REGULATORY PROCESSES IN THE CENTRAL NERVOUS SYSTEM: A STUDY OF G-PROTEIN ABUNDANCE AND OF SOLUBLE 5'-NUCLEOTIDASE

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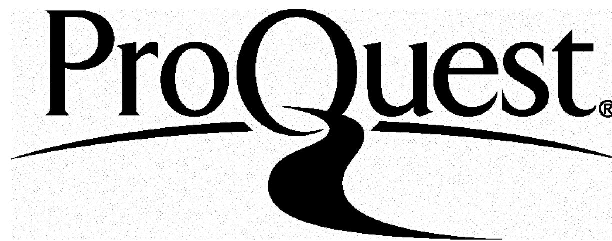

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ABSTRACT

The abundance of G-protein α-subunits were measured by quantitative immunoblotting. Hypothyroidism increased the abundance of Gi1α, Gi2α and Goα in synaptosomal membranes from the cerebral cortex and striatum. In the other brain regions some but not all of the G-proteins were up-regulated. In hyperthyroidism the abundance of Gi1α and Gi2α were halved in the cerebral cortex after only 3 days treatment with T3. Together these findings may in part contribute to physiological and behaviourial changes seen in thyroid abnormalities.

Soluble 5'-nucleotidase (EC 3.1.3.5) was assayed in six regions of the rat brain. ATP inhibited the activity in a complex fashion suggesting the presence of at least two soluble enzymes; one being strongly inhibited and another being ATP activated. The proportions of these two activities varied between brain regions, and activity changes seen in hypothyroidism suggest that they may be independently regulated. The ATP-inhibitable enzyme was purified 1770 fold to apparent homogeneity. It appeared to be a 230kDa glycoprotein composed of 53kDa subunits. The purified enzyme showed activity towards most nucleoside 5'-monophosphates, but not towards their 2'-deoxyribose counterparts. AMP and UMP were preferred substrates and the enzyme had a Km for AMP of 15μM. ADP and ATP were potent inhibitors of enzyme activity, ATP inhibition being of the mixed type with a Ki of 67μM. This ATP inhibition could be reversed by magnesium ions indicating that free ATP was the inhibiting species.

In the course of the purification, two enzyme populations were observed which exhibited different ATP and ADP inhibition characteristics. The differences appeared to be due to the presence of an unknown factor which appeared to be bound to a population of the enzyme hence altering its behaviour on ion exchange. Alone, the factor did not affect the enzyme, but did however greatly enhanced its ATP sensitivity. The factor could be stripped off the enzyme and reconstituted back in a saturable manner.

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ABBREVIATIONS

AR	Agonist-Receptor complex
CON A	Concanavalin A
DAG	Diacylglycerol
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
GppNHp	Guanylyl-imidodiphosphate
IC_{50}	50% maximum inhibition
IP_3	Inositol 1,4,5-trisphosphate
PIA	(-)-N^6-(R-phenyl-isopropyl)-adenosine
PIG	Phosphatidylinositol glycan
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIPLC	Phosphatidylinositol specific phospholipase C
PLC	Phospholipase C
PLD	Phospholipase D
PTU	6-n-propylthiouracil
T_3	Triiodothyronine
2D	2-Dimensional
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SAH	S-adenosyl-L-homocysteine hydrolase
SAM	S-adenosyl-L-methionine

CHAPTER ONE

INTRODUCTION

1.1 SIGNAL TRANSDUCTION

It is the ability of cells to respond to a variety of extracellular stimuli and consequently bring about an alteration in their behaviour that has sparked the interest and enthusiasm of many researchers for several years. Since few messengers actually enter their target cells, their information must be conveyed through the cell membrane by a process known as signal transduction. In some systems it is the same molecule that detects the stimulus and is additionally able to provoke the intracellular response. For example the binding of insulin to the extracellular domain of its receptor activates a tyrosine kinase activity on the intracellular domain of the same protein.

It is work from the last two decades however that has revealed a more complex system in which detection of the stimulus occurs on a quite separate entity to that which is eliciting the intracellular response. In these instances, binding of a ligand to its appropriate receptor activates its effector by means of a transducing molecule, that of the G-protein. The heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins) represent a subset of a large family of GTP binding proteins which includes the protein synthesis Tu elongation factor, some enzymes and a group of "small" G-proteins.

The first suggestions for the involvement of a G-protein in a transmembrane signalling pathway came from the study of Rodbell *et al.* (1971) who demonstrated a GTP requirement for glucagon to activate adenylyl cyclase in hepatocytes.

Further evidence was provided a few years later by Cassel and Selinger (1976) with the observation that β-adrenergic agonists stimulated a low Km GTPase activity in turkey erythrocytes; whilst Maguire *et al.* (1976) showed that guanine nucleotides were able to modulate the affinity of the receptor for agonists but not antagonists. The first direct evidence for the involvement of a G-protein however came from the studies of Ross and Gilman (1977) and Ross *et al.* (1978). They performed experiments using a mutant cell line (termed cyc^-) which lacked the GTP-dependant hormonal stimulation of adenylyl cyclase. By reconstituting adenylyl cyclase-free detergent extracts of plasma membranes with cyc^- membranes, they were able to show a restoration in the stimulation of cyclase by these agents. Subsequent work using reconstitution assays of this kind allowed the purification of the transducing protein now known to be G_s (Northup *et al.*, 1980). In 1983, Bokoch and co-workers managed to purify the transducer responsible for the receptor-mediated inhibition of adenylyl cyclase, thus providing the necessary explanation for the dual control of the enzyme.

Since these pioneering studies, the field has received much attention and nowadays it is known that a large array of cell surface receptors regulate their intracellular effector molecules through an ever increasing family of closely related heterotrimeric G-proteins (Citri and Schramm, 1980; Rodbell, 1980; Schramm and Selinger, 1984; Northup, 1985; Levitzki, 1988). Since the beginning of the 1980's, more than 1000 receptors have been anticipated to relay their

messages through one of at least 20 G-proteins. Table 1.1 outlines the various aspects of interactions between receptors, G-proteins and effectors which have been extensively reviewed over the last few years (Casperson and Bourne, 1987; Gilman, 1987; Allenoe, 1988; Lochrie and Simon, 1988; Neer and Clapham, 1988; Weiss *et al.*, 1988; Chabre and Deterne, 1989; Ross, 1989; Houslay & Milligan, 1990). Importantly, G- protein involvement provides the necessary means and amplification whereby cells are able to respond rapidly to very low concentrations of external stimuli. Amplification is achieved by the ability of a single receptor to activate many molecules of the respective G-protein. For example, one molecule of rhodopsin can activate as many as 500 transducin molecules almost simultaneously. Furthermore, the α subunit of activated G_s bound to adenylyl cyclase is capable of stimulating the synthesis of many molecules of cAMP before becoming deactivated. The intriguing aspect of transmembrane signalling of this kind comes from the specificity angle. Signals from different receptors can be integrated through one or more G-proteins or even to the same effector. For example, acetylcholine and adenosine in the heart can both activate the same potassium channel (Kurach *et al.*, 1986) and G_s can activate both adenylyl cyclase and dihydropyridine-sensitive calcium channels in skeletal muscle and cardiac tissue (Brown and Birnbaumer, 1988).

