of dry 1,8-bis-(dimethylamino)naphthalene (proton sponge) and 15 mL of trimethyphosphate were placed in a three-neck round bottom flask. The resulting mixture was stirred at room temperature under N₂ until the
nucleoside dissolved. The solution was cooled in an ice-water bath, and after 30 min, 205 μM (2.2 mmole) of POCl₃ was added dropwise through a septum. After 2 h of stirring at 0-4°C, a mixture of 0.5 M tri-n-butylammonium pyrophosphate in anhydrous DMF (15 mL, 5 equiv.) and tri-n-butylamine (1.5 mL) was quickly added and a dark purple solution resulted. After 2 h of stirring at 0-4°C, a mixture of 0.5 M tri-n-butylammonium pyrophosphate in anhydrous DMF (15 mL, 5 equiv.) and tri-n-butylamine (1.5 mL) was quickly added and a dark purple solution resulted. After 1 min, 0.2 M aqueous triethylammonium bicarbonate (TEAB, pH 7.5, 100 mL) was added to the reaction to destroy metaphosphates and stirring was continued at room temperature for 30 min. (faint pink solution). The solvent was then evaporated (25°C) under high vacuum, yielding a thick pink syrup. The residue was taken up in 10 mL of deionized water and extracted three times with 10 mL of diethyl ether. The aqueous layer was then lyophilized overnight to yield a fluffy white solid.

DEAE-Sephadex purification - The crude nucleoside 5′-triphosphate was purified by anion-exchange chromatography with DEAE-Sephadex A-25 (12 g, ~25 mequiv. swelled in 1.0 M NaHCO₃), using a 20x2 cm column eluted at room temperature. After equilibrating the column with deionized water (400 mL), the residue of the reaction mixture dissolved in water (8 mL) was applied. The column was washed with deionized water (400 mL), followed by a linear gradient of 0-700 mM TEAB buffer. The triphosphates were eluted in 1 L of buffer, and 100 (10 mL) fractions were collected and analyzed with a uv spectrometer (254 nm). The elution peak fractions were analyzed by TLC (1-propanol/ammonium hydroxide/water, 9:7:5) for the presence of triphosphates (Rₜ = 0.18). The fractions containing triphosphates were pooled and lyophilized. The residue was taken up in water (3 mL) and passed through a charcoal column (5 g, 60 mesh, 10x1 cm). The eluate was monitored by uv (254 nm) and appropriate fractions were pooled and lyophilized once again. The resulting fluffy white residue was analyzed by ³¹P-NMR. The spectra indicated the presence of the inorganic impurities pyrophosphate and metaphosphate. These impurities were removed by passing the crude nucleoside triphosphate through a second DEAE-Sephadex column as outlined above and successive lyophilization of the residue to remove TEAB afforded the triethylammonium salt of the nucleoside-5′-triphosphate (translucent glass). This triethylammonium salt was dissolved in methanol to give a concentration of 0.05 M, and five volumes of an acetone
solution of sodium perchlorate (15 equiv.) was added. The precipitated sodium salt was collected by centrifugation, washed with acetone (four 5 mL portions), and dried over phosphorus pentoxide under vacuum. This afforded the sodium salt of the nucleoside-5′-triphosphate (4) (102.2 mg, 0.17 mmole) (yield 15%) : 1H-NMR (200 MHz, D2O) δ 3.94 (m, 2H, 5′-H, 5′′-H, JH5,5′,p = 6.6 Hz), 4.39 (q, 1H, 4′′-H, JH4,5′,5′′ = *5.2 Hz), 4.71 (t, 1H, 3′-H, JH3,2 = 7.4 Hz), 6.53 (d, 1H, 1′-H, JH1,2 = 7.4 Hz), 8.00 (s, 1H, 2-H adenine); 31P-NMR (200 MHz, D2O, pH 7) δ -22.48 (br s, 1P, βP), -10.87 (d, 1P, αP, Jαβ = 20.2 Hz), -9.79 (d, 1P, γP, Jϕ = 18.3 Hz); FAB-MS calculated for C10H10O12N3P3 of the free acid [M+] = 517.206, found 519.9 [M+ + H+ + 2Na+], 541.9 [M+ + 2H+ + Na+] and 585.8 [M+ + 3Na+] (most intense peak is in bold)  

\*JH5,5′,5′′ = \(\frac{JH4,5′,5′′}{2}\)  

2.2.2 Preparation of 8,2′-thioanhydroadenosine-5′-monophosphate (5)  

8,2′-thioanhydroadenosine-5′-monophosphate (5)  

The procedure for the preparation of nucleoside monophosphates was adapted from Hoard and Ott (1965). The nucleoside (140 mg, 0.5 mmole) was first dried in an oven (100°C) for 4 h, dissolved in 4 mL of trimethylphosphate, and the resulting solution cooled to 0°C. POCl3 (95 μL, 1 mmole) was added dropwise maintaining the temperature at 0°C and allowed to react for 6 h. The resulting mixture was poured into ice water (10 mL) and stirred for another 1 h at 5°C. The solvent was removed in vacuo (35°C) to yield a yellowish syrup. This syrup was taken up in 5 mL of water and analyzed by TLC (1-propanol/ammonium hydroxide/water, 7:5:2) and the monophosphate was identified with an Rf of 0.61 along with some unreacted nucleoside (Rf = 0.86). An additional 5 mL of water was added and extracted three times with 10 mL of diethylether. The aqueous layer was lyophilized yielding a yellow-white residue. This residue was taken up in 5 mL of water and applied to a DEAE-Sephadex column as outlined for the triphosphate purification, with the exception that a linear gradient of 0-350 mM TEAB buffer was used and only one pass through the column was required to obtain the pure monophosphate as the triethylammonium salt. This ammonium salt was converted to the sodium salt as outlined previous affording the 8,2′-thioanhydroadenosine-5′-
monophosphate (5) (138 mg, 0.36 mmoles) (yield 72%): $^1$H-NMR (200 MHz, D$_2$O) $\delta$ 3.80 (m, 2H, 5'-H, 5''-H) 4.35 (q, 1H, 4'-H, $J_{H4,5'5''}$ = $^4$5.7 Hz), 4.66 (t, 1H, 3'-H, $J_{H3,4}$ = 3.8 Hz), 4.96 (dd, 1H, 2'-H, $J_{H2,3}$ = 3.0 Hz), 6.58 (d, 1H, 1'-H, $J_{H1,2}$ = 6.6 Hz), 8.05 (s, 1H, 2-H adenine); $^{31}$P-NMR (200 MHz, D$_2$O, pH 11, H-P coupled) $\delta$ 3.92 (d, 1P, 3'-P, $J_{H3,3'}$ = 7.7 Hz, %P$_{total}$ = 8%), 4.37 (t, 1P, 5'-P, $J_{H5,5'}$ = 5.4 Hz, %P$_{total}$ = 92%)

$^*$ $J_{H4,5'5''}$ = ($J_{H4,5'}$ + $J_{H4,5''}$)/2

2.2.3 Preparation of 2,2'-anhydouridine-5'-triphosphate (7)

2,2'-anhydouridine (6)

Uridine (5.0 g, 20.5 mmoles) was dissolved in 10 mL of dimethylformamide (DMF) and treated with diphenyl carbonate (5.7 g, 26.5 mmoles) and sodium bicarbonate (100 mg) according to Hampton and Nichol (1966). The mixture was heated at 150°C in an oil-bath for 30 min. and poured into diethyl ether (50 mL). The precipitated gum when crystallized from methanol afforded the 2,2'-anhydouridine (6) as a beige powder (2.7 g, 12.10 mmoles) (yield 59%, lit. (Hampton and Nichol, 1966) 59%) : (mp 230-235°C, lit. (Hampton and Nichol, 1966) mp 238-244°C); HPLC >99% purity, retention time 3.4 min; $^1$H-NMR (200 MHz, D$_2$O) $\delta$ 3.56 (d, 2H, 5'-H, 5''-H), 4.39 (q, 1H, 4'-H, $J_{H4,5'5''}$ = $^4$4.0 Hz), 4.66 (dd, 1H, 3'-H, $J_{H3,4}$ = 1.4 Hz), 5.46 (dd, 1H, 2'-H, $J_{H2,3}$ = 0.8 Hz), 6.18 (d, 1H, 5-H uracil, $J_{H5,5'}$ = 7.2 Hz), 6.53 (d, 1H, 1'-H, $J_{H1,2}$ = 5.8 Hz), 7.91 (d, 1H, 6-H uracil); FAB-MS m/z=227 corresponding to MH$^+$ (MW=226)