1.2 STRUCTURE AND ORGANIZATION OF G-PROTEINS

Following detergent solubilization from the plasma membrane,

Table 1.1 Integration of G-proteins, receptors and effectors

Abbreviations:

* indicates two splice variants

CTX:	Cholera toxin
PTX:	Pertussis toxin
α-AR:	α-adrenergic receptor
β-AR:	β-adrenergic receptor
M-cho:	Muscarinic cholinergic receptors
D2-Dop:	Dopaminergic receptors
Met-Enk:	Met-enkaphalin
ADO:	Adenosine
AC:	Adenylyl cyclase
PLC:	Phospholipase C
PLA2:	Phospholipase A2
PDE:	Phosphodiesterase

G-protein	Toxin	Tissue distribution	Representitive receptor	Effector response
$Gs_{(S)}$(X2)*	CTX	Ubiquitous	β-AR, Glucagon,	AC(+),
$Gs_{(L)}$(X2)*	CTX	Ubiquitous	TSH, ADO, others	Ca^{2+} channel(+)
G_{olf}	CTX	Olfactory neuro-epithelium	Odorant	AC(+)
Gi1	PTX	Ubiquitous	M2-Cho, α2-AR,ADO,	
Gi2	PTX	Ubiquitous	Somatostatin,	K^{+} channel(+),
Gi3	PTX	Ubiquitous	others	Ca^{2+} channels(-),
				AC(-)
				PLC(+)?
Go1	PTX	Brain, others	Met-Enk, α2-AR,	PLA2(+)?
Go2	PTX	Brain, others	D2-Dop, others	
Gt1	PTX/CTX	Retinal rods	Rhodopsin,	cGMP-specific
Gt2	PTX/CTX	Retinal cones	Cone opsins	PDE
Gz	-	Brain, others	M2-Cho(?), others(?)	AC(-)?, PLC(+)?, others(?)
Gq	-	Ubiquitous	M1-Cho, α1-AR,	β-types PLC(+)
G11		Ubiquitous	others	others?

G-proteins behave as entities (of approximately 100kDa) which can be resolved by SDS-PAGE into three separate subunits termed α (39-52kDa), β (35-36kDa) and γ (7-11kDa). It is the α subunit of each G-protein which although highly homologous appears to be the most diverse and consequently determines the relative specificity of each G-protein in terms of its appropriate receptor effector coupling. At present, more than 17 different genes that encode α, 4 that encode β and 7 that encode γ are known. This indicates that almost 1000 possible permutations could occur, although less are likely due to specific interactions between members of each of the groups. Furthermore it is the α subunit which contains the guanine nucleotide binding site and GTPase activity. With the exception of transducin (which can be washed from retinal disc membranes with GTP or a non-hydrolysable analogue) the heterotrimeric G-proteins can only be extracted from the plasma membrane with detergents. Whilst isolated α subunits appear to be essentially hydrophilic (Codina *et al.*, 1984; Neer *et al.*, 1984; Huff *et al.*, 1985; Sternweis, 1986) the tightly associated β and γ subunits remain highly hydrophobic and can only be separated from one another under denaturing conditions. These findings were confirmed by Sternweis (1986) with the observation that GDP-bound $G_i\alpha$ and $G_0\alpha$ behaved as soluble molecules even in the absence of detergent. The βγ unit on the other hand aggregated in the absence of surfactants but could readily associate with phospholipid vesicles. Although the α subunits showed no association with the phospholipid alone, they were able to

associate in a saturable manner with vesicles containing $\beta\gamma$, the effect of which could be reversed by the addition of GTPγS. It seems likely that the $\beta\gamma$ unit may anchor the α subunit to the membrane although it probably interacts with the membrane directly as well. It has been suggested (Navon and Fung, 1987; Neer *et al.*, 1988) that it is the amino terminal region of the α subunit which is involved in its interaction with the $\beta\gamma$ unit since its proteolytic cleavage results in a decrease in the interaction. Some α subunits ($G_i\alpha$ and $G_0\alpha$) appear to be myristoylated on their amino termini (Buss *et al.*, 1987; Lochrie and Simon, 1988; Mumby *et al.*, 1990) which may function to anchor the G-protein to the membrane presumably via $\beta\gamma$. $G_s\alpha$ however, which lacks the covalently bound lipid, may be anchored to adenylyl cyclase itself (Arad *et al.*, 1984; Levitzki, 1987). Linder *et al.* (1990,1991) provided good evidence for the involvement of myristoylation of $G_0\alpha$ in the interaction with the $\beta\gamma$ unit. By expressing recombinant $G_0\alpha$ in E.Coli, a low affinity for $\beta\gamma$ was observed with respect to the native protein. However, by co-expressing N-myrisoyl transferase with $G_0\alpha$, the authors observed a restoration in the affinity of the recombinant α subunit for the $\beta\gamma$. This aspect of membrane attachment is important since during G-protein activation, the α subunit dissociates from the rest of the protein. Without a means of membrane attachment, the hydrophilic α subunit would simply diffuse into the cytosol. In fact there is immunological evidence that some α-subunits are present in the cytosol (Milligan, 1990).

The α subunits also contain sites for NAD-dependant ADP ribosylation by bacterial toxins from *Vibrio cholera* or *Bordetella pertussis*. These toxins contain enzymes which transfer an ADP ribose group from NAD to specific acceptor sites on the α subunit (Gill and Meren, 1978; Katada and Ui, 1982; West *et al.*, 1985; Van Dop *et al.*, 1984). For example G_s and transducin can be modified by cholera toxin by ADP ribosylation of an internal arginine residue to result in their permanent activation. It appears that modification in this manner serves to reduce the GTPase activity of the α subunit thus prolonging their action (Cassel & Selinger, 1977; Abood *et al.*, 1982). On the other hand, G_0, transducin and all three forms of G_i are substrates for ADP ribosylation by pertussis toxin on a cysteine residue four amino acids from their carboxyl termini (West *et al.*, 1985; Hurley *et al.*, 1984; Ui, 1984). This results in an uncoupling of the G-protein from the receptor (Van Dop *et al.*, 1984; Ui, 1984; Hsia *et al.*, 1984). It is generally thought that the carboxyl terminus is involved in the receptor contact site (Sullivan *et al.*, 1987). Evidence to support this notion comes from the observation that synthetic peptides corresponding to the C-terminus of α subunits inhibit their interaction with receptors (Hamm *et al.*, 1988; Palm *et al.*, 1990) as does ADP ribosylation of G_s (Kahn and Gilman, 1984).

1.3 G-PROTEIN ACTIVATION CYCLE

G-proteins essentially function as on-off switches capable of transducing extracellular messages through the plasma

membrane to intracellular effector molecules. These effectors are then capable of generating many molecules of intracellular second message which in turn are able to initiate changes in the function of the cell. The G-protein activation/deactivation cycle has been extensively studied and has been summarised in many excellent reviews (Casey & Gilman, 1988; Gilman, 1987; Levitzki, 1987; Freissmuth *et al.*, 1989; Ross, 1989; Taylor, 1990; Kaziro *et al.*, 1991; Linder & Gilman, 1992).

The α subunit, apart from determining the relative function of each G-protein, also contains the nucleotide binding site and at least one high affinity binding site for magnesium (Gilman, 1987). The effects of magnesium are complex and have been fully described by Gilman (1987). The divalent cation is required for two important steps in the cycle. Firstly, nanomolar concentrations are required for GTP hydrolysis and secondly higher amounts (>10μM) required to maximise the activated receptor (AR)-catalysed nucleotide exchange (Brandt & Ross, 1986). The characteristics of the G-protein cycle are depicted in figure 1.1. When an agonist binds to its receptor, the receptor undergoes a conformational change and switches it to an active state. This activated form of the receptor has a high affinity for the G-protein trimer ($\alpha\beta\gamma$) and consequently the two associate. In the resting state, the α subunit contains tightly bound GDP. In the absence of AR, the dissociation of this nucleotide is rate limiting and is very slow. In fact the rate of GDP dissociation is approximately 10 fold

Figure 1.1 G-protein activation/deactivation cycle

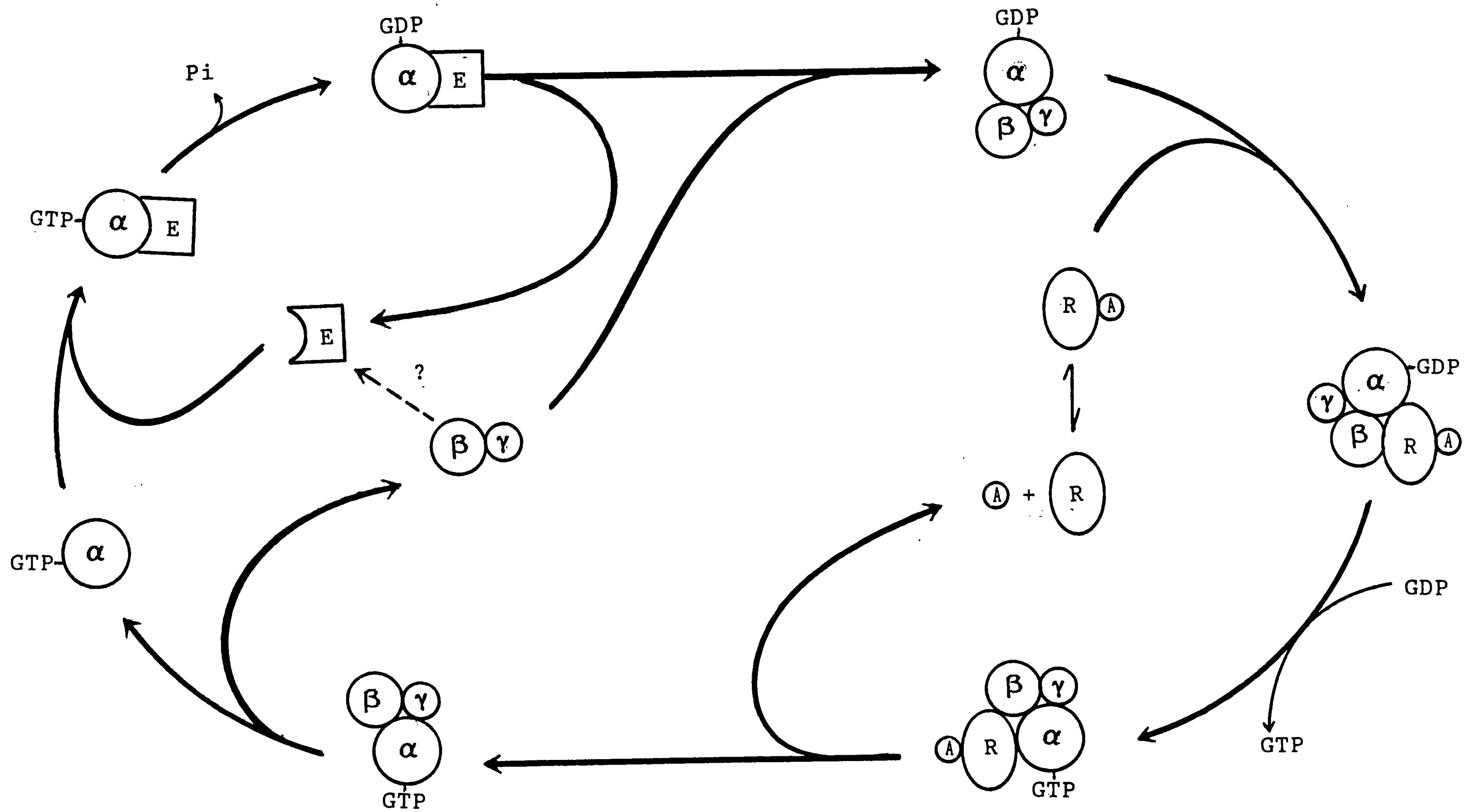
The general aspects of the G-protein cycle are discussed in depth in the text.

α, β and γ represent the appropriate subunits of the guanine nucleotide binding protein.

A Agonist

R Receptor

E Effector molecule which generates the second message, eg. enzyme or ion channel.


GDP
α
E
Pi
GTP
α
E
E
?
β
γ
GTP
α
β
γ
α
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GDP
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A
α
GDP
γ
β
R
A
A
+
R
GDP
GTP
β
γ
A
R
α
GTP

lower than the Kcat for GTP hydrolysis. Consequently the amounts of α subunit containing bound GTP are also very low and constitute $<1\%$ of the total G-protein population (Ross, 1989). The presence of activated receptor and magnesium however promotes its release by increasing the rate of GDP dissociation (Ui & Katada, 1989; Florio & Sternweis, 1989). With GDP absent from the binding site of the α subunit, a high affinity for the trimer and the receptor is established. GTP binds to the empty binding site within milliseconds (May & Ross, 1988) initiating three responses. Firstly it reduces the affinity of the receptor for the G-protein and promotes their dissociation. Secondly, GTP bound to the α subunit in the presence of magnesium lowers its affinity for $\beta\gamma$ and the two consequently dissociate thus releasing the active α subunit to activate the effector molecule. Thirdly, GTP binding induces a negative heterotrophic interaction of the receptor. This reduces its affinity for the agonist and thus initiates their dissociation. This permits the receptor to recycle, bind more agonist and activate multiple G-proteins. Incidently, this negative heterotrophic interaction of the receptor is not seen with antagonist binding (Casey & Gilman, 1988). The activated α subunit interacts with the effector, the duration of which can last up to a few seconds before deactivation occurs. The interaction with the effector is terminated following the hydrolysis of the bound GTP, a reaction catalysed by the intrinsic GTPase activity located on the α subunit. Typical Kcat values for GTP hydrolysis falls within the range 2-4 min^{-1} for various G-proteins

(Gilman, 1987). The α subunit thereby can be seen to be auto-regulatory in that it is capable of deactivating itself by hydrolysing its bound GTP. The GDP bound α subunit then reassociates with the $\beta\gamma$ complex and re-establishes the resting state of the G-protein. The role of the $\beta\gamma$ also appears to perform a role in the deactivation of α subunits by binding them and terminating their effects. For example the inhibition of adenylyl cyclase by activated G_i can result from the ability of the released $\beta\gamma$ complex to bind α_s subunits (released by G_s activation) thus terminating their stimulatory action on cyclase. A useful tool in the study of G-protein function is that of AlF_4^-. This complex activates the G-protein without the need for GDP dissociation. This ligand appears to bind alongside GDP and mimics the terminal phosphate group of GTP, thus promoting its activation (Bigay *et al.*, 1985).