$^*$ $J_{H4,5'5''}$ = ($J_{H4,5'}$ + $J_{H4,5''}$)/2

2,2'-anhydouridine-5'-triphosphate (7)

The reaction was carried out on 1.51 mmoles (0.342 g) of the nucleoside following the pyrophosphorylation procedure as outlined previous. In this reaction however 1.7 equiv. (2.57 mmoles, 0.551 g) of proton sponge was used. TLC (1-propanol/ammonium hydroxide/H$_2$O, 9:7:5) indicated the presence of the desired triphosphate product ($R_t$ = 0.17). Identical work-up and DEAE-Sephadex purification, yielded the triethylammonium triphosphate salt, which was converted to the sodium salt, to afford 2,2'-anhydouridine-5'-
triphosphate (7) as a off-white powder (148 mg, 0.27 mmoles) (yield 18%) : ^1^H-NMR (200 MHz, D_2O ) $\delta$ 4.05 (unres., 2H, 5'-H, 5''-H, J_{H5,5'..P} = 5.6 Hz), 4.59 (unres., 1H, 4''-H, J_{H4,5,5''..} = a 2.3 Hz), 4.85 (t, 1H, 3'-H, J_{H3,4..} = 0.8 Hz), 5.02 (s, 1H, 2'-H, J_{H2,3..} < 0.8 Hz), 6.29 (d, 1H, 5-H uracil, J_{H5,6} = 7.4 Hz), 6.60 (d, 1H, 1'-H, J_{H1,2..} = 5.8 Hz), 8.00 (d, 1H, 6-H uracil);

^3^P-NMR (200 MHz, D_2O, pH 7) $\delta$ -22.51 (t, IP, PP), -11.08 (d, 1P, $\alpha$P, J* = 19.4 Hz), -9.67 (d, 1P, $\beta$P, J* = 19.8 Hz); FAB-MS calculated for C_{16}H_{10}O_{12}N_{3}P_{4} of the free acid $[M^+] = 462.016$, found 464.9 $[M^+ + 2H^+]$, 486.9 $[M^+ + 2H^+ + Na^+]$ and 531.2 $[M^+ + 3Na^+]$ (most intense peak is in bold)

$^a$J_{H4,5,5''..} = (J_{H4,5..} + J_{H4,5..})/2

2.2.4 Preparation of 8-mercaptoadenosine (8)

8-mercaptoadenosine (8)

A mixture of 8-bromoadenosine (1) (2.0 g, 5.8 mmoles) and thiourea (0.9 g, 11.6 mmoles) in 100 mL of absolute ethanol was refluxed for 5 h. The ethanol was evaporated in vacuo to a volume of 5 mL and the resulting orange solution was analyzed by TLC. The TLC (ethylacetate/ethanol, 5:1) showed the presence of two spots, one with an Rf of 0.20 corresponding to the unreacted bromo compound and the other with an Rf of 0.54 which was identified as the thiol product. The thiol was purified by silica-gel column chromatography eluted with the above TLC solvent system. 50 (3 mL) fractions were collected and analyzed by TLC to determine content, the appropriate fractions were pooled (fractions 35-42) and evaporated to dryness under vacuum to yield an ochre gum. This residue was dissolved in a 1:1 mixture of ethanol/chloroform with heating. Chloroform was added to the hot clear yellow liquid, until the solution became slightly cloudy. After cooling for 10 h (4°C) the light-yellow crystals were filtered by suction, washed with chloroform and dried in a dessicator overnight, to afford 8-mercaptoadenosine (8) (0.87 g, 2.9 mmoles) (yield 50%) : (mp 171-173°C lit. (Ikehara and Yamada, 1971) mp 170°C) ; ^1^H-NMR (200 MHz, D_2O ) $\delta$ 3.85 (2x dd, 2H, 5'-H, 5''-H ribose), 4.25 (q, 1H, 4''-H, J_{H4,5,5''..} = a 2.9 Hz ), 4.45 (dd, 1H, 3'-H, J_{H3,4..} = 2.4 Hz), 5.02 (t, 1H, 2'-H, J_{H2,3..} = 5.4 Hz), 6.54 (d, 1H, 1'-H, J_{H1,2..} = 7.0 Hz), 8.06 (s, 1H, 2-H adenine).
2.2.5 Preparation of adenosine-2′,3′-carbonate (9)

This synthesis was adapted from Hampton and Nichol (1966). Adenosine (1.0 g, 3.74 mmoles) was dissolved in 5 mL of dimethylformamide (DMF) and treated with phenol (0.34 g, 0.36 mmoles) and diphenyl carbonate (1.2 g, 5.6 mmoles). The mixture was heated at 150°C in an oil-bath for 30 min and poured into diethylether (20 mL) while still hot. The fine buff-coloured precipitate was filtered off and washed with ether. The residue was taken up in 2 mL of DMF and applied to a TLC (ethylacetate); the carbonate product gave an intense spot with an Rf = 0.73. The crude mixture was run through a silica-gel column eluted with ethylacetate and the purified carbonate product was obtained, (0.99 g, 3.37 mmoles) (yield 90% lit. (Hampton and Nichol, 1966) 96%) : (mp 200-205°C lit. (Hampton and Nichol, 1966) mp 218-220°C) ; IR (KBr) v 3336, 3180, 2934, 1802 (carbonate), 1646, 1392, 1089 cm⁻¹; ¹H-NMR (200 MHz, DMSO-d₆) δ 3.60 (t, 2H, H-5′, H-5″), 4.42 (m, 1H, 4′-H, JH₄.₅-₆. = 6 Hz), 5.25 (t, 1H, 5′-OH), 5.50 (dd, 1H, 3′-H, JH₃.₄. = 3.6 Hz), 6.00 (dd, 1H, 2′-H, JH₂.₃. = 7.5 Hz), 6.50 (d, 1H, 1′-H, JH₁.₂. = 2.6 Hz), 7.40 (s, 2H, NH₂ adenine), 8.20 (s, 1H, 2-H adenine), 8.35 (s, 1H, 8-H adenine); ¹H-NMR (200 MHz, DMSO-d₆ + D₂O, exchange spectra) δ 3.76 (d, 2H, H-5′, H-5″), 4.43 (q, 1H, 4′-H, JH₄.₅-₆. = 5.2 Hz), 5.57 (dd, 1H, 3′-H, JH₃.₄. = 3.1 Hz), 5.98 (dd, 1H, 2′-H, JH₂.₃. = 7.6 Hz), 6.48 (d, 1H, 1′-H, JH₁.₂. = 2.5 Hz), 8.24 (s, 1H, 2-H adenine), 8.37 (s, 1H, 8-H adenine).

[a] JH₄.₅-₆. = (JH₄.₅. + JH₄.₆.)/2

2.3 Cell culture

PC12 cells (A and G) obtained from Dr. Lloyd Greene (New York University of Medicine) or from ATCC (American Tissue Culture Collection) were grown on 100 mm Falcon tissue culture plates in RPMI media supplemented with 5% fetal calf serum, 5% heat-inactivated donor horse serum, 2 mM L-glutamine, and 1% penicillin/streptomycin at 37°C with an atmosphere of 5% CO₂. Cells were passaged once every 5-7 days.
2.4 Adenosine transport assays