1.4 α-SUBUNIT DIVERSITY

Although α-subunits show high structural homology, it is this subunit that determines their relative specificity in terms of receptor effector coupling. Recent advances in molecular cloning however have provided the necessary key to their identification and study; which would pose an almost impossibility using classical biochemical techniques. The general properties of each of the major classes of α-subunits will be addressed separately, although the transducins will be omitted from the discussion as they are only involved in visual transduction and play no role in cellular signalling

in the CNS.

1.4.1 $G_s\alpha$

The involvement of $G_s\alpha$ in the hormonal stimulation of adenylyl cyclase has been known for many years. Moreover the ability of cholera toxin to covalently modify the α-subunit by ADP-ribosylation leads to its permanent activation and provides a useful tool for study. More recently, evidence has appeared for the involvement of $G_s\alpha$ in the opening of a calcium channel (Yatani *et al.*, 1987b; Brown & Birnbaumer, 1988). $G_s\alpha$ purifies as a mixture of two polypeptides which can be resolved by SDS-PAGE into two bands of 52 and 45kDa, the proportions of which vary amongst different tissues and cells. It is evident however that up to four different α-subunits exist, the cDNAs of which have been isolated from human brain (Bray *et al.*, 1986); a finding confirmed in 1988 by Kozasa and coworkers. By comparing the sequences of these cDNAs to the sequence of the human $G_s\alpha$ gene, it was concluded that the four forms arise from alternative splicing of a single gene product. It is moreover likely that all the forms of $G_s\alpha$ are capable of activating both adenylyl cyclase and the calcium channel (Mattera *et al.*, 1989), although the reason for several forms is not known. At the amino acid level, high structural homology exists amongst species in that only 1 out of all 394 amino acids differ between the rat and human α-subunit. In addition to $G_s\alpha$, another closely-related form of this α-subunit is exclusively expressed in olfactory sensory neurones (termed G_{0lf}). It is believed to

be responsible for signal transduction from odorant receptors, and like G_s is capable of stimulating adenylyl cyclase when expressed in S49 cyc^- cells which lack $G_s\alpha$ (Jones & Reed, 1989). Its molecular weight calculated from the cDNA sequence is 44,322, a value closely resembling the 45kDa form of $G_s\alpha$. However only 88% homology with G_s is observed in amino acid sequence suggesting their distinct natures.

1.4.2 $G_i\alpha$

$G_i\alpha$ exists in three distinct forms termed $G_i1\alpha$ (41kDa), $G_i2\alpha$ (40kDa) and $G_i3\alpha$ (41kDa). They show some 85% homology in amino acid sequence, and are the products of three quite separate genes. They all serve as substrates for ADP-ribosylation by pertussis toxin. Although $G_i2\alpha$ and $G_i3\alpha$ appear to have ubiquitous distributions, their relative amounts in various tissues vary. $G_i1\alpha$ on the other hand is more restricted and appears to be predominantly expressed in brain (Bray *et al.*, 1987; Brann *et al.*, 1988) although not exclusively localized to this tissue. The two $G_i\alpha$ subunits with molecular weights of 40 and 41kDa purified from porcine brain have been shown to be immunologically distinct by the use of selective antisera (Katada *et al.*, 1987). Their partial amino acid sequences moreover provide evidence that they represent $G_i2\alpha$ and $G_i3\alpha$ respectively (Itoh et al., 1988). The amino acid sequence of bovine $G_i1\alpha$ is identical to that of the human form, whilst the remaining two forms show greater than 98% homology amongst different mammalian forms. $G_i\alpha$ couples to adenylyl cyclase and attenuates its activity.

Direct evidence exists that it is capable of interacting directly with cyclase to inhibit its activity as shown by its ability to attenuate adenylyl cyclase in cyc$^-$ membranes (which lacks G_s)(Katada *et al.*, 1984; Roof *et al.*, 1985). However the $\beta\gamma$ complex released on activation of G_i also plays an important role in the attenuation of adenylyl cyclase by binding and hence removing active $G_s\alpha$ subunits. This issue will be dealt with in a later section (1.5). $G_i\alpha$ may also couple to phospholipase C and also to a potassium channel, although the specificity of each form is uncertain. Purified G_i from erythrocytes is moreover capable of activating a potassium channel in cardiac tissue resulting in the outward movement of this ion. This response is not selective to any one form of G_i in that all three forms are able to perform this function, as is $G_0\alpha$.

1.4.3 $\underline{G_0\alpha}$

The most abundant G-protein in nervous tissue is G_0 and constitutes up to 1% of total membrane protein. This pertussis toxin-sensitive G-protein was discovered during the purification of G_i from bovine brain (Neer *et al.*, 1984; Sternweis & Robishaw, 1984; Milligan & Klee, 1985), appearing as a 39kDa species. It is highly homologous amongst many species and shows greater than 98% amino acid identity. Recently it has been shown that two forms of $G_0\alpha$ exist. Molecular cloning techniques have enabled the isolation of two cDNAs from the mouse (Strathmann *et al.*, 1990), hamster (Hsu *et al.*, 1990) and rat (Tsukamoto *et al.*, 1991). They

are identical in amino acid sequence except for their C-terminal region, and appear to be generated by alternative splicing of a single $G_0\alpha$ gene product. There may however be as many as four types of $G_0\alpha$ (Inanobe *et al.*, 1990). Functionally, G_0 has been implicated in neuronal potassium (Van Dongen *et al.*, 1988) and calcium (Hescheler *et al.*, 1987; Ewald *et al.*, 1988) channel modulation.

1.4.4 $G_x\alpha$ and $G_q\alpha$

The α-subunit sometimes referred to as $G_x\alpha$ or $G_z\alpha$ is also mainly expressed in neuronal cells but represents a novel form of G-protein, quite distinct to the forms so far discussed. At best it shows only 60% identity with any other α-subunit ($G_i\alpha$) and fails to be covalently modified by either of the bacterial toxins. From the cDNA sequence it appears that there is an altered amino acid sequence in the highly conserved guanine nucleotide binding domain that is believed to be involved with the GTPase activity. The effect of this modified sequence has been confirmed (Casey *et al.*, 1990) by demonstrating the rate of GTP hydrolysis to be some 200 fold slower than that of other $G\alpha$ subunits. Although not confirmed, it may be involved in phoshpholipase C activation (Masters *et al.*, 1985) or potassium channel closure (Nakajima *et al.*, 1988).