All adenosine transport studies were conducted on attached PC12G cells grown in 3 mm 24 well Coster plates precoated with poly-D-lysine 37°C for 1-2 h. 1 h prior to the uptake experiments 100 μL of 0.5 M HEPES (pH 7.5) was added to each well so that the final concentration of HEPES in the culture medium was 20 mM. This was to maintain the pH at 7.5 after removal from the CO₂ environment. After removal of the culture medium, the cells were washed twice with 0.5 mL of standard incubation buffer (145 mM NaCl, 25 mM HEPES (pH 7.5), 20 mM glucose, 5 mM KCl, 1.25 mM CaCl₂, and 0.8 mM MgCl₂). Cells were preincubated with 0.2 mL of the standard buffer for 10 min at 37°C. In experiments where adenosine deaminase was inhibited, the cells were preincubated for the 10 min period with 10 μM EHNA. In experiments where further adenosine metabolism was inhibited by ATP depletion, the cells were incubated with 10 μM EHNA, 5 mM iodoacetate and 5 mM KCN for 10 min. For all ATP depletion studies the cells were preincubated in glucose-free standard buffer. In nucleoside analog inhibition studies the analog was also preincubated in this cocktail at the desired concentration. After aspiration of the standard buffer, transport was initiated by the addition of 0.2 mL of [³H]-adenosine containing assay buffer. This assay buffer contained all the components of the standard incubation buffer in addition to 10 μM [²⁻³H]-adenosine (21 μCi/mmole) (21 Ci/mmol; Amersham Life Science). Unless indicated otherwise, incubations were for a 2 min period at 37°C. The assay buffer was aspirated and transport terminated by the addition of 0.5 mL of ice-cold transport inhibitor-stop cocktail (standard incubation buffer containing 0.1mM DPR, an inhibitor of nucleoside transport). Following removal of this medium, cells were washed two additional times with ice-cold transport inhibitor-stop cocktail. Cells were digested with 0.4 NaOH (0.3 mL) at 37°C for 1-2 h or overnight at room temperature. A 50 μL aliquot was removed for protein analysis by the method of Lowry et al. (1951). 0.2 mL of the remaining solution was placed into a scintillation vial, with 8 μL of glacial acetic and 4.5 mL of scintillation fluid (Universol) and the radioactivity determined.
2.5 Determination of accumulated adenosine and metabolites

Accumulated adenosine and its metabolites were isolated and quantified using TLC. Adenosine transport assays were performed as described above except that a higher specific activity of [3H]-adenosine was used (105 µCi/mmole) and incubations were for 2 and 10 min. After removal of the transport inhibitor-stop cocktail, 0.2 mL of 5% trichloroacetic acid was added to each well and after 12 h at 4°C, cells were scraped off and transferred to 1.5 mL centrifuge tubes. Following centrifugation at 13,000 g for 5 min, the pellets were washed with 1.0 mL of acetone and digested with 0.1 mL of 1.0 M NaOH at 37° for 1-2 h. The protein was determined. 50 µL of supernatant was spotted onto Merck cellulose-precoated plastic TLC plates (Darmstadt, Germany) which were developed in 1-butanol/acetone/glacial acetic acid/ammonium hydroxide/H2O (14:10:6:1:8). Sample aliquots were "spiked" with non-radioactive adenosine, inosine and AMP (each at 2mM) for identification. The appropriate bands were cut out, the cellulose scrapped of the plate, placed into scintillation vials containing 0.5 mL of 0.1 M HCl. The vials were mixed well to extract the metabolites and radioactivity determined.

2.6 Measurement of [Ca2+]i in PC12 cells

Intracellular calcium ([Ca2+]i) was measured using the fluorescent calcium indicator Fura-2 (Gryniewicz et al., 1985) as outlined (Raha et al., 1993). PC12A cells are pre-loaded with Fura-2 in the standard incubation buffer supplemented with 1 mg/mL of BSA and 2 µM Fura-2-AM, and incubated for 30 min at 37°C. Following centrifugation, the cells were resuspended in buffer and stored in ice for up to 2 h. Fluorescence measurements (F) on cell suspensions were performed by exciting the dye at 340 nm and following the emission at 505 nm. Cells were equilibrated for 3 min at 37°C in a stirred thermostated cuvette prior to addition of compounds. Calculations of [Ca2+]i were made using the equation [Ca2+]i = Kd (F - Fmin)/(Fmax - F). Calibrations of the fluorescence signals were made at the end of each measurement. Fmax was the fluorescence following the addition of 15 µg/mL digitonin. Fmin was determined with 10 mM EGTA, pH >8. A value of 224 nM was used for the Kd.
2.7 Measurement of PC12 cell membrane potential with bisoxonol

Changes in the membrane potential of PC12A cells were monitored using the fluorescent potential-sensitive anionic dye, bisoxonol (bis[1,3-diethylthiobarbiturate]trimethine oxonol) (Rink et al., 1980) as outlined in de Souza et al., (1995). Cells were incubated with 50 nM bisoxonol for 5 min at 37°C prior to the addition of compounds. Fluorescence measurements were made at 540 nm (excitation) and 580 nm (emission).

2.8 Measurement of intracellular pH in PC12 cells

The intracellular pH was measured using the pH-sensitive dye 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF) (Graber et al., 1986) using the procedure described by de Souza et al. (1995). PC12A cells were harvested and loaded with 5 μM of BCECF-AM for 30 min at 37°C. Cells were washed and resuspended in incubation buffer (10 x 10⁶ cells/mL) and kept on ice for up to 2 h until use. Fluorescence measurements (at 1.5 x 10⁶ cells/mL) were made at 37°C (500 nm, excitation; 525 nm, emission). Intracellular pH was determined from a calibration curve as described previous (de Souza, 1995).

2.9 Uptake of adenosine and 8,2'-thioanhydroadenosine (3) measured by HPLC

PC12G cells (2.5 x 10⁶ cells/plate) were grown in 35 mm Coster plates precoated with polylysine. Media was removed and attached cells were washed 3 times with 1 mL of standard incubation buffer. Uptake experiments were initiated by addition of 3 mL standard incubation buffer containing 50 μM adenosine or 50 μM 8,2'-thioanhydroadenosine (3). 200 μL aliquots were removed at specified times, centrifuged for 1.5 min and supernatant was stored on ice. 20 μL samples were then analyzed by HPLC. All experiments were performed in triplicate.
III. RESULTS AND DISCUSSION

3.1 Synthetic aspects

3.1.1 Synthesis of 8,2′-thioanhydroadenosine-5′-triphosphate (4)

Although the synthesis of 8,2′-thioanhydroadenosine (3) has been described previously by Ogilvie et al., (1972) this thesis is the first report of the synthesis of the triphosphate derivative. Ogilvie et al. (1972) synthesized 8,2′-thioanhydroadenosine (3) on a microscale, and modifications had to be made for large scale synthesis. Ogilvie et al. (1972) did not characterize the intermediate nucleosides and this work thereby represents the first complete detailed analysis of these compounds. The reaction scheme for the synthesis of 8,2′-thioanhydroadenosine-5′-triphosphate (4) is summarized (scheme 5).

The first step involves bromination of the C(8) position of the adenine base in adenosine. The mechanism involves electrophilic substitution of hydrogen by bromine at an sp² carbon. It is of interest to compare substitution at the C(8) position of adenine with the possible competing reaction for substitution at the C(2) position. Close examination of the adenine ring reveals that the position of the NH₂ group, resembles the orientation in aniline. Since the NH₂ substituent is an ortho and para directing activator, the meta position C(2) is not stabilized by electron donation from this nitrogen. Comparison of the ¹H-NMR spectral data of 8-bromoadenosine (1) with adenosine confirms the favourable proton substitution at C(8) by bromine. The signal for the proton at C(8) of adenosine (s, 8.3 ppm) is absent in the spectra of 8-bromoadenosine (1).

The next step in the synthesis involves the formation of the cyclic carbonate linking the 2′ and 3′-hydroxyls of the ribose sugar. The reaction involves heating the nucleoside in DMF in the presence of diphenyl carbonate and a base catalyst. The mechanism involves nucleophilic attack by ribose hydroxyls at the reactive carbonyl group of diphenyl carbonate (Ogilvie et al., 1972). The reaction is catalyzed by base deprotonation of the hydroxyl during the formation of the tetrahedral intermediate. The reaction is also accelerated by loss of the good leaving group, the phenoxide ion (Hampton and Nichol, 1966). Of mechanistic interest
Scheme 5. Synthesis of 8,2'-thioanhydroadenosine-5'-triphosphate (4)
(i) Br₂/H₂O, NaOAc, rt, 3 h (ii) (C₆H₅O)₂CO, DMF, NaHCO₃, 150°, 30 min (iii) Thiourea, n-butanol, reflux, 5 h (iv) (a) POCl₃, proton sponge, PO(OMe)₃, 0°, 2 h (b) H₃P₂O₇³⁻ (Bu₃NH)₃⁺, DMF, 0°, 1 min (c) (Et₃NH)⁺HCO₃⁻(aq), rt, 30 min

in the reaction is the possible formation of 3',5' or 2',5' cyclic carbonates. These cyclic carbonates are not favoured possibly due to steric restraints imposed by the ribose ring. Also six-membered cyclic carbonate esters (3',5' or 2',5'-carbonates) are predicted to be less stable than the corresponding five-membered rings (Hampton and Nichol, 1966). Therefore
protection of the 5'-hydroxyl of ribose is not required. Similarly, protection of the amino (NH₂) group of the adenine ring is not required since the nucleophilicity of this nitrogen is reduced by donation of its lone pair of electrons to the adenine ring system (Ogilvie et al., 1972). FT-IR analysis of 8-bromoadenosine-2',3'-carbonate (2) confirmed the presence of the cyclic carbonate with a C=O stretch at 1811 cm⁻¹. Comparison of the ¹H-NMR (200 MHz, DMSO-d₆) spectral data of the carbonate product with adenosine confirms substitution at the 2',3'-hydroxyls due to the observed downfield shifts of the 2' and 3'-H signals. The 2'-H is shifted downfield from 5.1 ppm (adenosine) to 6.26 ppm (difference of 1.1 ppm) and the 3'-H is shifted downfield from 4.2 ppm (adenosine) to 5.6 ppm (difference of 1.4 ppm). This downfield shift in the signals for the 2' and 3' protons can be attributed to the deshielding effect experienced from the strong electron withdrawing carbonate functional group.