A new family of pertussis toxin insensitive G-proteins has recently received much attention. There appear to be five homologous forms reported and termed G_q and G_{11-16} inclusive. Of these, G_q has been the most extensively

studied, and like G_{11} is involved in the activation of the β subtypes of phospholipase C (see section 1.6). Although G_q and G_{11} are homologous and activate the same effector molecule, they only show some 88% identity at the amino acid level. The differences in sequence moreover are located in the amino terminal end of the structure and may reflect differences in receptor recognition. The role of the other forms is uncertain, but the possibility arises that they may regulate the other isoforms of phospholipase C (Simon *et al.*, 1991).

1.4.5 **βγ COMPLEX**

Less studies have been directed towards the β and γ subunits as they were originally believed to be functionally interchangeable (and consequently non-specific), and only function to anchor the α-subunit to the membrane (Sternweis, 1986). In fact the βγ complexes purified from G_s, G_i and G_0 can all be functionally exchanged and work equally well. Recent studies however have revealed their involvement in presenting the α-subunit to receptors as well as functioning in effector modulation both directly and indirectly. Contrary to initial belief, there appear to be 4 distinct forms of β and at least 7 forms of γ, all of which are products of separate genes. However, they are always tightly associated and can only be separated under denaturing conditions. Initial studies on purified β revealed the presence of 2 forms which could be resolved on gels into 35 and 36kDa bands (Sternweis & Robishaw, 1984) and appeared to

be immunologically distinct (Roof *et al.*, 1985; Evans *et al.*, 1987). Their distinct natures were consequently confirmed by amino acid sequencing and displayed a striking 90% identity at this level (Fong *et al.*, 1987; Gao *et al.*, 1987). In fact all 4 forms which have been purified to date display 80-90% amino acid identity (Hepler & Gilman, 1992). They appear to be ubiquitously expressed, although retinal cells only contain the 36kDa form of β. The γ subunits are the smallest of the subunits appearing as 6-10kDa polypeptides on SDS-gels, and appears to be prenylated which may function to tightly anchor them to the membrane. They too are nearly ubiquitously distributed, although γ1 is localised to retinal cells, and is immunologically distinct from those originating from G_i and G_s (Neer & Clapham, 1988). At the amino acid level, γ2-6 are highly homologous but show only 25-30% identity compared to γ1. Interactions between β and γ however appear to be selective. In a recent study, Pronin & Gautam (1992) provided evidence that γ1 showed no association with β2, whilst β3 would not associate with either γ1 or γ2 in transfected cell assay systems. However more studies need to be performed on the selective interactions of these two subunits. Functionally, the βγ complex has been shown to be required for interaction of α with receptors, inhibition of α action by binding to it and blocking its action, and modulation of adenylyl cyclase activity (see section 1.5). Other functions have been postulated, for example the regulation of phospholipase A2 activity (Kim *et al.*, 1989) as well as modulating ion channel

activity either directly or indirectly (see section 1.7).

1.5 DUAL CONTROL OF ADENYLYL CYCLASE

Adenylyl cyclase is the membrane bound enzyme which generates cAMP, a potent second messenger. For many years it has been known to be under dual control regulated by extracellular receptors. These extracellular signals are transduced through the plasma membrane by the G-protein subtypes G_s and G_i resulting in a stimulation or inhibition of the enzyme respectively. cAMP then modulates many intracellular events by activating a cAMP-dependent kinase resulting in the phosphorylation of key intracellular proteins. The current mechanism for the control of adenylyl cyclase is more complex than first imagined. To date at least 5 isoforms of the enzyme have been studied, all of which are activated by G_s. The original G-protein dissociation model (see section 1.3) appears inconsistent with respect to several lines of evidence for the activation of adenylyl cyclase by G_s however. Firstly, many purified preparations of the enzyme contain tightly associated G_s, although some appear to be devoid. This may however represent purification of differing isoforms of adenylyl cyclase, although this has not been experimentally determined. In fact G_s-associated adenylyl cyclase can only be resolved into its separate entities by treatment with detergents and high ionic strength (Levitzki, 1990). Secondly, first order kinetics are observed with respect to adenylyl cyclase activation by guanine nucleotides and hormone stimulated receptor, with an activation directly

proportional to activated receptor concentration. If α_s and adenylyl cyclase were separate entities, more complex kinetics would be observed. It is likely therefore that at least some populations of adenylyl cyclase (perhaps differing isoforms) are constantly associated with cyclase itself, and thus the original G-protein activation model must be modified to allow for these discrepancies (see figure 1.2).

Inhibition however appears to be mediated by two mechanisms. Firstly, α_i is capable of directly interacting with cyclase and inhibiting its activity as demonstrated by the ability of α_i to inhibit adenylyl cyclase in cyc^- membranes (Roof *et al.*, 1985). The second mechanism which has received more interest recently is the role of the βγ unit. Although it was first thought that this complex could inhibit cyclase activity (presumably by binding α_s and hence removing stimulation), this idea was questioned due to the relative non-specificity of βγ's arising from the fact that they are functionally interchangeable between different α-subunits. However, the large excess of G_i over G_i within the membrane could serve to provide a means of increasing free βγ concentration. This pool of βγ could then buffer αs or bind to αs-associated cyclase hence terminating the stimulatory effect. In addition, βγ is more effective at inhibiting adenylyl cyclase than α_i. It is however possible that αs and α_i may both be capable of binding cyclase simultaneously, presumably at different sites. The different isoforms of adenylyl cyclase appear to be differentially regulated by βγ in a direct manner (Tang & Gilman, 1991). These authors

Figure 1.2 The dual control of adenylyl cyclase

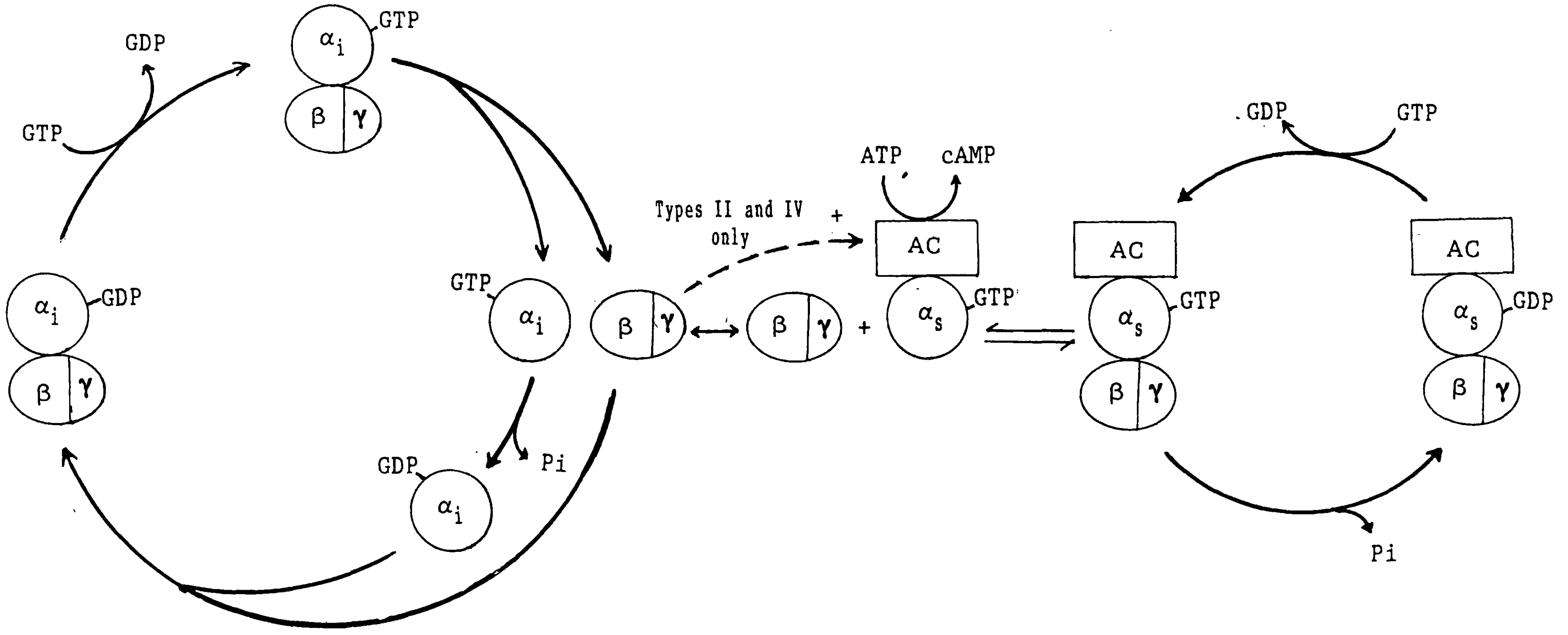
This modified G-protein activation cycle has been described in detail in the text.

α_i α-subunit of the inhibitory G-protein G_i. This α-subunit can interact directly with adenylyl cyclase, although this is not depicted in the figure.

α_s α-subunit of the stimulatory G-protein G_s is permanently associated with adenylyl cyclase

$\beta\gamma$ This complex appears important in modulation of α-subunit activity as well as direct stimulation of types I and IV adenylyl cyclase in the presence of α_s.

AC The enzyme adenylyl cyclase when activated by α_s results in the production of the second messenger, cAMP.


GDP
GTP
α_i
GTP
β
γ
GTP
α_i
GDP
β
γ
GTP
α_i
GDP
α_i
Pi
β
γ
Types II and IV
only
+
β
γ
+
ATP
cAMP
AC
α_s
GTP
GDP
GTP
AC
α_s
GTP
β
γ
AC
α_s
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Pi

concluded that type I adenylyl cyclase was inhibited by βγ, presumably by removing active αs from the system, since it had no effect alone. Surprising results were seen on the other hand by the observation that βγ could stimulate types II and IV adenylyl cyclase activity, although only in the presence of activated αs. This finding has recently been confirmed by Gao & Gilman (1991) and Taussig *et al.* (1993). These observations were further demonstrated by Federman and coworkers (1992) who additionally concluded that the βγ responsible probably originated from G_i or G_0, by the ability of pertussis toxin to attenuate the response. Furthermore the α-subunit of transducin also blocked the effect by binding to and removing βγ (alone transducin had no effect on cyclase). Interestingly, adenylyl cyclase type II is predominantly expressed in the central nervous system (Feinstein *et al.*, 1991) and this modulatory effect may be of particular importance in this tissue. Type I adenylyl cyclase is activated by calcium-calmodulin. The ability of G-proteins to modulate intracellular calcium concentration (see section 1.7) complicates the model further by introducing the possibility that separate signalling pathways may ultimately result in the activation of this form of cyclase.

These results demonstrate that adenylyl cyclase modulation by G-proteins is far more complicated than first imagined and interconnections between different signal transducing pathways appear likely to be important in the regulation. More work will undoubtedly further our knowledge

concerning the modulation of this important second messenger system.

1.6 INOSITOL PHOSPHATE METABOLISM

Recent interest has been directed towards the second messenger system controlled by inositol phosphate metabolism. In common with other transmembrane signalling systems, this pathway seems to be under G-protein control although a more complex system of regulation is apparent. In this case the effector molecule is the enzyme phosphatidylinositol-specific phospholipase C (PLC). It catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing two potent second messengers namely inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These two messenger species consequently bring about the mobilization of calcium from intracellular stores (the endoplasmic reticulum) and activation of protein kinase C (Berridge & Irvine, 1984). Cellular regulation is then modulated in common with the cAMP system by the activation of specific protein kinases resulting in phosphorylation of key metabolic and structural proteins and enzymes within the cell. PLC appears to exist in forms both bound to the plasma membrane and free in the cell cytosol (Baldassare & Fisher, 1986; Deckmyn *et al.*, 1986). It is reported that both of these forms can be activated by guanine nucleotides (Litosch, 1987; Baldassare & Fisher, 1986; Deckmyn *et al.*, 1986). Stimulation of the enzyme seems to be mediated by the ability of these nucleotides to lower the amounts of calcium required to

stimulate the enzyme. Further stimulation by this ion could occur following its influx, another response which can also be G-protein mediated (see section 1.7), again suggesting interconnections between transmembrane signalling pathways.

The first demonstration for G-protein involvement in this pathway came from the observation that GTPγS was capable of stimulating PLC activity in permeabilized platelets (Haslam & Davidson, 1984), a finding confirmed in 1987 by Litosch using membrane and permeabilized cell models, and subsequently by several other authors (see Litosch, 1990). In 1985 the first direct evidence for agonist stimulation of PLC was demonstrated in membranes isolated from blowfly salivary glands (Litosch *et al.*, 1985).

It is now appreciated that stimulation of PLC occurs by two separate transducing systems, one sensitive and the other insensitive to blockage by pertussis toxin (Litosch, 1990; Sternweis, 1992). The presence of a pertussis toxin-sensitive component suggests the involvement of G_i or G_0. In fact evidence to support this idea comes from reconstitution experiments using human leukaemic HL-60 cells (Kikuchi *et al.*, 1986). Initially it was observed that pertussis toxin reduced agonist stimulation of PLC, an effect which could be reversed by the addition of purified G_i or G_0. Moreover, these purified G-proteins were ineffective if pre-treated with pertussis toxin prior to reconstitution. Using partially purified PLC from platelet membranes and reconstituting with G_i or G_0, Banno and coworkers (1987) were able to demonstrate PLC stimulation, a finding however which

unfortunately could not be repeated with a homogeneous preparation of the enzyme (Banno *et al.*, 1988). More recently, the involvement of βγ in PLC modulation has been observed (Camps *et al.*, 1992). Although direct activation by αi or αo has not been demonstrated, purified βγ is capable of activating the enzyme, which may in turn result from G_i or G_0 activation (and hence dissociation from their α subunits). The α subunits thus released could activate their respective effectors whilst the βγ modulates PLC activity (Sternweis, 1992). Thus multiple pathways could exist, although βγ subunits are non-specific and the question of specificity arises. Although their involvement is unquestionable they probably only represent a part of the regulation of PLC, and various forms of PLC may be regulated by both α and βγ.