The coupling constant for the 1' and 2'-protons (J₁H₂) of the carbonate was determined to be -0.8 Hz. Such a small J₁H₂ for the carbonate product in unique among nucleosides, since this coupling constant is normally between the range of 4 to 7.6 Hz (Davies and Danyluk, 1974). This may be attributed to ribose ring constraints imposed by the formation of the 2',3'-cyclic carbonate. Examination of the Newman projection along the C(1')-C(2') bond axis predicts a dihedral angle between the 1'-H and 2'-H on the order of 80 to 100°, and therefore according to the Karplus relationship this would correspond to a coupling constant between 0 and 1.0 Hz, as is observed (Fig. 17).

The third step in the synthesis involves formation of the sulphur bridge. The first part of the mechanism involves nucleophilic attack from the sulphur of thiourea displacing bromine. This leads to the formation of the stable alkylisothiourea salt. The next step in the mechanism involves sulphur nucleophilic attack at the C(2') position of ribose sugar. This leads to the release of the good leaving group CO₂ and simultaneous displacement of the alkylisourea salt yielding the sulphur bridged adenosine derivative locked in the anti-conformation.

The reaction may be entropy driven since the stable products CO₂ and alkylisourea salt are formed. The attack of the sulphur is at the C(2') position as opposed to the C(3') position and in agreement with Ogilvie et al. (1972), the formation of the 3'-S-cycloadenosine was not
Figure 17. Newman projection along the C(1')-C(2') bond axis of 8-bromoadenosine-2',3'-carbonate (2)

observed. Ikehara and Kaneko (1970) obtained both the 2' and 3'-isomers in appreciable yields (59% and 43%, respectively) by different synthetic methods. Ikehara and Kaneko (1970) selectively sulfonated either the 2' or 3'-hydroxyl of 8-bromoadenosine (1) with TPS-
Cl (2,4,6-tri-isopropylbenzene-sulfonylchloride) in the presence of NaH. Cyclization was achieved by NaSH, which subsequently displaced the bromine and formed the sulphur bridge upon release of the sulfonate.

In the $^1$H-NMR (200 MHz, D$_2$O) spectral data of 8,2'-thioanhydroadenosine (3) the upfield shift of the signals for the 2' and 3' ribose protons (4.95 and 4.56 ppm, respectively) back to the original positions as seen in 8-bromoadenosine (1), confirms the removal of the cyclic carbonate. Similarly the unusual coupling constant for the 1' and 2' protons ($J_{H1,2'}$) is lost and instead the more characteristic alkane ring coupling constant of 6.6 Hz is found.

In order to prove the formation of the 2'-isomer, a $^1$H-NMR (200 MHz) spectra was performed in DMSO-d$_6$, since under such conditions hydroxyl protons do not exchange. The signal for the 2'-OH proton usually occurring at 5.1 ppm (adenosine), is not observed, instead the downfield shifted 3'-OH signal is observed at 5.90 ppm. Comparison of $^1$H-NMR spectra of adenosine and deoxyadenosine in DMSO-d$_6$ reveals loss of a signal at 5.12 ppm in the latter corresponding to 2'-OH proton (Gatlin and Davis, 1961).

The formation of the desired triphosphate product was accomplished in a “one pot” synthesis starting with the unprotected nucleoside. The reaction was adapted from Zimmet et al. (1993). Maintaining strictly anhydrous conditions is crucial since water is a prime competitor for nucleophilic attack at the 5'-monophosphate. Also addition of a large excess of pyrophosphate (5 equivalents) is important since the thermodynamic equilibrium of the reaction is toward the formation of the triphosphate product. The formation of the triphosphate is rapid, since the reaction is terminated exactly 1 minute after the addition of the pyrophosphate. Finally the selective phosphorylation of the primary 5'-hydroxyl is achieved by maintaining low temperatures (0°C). The DEAE-Sephadex elution profile for the triphosphate and other by products is shown (Fig. 18).

Peak I contains the unreacted nucleoside, 8,2'-thioanhydroadenosine (3). Peak II and III contain the corresponding mono- and di-phosphates, respectively. Peak IV afforded the desired nucleoside-triphosphate, whereas peak V contains nucleoside-polyphosphates (ie. tetra). The $^{31}$P-NMR spectrum of peak IV indicates two inorganic phosphate impurities, the pyrophosphate (-7.99 ppm) and a cyclic metaphosphate (-20.87 ppm).
Figure 18. Separation of nucleoside phosphates on DEAE-Sephadex eluted with TEAB (0-700 mM)
Figure 19. $^{31}$P-NMR spectrum of nucleoside-triphosphate (peak IV)

-10.96 ppm α P of nucleoside triphosphate
-9.55 ppm γ P of nucleoside triphosphate
-22.51 ppm β P of nucleoside triphosphate

-7.99 ppm PP$_i$
-20.87 ppm cyclic metaphosphate
The molar ratio of these phosphates is 34:62:4 for the nucleoside triphosphate, pyrophosphate and cyclic metaphosphate, respectively. Metaphosphates are a class of compounds with the molecular formula (PO₃)₆. A subsequent DEAE-Sephadex purification was performed, and the molar phosphorus ratio obtained was 90:9:1 for the three phosphate species as outlined above. The ¹H-NMR spectra of 8,2'-thioanhydroadenosine-5'-triphosphate (4) was devoid of any nucleotidyl contaminants, and thus the compound was utilized for biological assays as is, in 90% purity, with impurities identified as inorganic phosphate salts with no biological activities.

The percentage yield of 8,2'-thioanhydroadenosine-5'-triphosphate (4) was 15% which is within the range obtained by Fischer et al. (1993) (14-45%) for a similar “one-pot” synthesis of other nucleoside triphosphates. Thus the total overall yield of the triphosphate analogue is 5%. The ¹H-NMR (200 MHz, D₂O) spectral data was identical to the corresponding nucleoside except for the apparent downfield shift of the 5'/5''' and 4'-proton signals. The 5'/5'''-proton signal occurred at 3.94 ppm (difference of 0.37 ppm) and the 4'-proton signal at 4.39 ppm (difference of 0.13 ppm). This shift can be attributed to the deshielding effect experienced by these protons due to the presence of the electron-withdrawing 5'-triphosphate moiety. Also the coupling constant for J₅,₅'-P is 6.6 Hz, which is comparable to value in ATP (4.8 Hz). The ³¹P-NMR spectral data displays three distinct phosphate signals, αP (d, -10.87 ppm, Jₘα = 20.2 Hz), βP (br s, -22.48 ppm) and the γP (d, -9.79 ppm, Jₚγ = 18.3 Hz) as seen for ATP. The FAB-MS data identified four detectable species with the [M⁺ + 2H⁺ + Na⁺] (541.9 g/mole) as the most intense m/z peak (MW calculated for the free acid [M⁺] is 517.206 g/mole).