The search for the pertussis toxin insensitive component of the pathway has recently been successful due to the finding of at least 8 toxin insensitive α-subunits, all of which represent likely candidates (Hepler & Gilman, 1992). A novel pertussis toxin insensitive G-protein preparation has been isolated from bovine brain (Pang & Sternweis, 1990) which has been shown to stimulate PLC (Smrcka *et al.*, 1991). The preparation contains two homologous proteins, one of which has been studied further and appears identical in amino acid sequence to that of the new G-protein G_q (Hepler & Gilman, 1992; Sternweis & Smrcka, 1992). Moreover the ability of α_q to stimulate the enzyme (Smrcka *et al.*, 1991; Taylor *et al.*, 1991; Waldo *et al.*, 1991) can be inhibited by antibodies directed against this α subunit, providing

further evidence for the involvement of αq in the pathway (Sternweis & Smrcka, 1992). Although these experiments have been demonstrated using the β-subtype of PLC, other forms also exist which may be regulated in a similar fashion. It is also possible that PLC could be under dual control like the adenylyl cyclase system, since it may be additionally subject to inhibitory G-protein regulation. This has been indicated by the ability of guanine nucleotides to inhibit PLC in membrane preparations or extracts (Litosch, 1989; Gutowski *et al.*, 1991; Geet *et al.*, 1990; Bizzari *et al.*, 1990). These regulatory events have been summarised for clarity in figure 1.3.

1.7 G-PROTEIN REGULATION OF ION CHANNELS

The ability of G-proteins to modulate ion channels is of particular importance in the brain. Changes in potassium channels can lead to modulatory effects on neuronal activity whilst that of calcium playing an important role in neurotransmitter release and hence synaptic transmission. Presynaptic transmitter release is dependent on calcium influx through a voltage-gated channel, and many transmitter substances or neuromodulators (eg. adenosine) can act presynaptically to prevent further transmitter release. In addition, calcium channels by allowing influx of this ion can integrate with the inositol phosphate messenger system to modulate PLC activity as well as activate specific protein kinases to control intracellular phosphorylation events.

Studies are generally performed using patch clamped

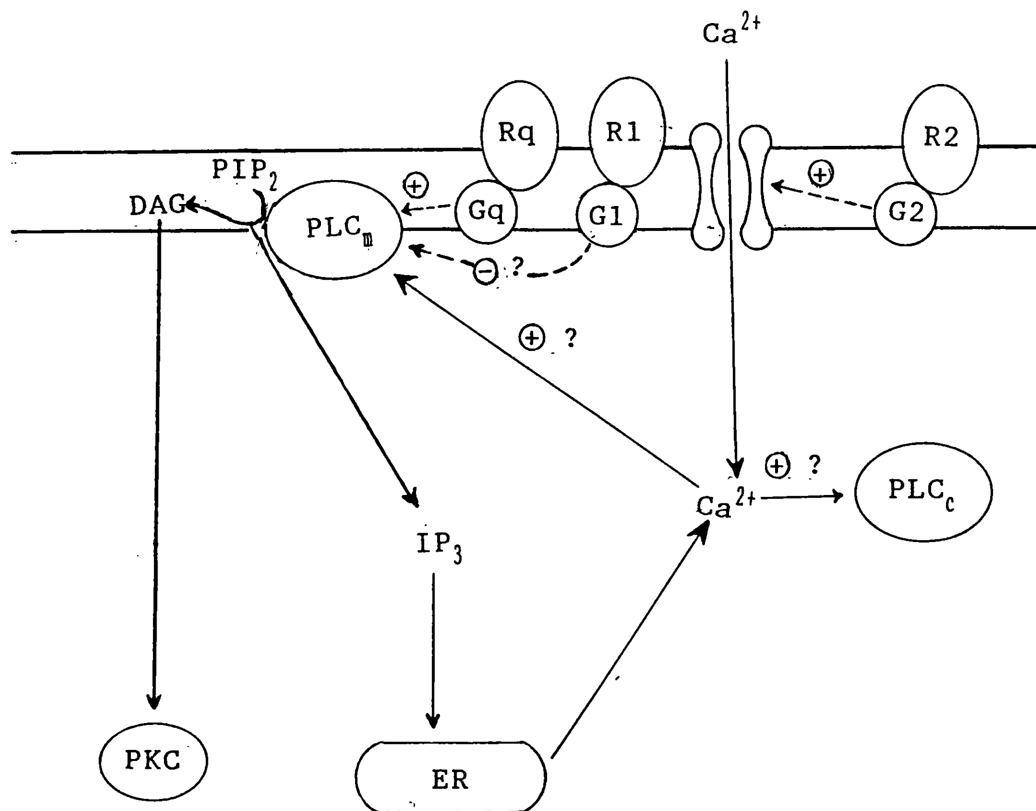


Figure 1.3 G-protein modulation of inositol phosphate metabolism

R1 Receptor mediating PLC inhibition

G1 G-protein responsible for inhibition of PLC

Rq Receptor mediating PLC activation

Gq G-protein responsible for PLC activation

R2 Receptor mediating calcium channel opening

G2 G-protein responsible for calcium channel opening

PLC_m Membrane associated phospholipase C (PLC)

PLC_c Cytosolic phospholipase C (PLC)

PKC Protein kinase C

ER Endoplasmic reticulum

PIP_2 Phosphatidylinositol 4,5-bisphophate

IP_3 Inositol 1,4,5-trisphosphate

DAG Diacylglycerol

membranes and measuring ion trafficking with recording electrodes. Problems frequently encountered when attempting to demonstrate a direct G-protein effect on a channel involves channel modulation by second messengers produced by other signalling pathways. For example both G_s and G_i regulate the production of cAMP. This in turn activates cAMP-dependent protein kinase which is then capable of phosphorylating calcium channels and hence regulating them (Dolphin, 1990). Despite these drawbacks, direct modulation of ion channels has been demonstrated by G-proteins. In the nervous system, neurotransmitters have been shown to cause hyperpolarization by activating a potassium channel. In fact the channel can even couple to more than one receptor. In 1988, Van Dongen and coworkers demonstrated the ability of purified G_0 to activate a potassium channel in patches of cultured hippocampal neurones. This observation has additionally been seen by G_i in cardiac cells (Yatani *et al.*, 1987a) and consequently results in membrane hyperpolarization. In fact the G-protein described as G_k which mediates this effect has been shown to be G_i3 by comparison of the amino acid sequences of the purified preparations. However, G_i1 and G_i2 are also able to mediate this response equally well. It has also been suggested that the βγ complex may play a role (Logothetis *et al.*, 1987) by the observation that it could also effect potassium channel activity. However, it has not been ascertained whether these effects are mediated directly by βγ or purely as a result of βγ affecting another pathway.