Preliminary attempts to synthesis the 8,2'-thioanhydroadenosine-5'-triphosphate (4) were carried out by first synthesizing 8,2'-thioanhydroadenosine-5'-monophosphate (5) followed by pyrophosphorylation according to the procedure described by Hoard and Ott (1965). This procedure was reported to give higher yields. However, the synthesis, involved reacting the unprotected nucleoside with POCl₃ yielding a mixture of 3' and 5' monophosphates (8% and 92%, respectively). The monophosphates were difficult to separate by chromatography and no further pyrophosphorylation was attempted.
3.1.2 Synthesis of 2,2'-anhydouridine-5'-triphosphate (7)

Although the synthesis of the 2,2'-anhydouridine (6) was reported previously (Hampton and Nichol, 1966) this is the first synthesis of the triphosphate analogue. The reaction scheme for the synthesis of 2,2'-anhydouridine-5'-triphosphate (7) is outlined.

Scheme 6. Synthesis of 2,2'-anhydouridine-5'-triphosphate (7)

(i) (C₆H₅O)₂CO, DMF, NaHCO₃, 150°, 30 min (ii) (a) POCl₃, proton sponge, PO(OMe)₃, 0°, 2 h (b) H₃P₂O₇(Bu₃NH)₃⁺, DMF, 0°, 1 min (c) (Et₃NH)⁺HCO₃(aq), rt, 30 min

The synthesis involves the cyclization reaction forming the oxygen bridge between the sugar C(2') and base C(2) positions of uridine. This is accomplished by reacting uridine with diphenyl carbonate in the presence of a catalytic amount of base. The mechanism is identical to that for the formation of the 8-bromoadenosine-2',3'-carbonate (2). The cyclic nucleoside it then formed by nucleophilic attack at the C(2') of uridine-2',3'-carbonate by the C(2) oxygen on the uracil base moiety (lactim tautomer). This displaces CO₂ and forms the oxygen bridge.

Comparison of the ¹H-NMR (200 MHz, D₂O) spectral data of the cyclic product with uridine is of interest. The signal for the 2'-H is shifted downfield from 4.35 ppm (uridine) to 5.46 ppm (cyclic uridine) for a difference of 1.11 ppm. This is due to deshielding experienced on the 2'-H by the electron-withdrawing bridging-oxygen. The apparent downfield shift of the 1'-H signal, from 5.90 ppm (uridine) to 6.53 ppm (cyclic uridine) a difference of 0.63 ppm
can be attributed to the close proximity of the electron-withdrawing uracil heterocycle to the 1'-H.

Differences are also apparent in the coupling constants for the two nucleosides. The $J_{H5',H4'}$ differs by +1.7 Hz (from 2.3 Hz (uridine) to 4.0 Hz), $J_{H3',H4'}$ differs by -3.2 Hz (from 4.6 Hz (uridine) to 1.4 Hz) and the $J_{H2',H3'}$ differs by -4.2 Hz (from 5.0 Hz (uridine) to a minuscule 0.8 Hz in the cyclic uridine). Such large changes can be attributed to the constraints imposed on the arabinose sugar by the oxygen bridging. These differences were also observed upon comparison of the $^1$H-NMR (200 MHz, DMSO-$d_6$) spectral data of 8,2'-thioanhydroadenosine (3) and adenosine although not as dramatic. This less pronounced effect may be due to the fact that the oxygen-bridge is more strained than the sulphur-bridge, since sulphur is a larger atom and can adopt a wider range of bond angles. The $J_{H5',H4'}$ differs by +1.8 Hz (from 3.2 Hz (adenosine) to 5.0 Hz), $J_{H3',H4'}$ differs by -0.3 Hz (from 3.5 Hz (adenosine) to 3.2 Hz) and the $J_{H2',H3'}$ differs by -2.6 Hz (from 5.2 Hz (adenosine) to 2.6 Hz in the cyclic adenosine). Conformational analysis (crystal structures) of the arabinose sugars of these two constrained analogues may clarify these similarities which may be unique to ribose sugars “locked” in the S-type configuration.

The triphosphate moiety is then introduced to the 5'-hydroxyl. Since the cyclic nucleoside, 2,2'-anhydouridine is susceptible to acid hydrolysis at the bridging oxygen, the reaction is carried out in the presence of a “proton sponge”, 1,8-bis-(dimethylamino) naphthalene. The proton sponge contains a pair of tertiary amino groups that can donate their lone pair of electrons to the aromatic ring system and this stabilizes ammonium formation to a greater extent than the unprotonated form.

The yield of 2,2'-anhydouridine-5'-triphosphate (7) was 18% and the overall yield is therefore 11%. The $^{31}$P-NMR identified the inorganic phosphate contaminants in the ratio 89:9:2 for the nucleotide triphosphate, the pyrophosphate and the cyclic metaphosphate, respectively. $^1$H-NMR spectra reveals no nucleoside contaminant and the compound was used as is (89% purity by phosphate) in subsequent biological studies. The $^1$H-NMR (200 MHz, D$_2$O) spectral data was identical to the corresponding nucleoside except for the apparent downfield shift of the 5'/5'' and 4'-proton signals. The 5'/5'',4'-proton signal
occurred at 4.05 ppm (difference of 0.46 ppm) and the 4'-proton signal at 4.59 ppm (difference of 0.20 ppm). This shift is analogous to those observed in 8,2'-thioanhydroadenosine-5'-triphosphate (4).

3.1.3 Synthesis of 8-mercaptoadenosine (8)

The adenosine analogue, 8-mercaptoadenosine (8) was afforded by reacting 8-bromoadenosine (1) with thiourea. The mechanism involves displacement of the halide ion to yield an intermediate alkylisothiourea salt, subsequent hydrolysis with alcohol yields the desired thiol. The yield of the reaction was 50% slightly better than that reported by Ikehara and Yamada (1971) (44%) using NaSH as the nucleophile. The mp of 171-173°C is comparable to the literature (170°C) (Ikehara and Yamada, 1971). The authenticity was confirmed by 1H-NMR analysis.

3.1.4 Synthesis of adenosine-2',3'-carbonate (9)

The synthesis of adenosine-2',3'-carbonate (9) utilizes diphenyl carbonate, to introduce the 2',3'-protecting group in the presence of phenol catalyst. The mechanism is identical to that already discussed for the formation of 8-bromoadenosine-2',3'-carbonate (2). The yield of the cyclic carbonate product was 90% which is comparable to that obtained by Hampton and Nichol (1966) (96%). The FT-IR data displayed the C=O stretch at 1802 cm⁻¹, which is characteristic of the carbonate functional group.

This study reports the first authentic 1H-NMR data for this compound. Previous published 1H-NMR spectral data reported by Law et al. (1971) are suspect since the chemical shift values are identical to those of the unmodified nucleoside, adenosine. Law et al. (1971) recrystallized the cyclic carbonate from hot methanol, conditions which I found hydrolyzes the carbonate to the corresponding nucleoside. My 1H-NMR (200 MHz, DMSO-d₆+D₂O) spectral data, reveals a similar pattern of signal shifting and comparable coupling constants as those observed for 8-bromoadenosine-2',3'-carbonate (2).
3.2 Screening of nucleosides in PC12 cells using the nucleoside transport assay

The nucleoside analogues were tested as potential inhibitors of adenosine transport. The transport studies involved measuring uptake of radioactive [2-^3^H]-adenosine in PC12G cells. Preliminary studies were carried out to determine the time course of the transport.

3.2.1 Determination of time course for adenosine uptake

A typical profile for adenosine transport (Fig. 20), involves an initial rapid uptake within the first minute, followed by an approach to plateau. [^3^H]-adenosine uptake is inhibited by 90% by the well known inhibitor of NT, dipyridamole (DPR) (0.1 mM) indicating that NT in PC12 cells is facilitated. The rate of adenosine uptake is linear within the first 2 min. The inhibitor studies were thus performed under the same conditions using 2 min as the point of study. Three nucleosides, uridine, thymidine and 8,2'-thioanhydroadenosine (3) were examined to determine their affects on the uptake of adenosine. Figure 21, compares the uptake of adenosine in the presence and absence of the three nucleosides (300 μM each).

Uridine and thymidine are expected to compete with adenosine and are tested as positive controls. These permeants of the es and ei-transporters have been shown to inhibit adenosine uptake in the range of 30-50% at approximately 300 μM (Thampy and Barnes, 1983). In PC12 cells, however, uridine and thymidine only inhibited by 15% and 8,2'-thioanhydroadenosine (3) was ineffective. There are two possible explanations for this lack of inhibition by uridine and thymidine. Either PC12 cells have a NT system which is unique and does not transport these nucleosides (unlikely). Alternatively the measured “initial rate” is not the true rate, and is complicated by the further metabolism of [^3^H]-adenosine once inside the cell. Transport must therefore be determined under conditions where the intracellular [^3^H]-adenosine is not removed by either deamination or phosphorylation. This was accomplished as described below.
Figure 20. Time course of $[^3H]$-adenosine uptake into PC12 cells in the presence and absence of dipyridamole (DPR) (0.1 mM). Values were mean ± SEM, $n \geq 3$. 

well = $1 \times 10^6$ cells
Figure 21. Adenosine transport in PC12-cells in the presence of various nucleosides (1 x 10^6 cells incubated with 10 μM adenosine for 2 min.). Values were mean ± SEM, n ≥ 6.

3.2.2 Adenosine metabolism in PC12 cells

To determine the extent of adenosine metabolism, adenosine and its radioactive metabolites (inosine, AMP, ADP and ATP) were separated by cellulose-TLC as described in experimental. These studies were performed in the presence and absence of inhibitors of the key enzymes involved in the metabolism of adenosine, namely adenosine deaminase (AD) and adenosine kinase (AK). AD was inhibited by 10 μM EHNA, a classical non-nucleoside that is very specific for this enzyme. AK activity was inhibited by depleting the cells of ATP, using 5 mM iodoacetate and 5 mM KCN. KCN blocks cytochrome oxidase, a key enzyme in the electron transport chain, thus reducing the production of ATP in the cell (Voet and Voet,
1995). Iodoacetate acts on glyceraldehyde-3-phosphate dehydrogenase, an enzyme required for glycolysis, by reacting with an active site cysteine sulfhydryl group and rendering the enzyme inactive (Voet and Voet, 1995). The conditions for the effective “ATP depletion” of typical mammalian cells was originally determined by Plagemann et al. (1976). Incubation of Novikoff rat hepatoma cells (subline N1S1-67) with 5 mM iodoacetate and 5 mM KCN for 10 min at 37°C, effectively depleted intracellular levels of ATP by 99% (Plagemann et al., 1976).

The downstream affect of “ATP depletion” is an accumulation of ADP and AMP in the cell. AK catalyzed the transfer of the terminal phosphate of ATP to adenosine to yield AMP and ADP. Without any available substrate (ATP) the reaction cannot proceed. Elevated AMP and ADP can also act as feed-back product inhibitors.

In order to obtain accurate transport results, it is important to determine if the addition of the toxins KCN and iodoacetate is deleterious to the cells during the assay period. Cells were incubated for 30 min in the presence of trypan blue (a vital stain) with and without KCN and iodoacetate. Trypan blue stains any dead cells a dark blue colour whereas live cells exclude the dye. Cell viability in control and KCN/iodoacetate treated cells were identical (93%). These results are in accordance with Plagemann et al. (1976), who performed the analogous experiment on N1S1-67 cells and reported no increase in mortality upon treatment with KCN and iodoacetate.

The radioactive purines isolated in PC12 cells in the presence and absence of inhibitors of AD and AK are shown in Figure 22 for 2 and 10 min. As expected, intracellular [3H]-adenosine accumulation (A) increased in control cells and reached 85±9 pmoles/mg protein at 10 min. In the presence of EHNA alone, adenosine accumulation was indistinguishable from the control (88±15 pmoles/mg protein). In the presence of both AD and AK inhibitors the accumulation was inhibited by approximately 55% (38±5 pmoles/mg protein).

[3H]-Inosine production (B) in control cells increased with time and reached a maximum of 507±167 pmoles/mg protein. This is nearly six times the level of [3H]-adenosine. As expected, the addition of EHNA reduced inosine production to 27±7 % of the control.
absence of inhibitors of adenosine metabolism. Values are mean ± SEM, n ≥ 4.

Figure 22. Accumulation of radioactive [3H]purines in P12 cells in the presence and absence of inhibitors of adenosine metabolism. Values are mean ± SEM, n ≥ 4.
Conversion to adenine nucleotides (AMP, ADP and ATP) (C) in control cells reached a maximum of 314±35 pmoles/mg protein. In the presence of EHNA alone (460±70 pmoles/mg protein), there is an “over accumulation” of nucleotides. This may be due to the fact that inhibition of AD allows more adenosine to be phosphorylated by AK. The accumulation of adenine nucleotides is considerably reduced in the presence of both AD and AK inhibitors (maximum 89±24 pmoles/mg protein or 29 % of control).

These results show that adenosine metabolism in PC12 cells is robust. This is not surprising since PC12 cells are rapidly proliferating tumor cells that are very active in AD and especially AK activity. These are essential processes in nucleotide salvage pathways.

In order to determine “true” transport under conditions where metabolism is minimal, conditions require that the formation of inosine and nucleotides be at a minimum. In other words, one needs to maximize the intracellular radioactivity in the form of [3H]-adenosine, not metabolities. Figure 23, shows the data from figure 22, normalized to control (100%). For the 2 min. time point with both AD and AK inhibitors, the radioactive adenosine is at 60% of control, while the radioactive inosine and nucleotides are reduced to 22 and 9% respectively. These conditions satisfy the criteria .

[3H]-adenosine transport was now measured in the absence and presence of AD and AK inhibitors at the specified time and the time course was obtained. Figure 24a shows an expanded scale of the time course for the transport with both pathways inhibited. As expected, the uptake is linear over the first 2 min span (correlation coefficient = 0.999) and the transport is 398±21 pmole/mg protein/2 min. This is substantially less than the “apparent” transport of 2432±54 pmole/mg/2 min in the absence of inhibitors (Fig. 24b). This data confirms the dependance of the “apparent” transport on metabolism.
Figure 23. Purine accumulation in PC12 cells (% of control) in the presence and absence of adenosine metabolism inhibitors. Values are mean ± SEM, n ≥ 4.
Figure 24a. Time course of adenosine uptake in PC12 cells in the presence of inhibitors of adenosine metabolism. Values are mean ± SEM, n ≥ 3.
Figure 24b. Time course of adenosine uptake in PC12 cells in the presence and absence of inhibitors of adenosine metabolism. Values are mean ± SEM, n ≥ 3.
3.2.3 Inhibitor studies of NT processes in PC12 cells

The effects of various natural and synthetic nucleosides on the transport of adenosine in PC12 cells was determined and the data is summarized (Fig. 25).

The novel "anti-locked" adenosine, 8,2'-thioanhydroadenosine (3) was ineffective in inhibiting transport. Thus the computer modeling (QSAR) study by Viswanadhan et al. (1990) which proposed the favored anti-conformation as the transported nucleoside is not supported in these findings. Nevertheless, this does not rule out the selectivity for adenosine in the anti conformation. It may be that permeants or inhibitors require some flexibility in the glycosidic bond in order to be recognized by the transporters. 8,2'-thioanhydroadenosine (3) locks the adenine base in a rigidly constrained anti conformation with a $X_{CN}$ of 122° (Tomita et al., 1972). The loss of the 2'-hydroxyl in 8,2'-thioanhydroadenosine (3) cannot be considered at fault since 2'-deoxyadenosine inhibits adenosine uptake (37±2 % of the control).

The "syn-locked" uridine, 2,2'-anhydrouridine was also ineffective at inhibiting transport. This further attests to the fact the transporters may favour nucleosides with flexibility in the base moiety or it may indicate that pyrimidines are not recognized in the less favourable syn conformation. Since both these constrained nucleosides exhibit the S-type configuration of the arabinose sugar, it can be concluded that the transporters do not favour the more stable sugar pucker.

Contrary to the results previously observed in the absence of AK and AD inhibitors (Fig. 21) thymidine and uridine did inhibit transport (68±5 % and 64±5 % of control, respectively) in the presence of inhibitors of adenosine metabolism. Similarly the well known potent inhibitors of es and ei-transport systems, NBTI and DPR, also inhibited adenosine uptake (19±2 % and 35±2 % of the control, respectively). These inhibitors are expected to reduce transport to nearly 0%; residual transport may be explained by the presence of insensitive transporters or alternatively incomplete blockage of adenosine metabolism.

The synthetic nucleosides 8-bromoadenosine (1) and 8-mercaptoadenosine (8) also inhibited adenosine uptake. These results indicate that the transporters may tolerate some
Figure 25. Adenosine uptake in PC12 cells in the presence of various natural and synthetic nucleosides (300 μM). Values are mean ± SEM, n ≥ 6.
substitutions in the base moiety, particularly at the C(8) position. This is not surprising since it is known the es and ei transporters accept a broad range of nucleosides as permeants.

The most interesting analogue is adenosine-2',3'-carbonate (9), since, surprisingly this nucleoside was more potent (27±1 % of the control) than DPR. The relative potency of the cyclic carbonate was analyzed in more detail, Figure 26 shows a dose-response curve.

\[ \text{Adenosine Uptake (\% of control)} \]

\[ \text{IC}_{50} = 10 \mu \text{M} \]

\[ \text{Adenosine-2',3'-carbonate (\mu M)} \]

*Figure 26.* Inhibition of adenosine uptake in PC12 cells by adenosine-2',3'-carbonate (9). Values are mean ± SEM, n ≥ 4.
The curve is biphasic and predicts a high affinity and low affinity site. An IC₅₀ of 10 µM was estimated for the cyclic carbonate for the low affinity transporter. The IC₅₀ of the high affinity site could not be estimated but it is less than 10 nM. Further studies are required to confirm this model. The exact nature of inhibition has yet to be established. It is possible that the unusual configuration of the sugar may interact with the transporters possibly through hydrophobic interactions. Similarly the carbonate functional group may interact with some amino acid residues in the active site of the transporters possibly through some H-bonding capabilities.

An HPLC study was performed to determine if 8,2'-thioanhydroadenosine (3) was being transported and metabolized in PC12 cells. This is important since the uptake experiments cannot distinguish the mode of inhibition, which can either be by active-site blockage or competition for transport. Cells were treated with a known concentration of nucleoside (50 µM), and aliquots of the extracellular fluid were removed at specified times and subjected to HPLC to determine uptake (Fig. 27).

The extracellular adenosine concentration decreased with time since it was actively being taken-up by the transporters. In accordance with this finding, is the increase in extracellular inosine concentration, due to the metabolism (deamination) of adenosine by AD. The transporters are equilibrative and inosine accumulation in the cell is accompanied by efflux into the medium. It is apparent that 8,2'-thioanhydroadenosine (3) is not transported into the cells since it's concentration is maintained throughout the course of the experiment. 8,2'-thioanhydroadenosine (3) is a substrate for AD with a relative activity of 7-29% that of adenosine (Ogilvie et al., 1971). Thus if 8,2'-thioanhydroadenosine (3) was taken-up by the cells the appearance of the metabolite, 8,2'-thioanhydroinosine in the medium would be expected if transport occurred. These data suggest that 8,2'-thioanhydroadenosine (3) is not an inhibitor nor is it transported by an alternative pathway.
Figure 27. Purine uptake and metabolism in PC12 cells. Values are mean ± SEM, n ≥ 3.
3.3 Studies on P₂-purinoceptors in PC12 cells

P₂-purinoceptors are a widely distributed family of cell surface receptors that mediate signal transduction pathways in many mammalian cells. The natural agonists are ATP, ADP or UTP depending on the receptor class. There is considerable interest in the pharmaceutical industry in designing new nucleotide analogues that may show tissue and receptor selectivity. Such molecules could be important in various clinical applications such as in the treatment of stroke, cancer and cardiovascular disease.

The nucleotide analogues described in this thesis were designed and synthesized with this goal in mind. The studies outlined below describe the screening assay used to determine bioactivity. Three specific tests were carried out, all of which involve measurement of P₂-dependant activity. These include a P₂-activated increase in intracellular Ca²⁺; a P₂-mediated cell depolarization; and a P₂-mediated change in pH homeostasis.

3.3.1 The effects of conformationally constrained nucleoside triphosphate analogues on P_{2Y2}-purinoceptor response in PC12 cells

The addition of micromolar concentrations of ATP to PC12 cells in suspension evokes a transient increase in intracellular [Ca²⁺] which is mediated by P_{2Y2}-purinoceptors (Raha et al., 1993). [Ca²⁺], is measured using the calcium sensitive dye, Fura-2. The fluorescence properties of Fura-2 changes upon chelation with Ca²⁺. Fluorescence measurements are simplified since the excitation wavelength of the dye (340 nm) does not interfere with its emission wavelength (505 nm) (Gryniewicz et al., 1985). Figure 28a illustrates the typical response elicited by 50 μM ATP (control). Intracellular [Ca²⁺] increased rapidly from a resting level of ~60 nM to a peak at ~500 nM (diff. +440 nM) within 6-8 sec, subsequently declining back to basal levels within 2 min.

Figure 28b depicts the same experiment in the presence of 8,2'-thioanhydroadenosine-5'-triphosphate (4) (50 μM). Addition of this triphosphate evokes no appreciable increase in intracellular [Ca²⁺] and therefore this analogue is not an agonist of the P_{2Y2}-purinoceptor. Also, the analogue is not a competitive inhibitor for the ATP response or an antagonist of the
**Figure 28a.** Measurements of $[Ca^{2+}]_i$ in PC12 cells: control (50 µM ATP at 60 sec)
Figure 28b. Measurements of $[Ca^{2+}]_i$ in PC12 cells: (50 μM 8,2'-thioanhydroadenosine-5'-triphosphate (4) at 60 sec and 50 μM ATP at 140 sec)
Figure 28c. Measurements of $[\text{Ca}^{2+}]_i$ in PC12 cells: (50 μM 2,2'-anhydouridine-5'-triphosphate (7) at 60 sec and 50 μM ATP at 160 sec)
P<sub>2Y2</sub>-purinoceptor, since addition of ATP at 140 sec. elicits a response profile which is indistinguishable from the control (Fig. 28a).

Figure 28c illustrates the P<sub>2Y2</sub>-purinoceptor response in the presence of 2,2′-anhydouridine-5′-triphosphate (7) (50 μM). Addition of this triphosphate at 60 sec. evokes a smaller but significant transient increase in intracellular [Ca<sup>2+</sup>] from a resting level of ~145 nM to a peak of ~290 nM (diff. +145 nM) within 10-13 seconds. Therefore this triphosphate analogue can be classified as a weak agonist of the P<sub>2Y2</sub>-purinoceptor. 2,2′-anhydouridine-5′-triphosphate (7) does not appear to competitively inhibit the ATP response or be an antagonist for the P<sub>2Y2</sub>-purinoceptor, since addition of ATP at 160 sec. displays a P<sub>2Y2</sub>-purinoceptor activation profile analogous to that in the control experiment.

3.3.2 The effects of conformationally constrained nucleoside triphosphate analogues on P<sub>2X2</sub>-purinoceptor response in PC12 cells

3.3.2a Measurement of PC12 membrane potential with bisoxonol

Membrane depolarization is mediated by activation of P<sub>2X2</sub>-purinoceptors leading to a transient increase in intracellular [Na<sup>+</sup>]. P<sub>2X2</sub>-purinoceptors are non-specific cation channels. Changes in membrane potential were measured using the lipophilic anionic fluorescent dye, bisoxonol. Negatively charged oxonol dyes partition across the cell membrane according to the membrane potential, and an increase in fluorescence intensity indicates cell depolarization (Rink et al., 1980). The mechanism by which ATP triggers this Na<sup>+</sup> influx and subsequent depolarization has yet to be established.

Figure 29a shows the typical response elicited by the addition of 10 μM ATP to a suspension of PC12 cells in the presence of 50 nM bisoxonol (control). The fluorescence intensity increases from ~5230 to a peak at ~5800 (diff. +570) within 10 sec. and gradually returns to resting level in 3-5 min., a process which reflects ATP hydrolysis and removal of the agonist.

Figure 29b depicts the same experiment in the presence of 8,2′-thioanhydroadenosine-5′-triphosphate (4). Addition of the triphosphate analogue (100 μM) at 60 sec. evokes a
small increase in fluorescence intensity (~5230 to ~5350) (diff. +120). No measurable response was seen with 10 μM analogue. Therefore this analogue is a very weak agonist of the P2X2-purinoceptor. 8,2’-thioanhydroadenosine-5’-triphosphate (4) also does not appear to inhibit the response evoked by ATP and is therefore not an antagonist of the P2X2-purinoceptor.

UTP also evokes membrane depolarization in PC12 cells, although at a much higher concentration (10x) than that of ATP. Figure 30a shows the typical response elicited by the addition of 100 μM UTP to a suspension of PC12 cells in the presence of 50 nM bisoxonol (control). The fluorescence intensity increases from ~5240 to a peak at ~5720 (diff. +480) within 10 sec. and maintains a depolarized state with a fluorescence intensity of ~5650. Compared with the ATP profile (Fig. 29a), the UTP response exhibits a prolonged depolarization response which may reflect differences in metabolism or desensitization.

Figure 30b depicts the same experiment using 2,2’-anhydrouridine-5’-triphosphate (7). Addition of the triphosphate analogue (100 μM) evokes an increase in fluorescence intensity (~4950 to ~5420) (diff. +470). The final membrane potential (i.e. fluorescence change) is comparable but the rate of depolarization is substantially less with the analogue. Therefore 2,2’-anhydrouridine-5’-triphosphate (7) is an agonist for this response, although weaker than UTP.

3.32b Measurements of intracellular pH

P2X2-purinoceptors in PC12 cells also mediate changes in intracellular pH (Moore and Reed, 1997). The intracellular pH of PC12 cells was measured using the fluorescent pH indicator dye BCECF. A decrease in fluorescence intensity of BCECF reflects a decrease in intracellular pH (Graber et al., 1986).

Figure 31a shows that addition of 50 μM ATP to PC12 cells in suspension results in an initial rapid acidification (pH ~7.30 to ~7.22) (diff. -0.08 pH units), followed by a gradual alkalization (control). 8,2’-thioanhydroadenosine-5’-triphosphate (4) does not evoke any changes in [pH], nor does this analog inhibit the ATP induced changes in pH (Fig. 31b).
Figure 29a. Measurements of PC12 membrane potential with 50 nM bisoxonol in the presence of ATP (10 μM)
Figure 29b. Measurements of PC12 membrane potential with 50 nM bisoxonol in the presence of ATP (10 μM) and 8,2'-thiohydroadenosine-5'-triphosphate (4) (100 μM).
Figure 30a. Measurements of PC12 membrane potential with 50 nM bisoxonol in the presence of UTP (100 μM).
Figure 30b. Measurements of PC12 membrane potential with 50 nM bisoxonol in the presence of UTP (100 μM) and 2,2'-anhydrouridine-5'-triphosphate (7) (100 μM).
Figure 31a. Measurements of intracellular pH in PC12 cells in the presence of ATP (50 μM).
Figure 31b. Measurements of intracellular pH in PC12 cells in the presence of ATP (50 µM) and 8,2'-thioanhydroadenosine-5'-triphosphate (4) (100 µM).
These results confirm that 8,2′-thioanhydroadenosine-5′-triphosphate (4) is neither a potent agonist nor an antagonist of the P₂X₂-purinoceptor. Table 3 summarizes the results of these studies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Purinoceptor tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P₂Y₂ ([Ca²⁺],)</td>
</tr>
<tr>
<td>8,2′-thioanhydroadenosine-5′-triphosphate (4)</td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>agonist</td>
</tr>
<tr>
<td></td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>antagonist</td>
</tr>
<tr>
<td>2,2′-anhydouridine-5′-triphosphate (7)</td>
<td>weak</td>
</tr>
<tr>
<td></td>
<td>agonist</td>
</tr>
<tr>
<td></td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>antagonist</td>
</tr>
</tbody>
</table>

Table 3. Summary of the bioactivity of conformationally “locked” nucleoside triphosphate analogues (ND = not determined)

The novel “anti” locked adenosine analogue, 8,2′-thioanhydroadenosine-5′-triphosphate (4) is not recognized by the P₂Y₂-purinoceptor. This suggests that natural ATP may not bind to the P₂-purinoceptor in the favoured anti conformation about the glycosidic bond. On the other hand this cannot be equivocally stated since this analogue is rigidly locked with an XCN bond angle of 122°, whereas ATP can adopt a wider range of XCN bond angles (Fig. 7). The data also suggests that ATP does not bind to the receptor in the favoured S-type
sugar pucker which is the preferred state of this analogue. Alternatively the lack of bioactivity may be attributed to the constraints imposed by the sulphur bridge between C(8) and C(2'). These constraints may distort the molecule, especially the sugar pucker.

The "syn" locked uridine analogue, 2,2'-uridine-5'-triphosphate (7) acts as a weak agonist of the P_{2Y2}-purinoceptor. This suggests that UTP may bind to the receptor in the "syn" conformation. The weak bioactivity may also be attributed to the configuration of the sugar in the constrained analogue, which adopts the favoured S-type configuration.

Comparison of the response elicited by the uridine analogue with respect to the adenosine analogue, suggests that the receptor's specificity for ATP maybe very strict, more so than that for UTP. Thus the smaller size of the uridine triphosphate analogue may allow more flexibility for it to fit into the active site of the P_{2}-purinoceptor and elicit the observed response, whereas the larger adenosine triphosphate analogue may be more constrained. Thus in summary the agonist "potency" profile for the P_{2Y2}-purinoceptor is ATP > 2,2'-uridine-5'-triphosphate >> 8,2'-thioanhydroadenosine-5'-triphosphate. The agonist "rank order potency" for the observed depolarization of PC12 cell membranes is ATP >> UTP > 2,2'-anhydrouridine-5'-triphosphate > 8,2'-thioanhydroadenosine-5'-triphosphate.

3.4 Future prospects and conclusions

This work outlines the synthesis of two new conformationally constrained nucleotides, 8,2'-thioanhydroadenosine-5'-triphosphate (4) and 2,2'-anhydrouridine-5'-triphosphate (7) and present the first biological studies of conformationally constrained nucleosides and nucleotides in PC12 cells. The two main targets of the study were the equilibrative nucleoside transport (NT) proteins and the P_{2}-purinoceptors.

The equilibrative nucleoside transport (NT) studies presented some technical problems because of the aggressive metabolism of adenosine in PC12 cells. By reducing the metabolism by the addition of adenosine deaminase (AD) and adenosine kinase (AK) inhibitors it was possible to measure adenosine transport that displayed expected sensitivities to known transport inhibitors and other nucleosides. The transient assay could be improved by using a shorter sampling time (20 sec).
Nucleosides with "locked" glycosidic bonds (ie. 8,2'-thioanhydroadenosine (3) (anti "locked" adenosine) and 2,2'-anhydouridine (6) (syn "locked" uridine)) were not inhibitors of the NT processes. This suggest that the corresponding natural nucleosides are likely not transported in these conformations. Both molecules are constrained analogues that are rigid and adopt a single $X_{CN}$ bond angle. Similarly it can be concluded that the natural nucleosides are not recognized in the S-type configuration of the sugar, since both analogues adopt this favoured sugar pucker. The synthesis and screening of nucleoside analogues “locked” in other $X_{CN}$ angles about the glycosidic bond and sugar puckers may clarify the nucleoside conformational requirements of the NT proteins. One molecule that could be tested in the future is the anti locked adenosine, 8,3'-thioanhydroadenosine. This molecule adopts an $X_{CN}$ bond angle of 75° (pure anti) and the sugar pucker exists in the N-type configuration (Tomita et al., 1972).

Of particular interest in this work is the apparent potency of adenosine-2',3'-carbonate (9) at inhibiting the NT process. The adenosine-2',3'-carbonate (9) is frequently used as a protecting group in nucleoside synthesis but it has never before been tested for biological activity. It’s inhibitory effect is therefore surprising and further screening is required to elaborate upon these results.

The novel nucleoside triphosphate analogues used to elicit P₂-purinoceptor responses in PC12 cells tested the conformational requirements of the active ligand. To date the search for potent antagonists selective for specific P₂-purinoceptors has yielding no positive results. The anti "locked" adenosine analogue, 8,2'-thioanhydroadenosine-5'-triphosphate (4) was shown to be a very weak agonist of the P₂X₂-purinoceptor and inactive toward P₂Y₂-purinoceptor activation. The syn "locked" uridine analogue, 2,2'-anhydouridine-5'-triphosphate (7) is a weak agonist of the P₂Y₂-purinoceptor and an agonist for Na⁺-dependant depolarization of PC12 cell membranes. Therefore this molecule may be a good starting point for the synthesis of novel UTP analogues. Future work may involve the synthesis of similarly constrained nucleoside triphosphate analogues locked in other $X_{CN}$ bond angles about the glycosidic bond (ie. synthesis of 8,3'-thioanhydroadenosine-5'-triphosphate).
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