The neurotransmitters which activate potassium channels are also capable of blocking calcium currents, an effect mediated by a pertussis toxin sensitive G-protein. It is not certain whether these events occur simultaneously however, or if the same G-protein is involved in the modulation of both channels. A series of experiments have provided us with unquestionable evidence of G-protein involvement. By measuring calcium currents in pertussis toxin pretreated membrane patches, it was demonstrated that exogenously added G-proteins could restore the ability of neurotransmitters to inhibit the channel (Hescheler *et al.*, 1987; Ewald *et al.*, 1988). Moreover, G_0 was more effective than G_i and antibodies directed towards this G-protein were able to reverse the effect (Ewald *et al.*, 1988; Harris-Warwick *et al.*, 1988; McFadzean *et al.*, 1989). G_s also modulates calcium channels and the demonstration of its stimulation in skeletal muscle (Brown & Birnbaumer, 1988) and heart membranes (Yatani *et al.*, 1987b, 1988) has been reported. Although direct activation by G_s occurs, a second response is apparent and represents long term regulation mediated by channel phosphorylation by cAMP-dependant protein kinase. There is in addition evidence that $\beta\gamma$ indirectly opens a potassium channel. It is thought that $\beta\gamma$ serves to activate phospholipase A2, and the arachadonic acid thus produced, or its metabolites, consequently modulates the channel.

1.8 REGULATION OF G-PROTEIN ABUNDANCE

With the availability of specific antibodies to the

individual G-proteins as well as oligonucleotide probes for the respective mRNA's, quantitation of these molecules has become easier and more reproducible. In recent years, the use of such reagents has enabled in-depth studies of G-protein abundance under different physiological and pathological states to be performed. These results, in conjunction with sensitive receptor binding studies, have provided insight into the dysfunction of certain cellular signalling pathways under these conditions. It is known that certain hormone concentrations in the blood can govern the levels of various G-proteins within the membrane, and many studies have been performed using appropriate animal models which mimic different physiological and disease states. For example prolonged exposure of adipocytes to the adenosine A1 receptor agonist N^6-L-phenylisopropyladenosine (PIA) has demonstrated the down-regulation of the α-subunit of G_i. β subunits were also reduced whilst G_s was unaltered (Green *et al.*, 1990). This effect was also observed *in vivo*, and interestingly, mRNA levels were unaltered indicating that the regulation was post-transcriptional. Moreover, removal of the agonist resulted in a restoration of the G-protein levels. Under some circumstances however, regulation can occur at the transcription level for example the ability of long term ethanol exposure to down regulate $G_s\alpha$ with a parallel decrease in the mRNA (Mochly-Rosen *et al.*, 1988). This effect may however be indirect in that ethanol can inhibit the nucleoside transporter resulting in increased levels of extracellular adenosine. The prolonged action of

adenosine at adenosine A2 receptors which couple to adenylyl cyclase by G_s may then cause the down regulation observed.

In the diabetic state, significantly reduced levels of G_i have been reported in hepatocyte membranes (Bushfield *et al.*, 1990a; Gawler *et al.*, 1987) and increased amounts in adipocyte plasma membranes (Saggerson *et al.*, 1991). No such studies have been performed in nervous tissue. However, altered G-protein coupled receptors and adenylyl cyclase activity have been reported in the central nervous system (Lynch & Exton, 1992) as has the loss of G_i-mediated cyclase inhibition. More studies should thus be directed towards direct quantitation of G-protein abundance in the brain in this perturbed metabolic state.

Several studies have been performed on altered signalling in the hypothyroid state (see Saggerson, 1992 for review). Adipose tissue isolated from hypothyroid rats shows both an enhanced sensitivity to hormones which inhibit adenylyl cyclase and also decreased sensitivity towards the stimulatory pathway. Consequently, these membranes show increased levels of $G_i\alpha$ (Ros *et al.*, 1988; Milligan & Saggerson, 1990) as well as increased levels of β-subunits (Milligan *et al.*, 1987). In contrast, $G_s\alpha$ was unaltered (Milligan & Saggerson, 1990). Within the central nervous system, indirect evidence for altered signalling pathways was obtained by Mazurkiewicz & Saggerson (1989a). It was observed that forskolin-stimulated adenylyl cyclase in synaptosomal membranes isolated from hypothyroid rats was inhibited by 10-100μM GTP, an effect not seen in control

membranes. Furthermore, an enhancement by the adenosine A1 agonist PIA to inhibit cyclase was also reported in hypothyroid membranes, despite no change in receptor number and affinity for the agonist. Together these results indicated that post- receptor regulation was apparent, possibly by the up- regulation of G_i. A confirmation of these findings were provided by Orford *et al.* (1991a) who provided direct immunological evidence for this up-regulation of G_i in 6 anatomically distinct regions of rat brain in the hypothyroid state (see also this thesis). The α-subunit of G_0 was additionally increased under these conditions. Neurotransmitter release can be inhibited by hormones, neurotransmitters or neuromodulators presynaptically by the G_i-coupled pathway. The fact that hypothyroidism is accompanied by up regulation of these inhibitory G-protein α-subunits may account for some of the observations seen in hypothyroidism (see section 1.11). In the hyperthyroid state however, opposite effects are seen to hypothyroidism (see section 1.11). These may be related to down-regulation of these inhibitory G-proteins, as indicated in a preliminary study performed by Orford *et al.* (1992) and described later in this thesis. Moreover, increases in the G_s subtype may be apparent, although no such studies have been carried out to date.

The evidence so far discussed relates to changes in G-protein abundance arising as a result of altered hormonal status. A further mechanism however has been implicated that the effector molecule may also modulate G-protein levels, and

Hadcock *et al.* (1990) provided evidence to support this notion. Stimulation of adenylyl cyclase by β-adrenergic agonists, or direct stimulation of cyclase by forskolin resulted in a decrease in $G_s\alpha$ with a parallel increase in $G_i2\alpha$. Moreover, the mRNA for $G_i2\alpha$ was also increased, but its production preceded that of the protein. This effect was not seen in the mutant S49 cells (KIN^-) which lacks cAMP-dependant protein kinase. It was concluded that the kinase may possibly induce translation of the $G_i2\alpha$ gene thus enhancing its production, or possibly by stabilizing the relevant mRNA.

Evidence also exists that signalling pathways may further be regulated by G-protein phosphorylation (Jakobs *et al.*, 1985; Katada *et al.*, 1985; Pyne *et al.*, 1989; Bushfield *et al.*, 1990b). For example in diabetes, $G_i2\alpha$ is phosphorylated and consequently non-operational (Bushfield *et al.*, 1990a).

Many studies are needed to confirm and expand on those reported to date and provide further support for the involvement of G-proteins in altered cell signalling in many physiological and pathological states.

1.9 ADENOSINE - GENERAL ASPECTS

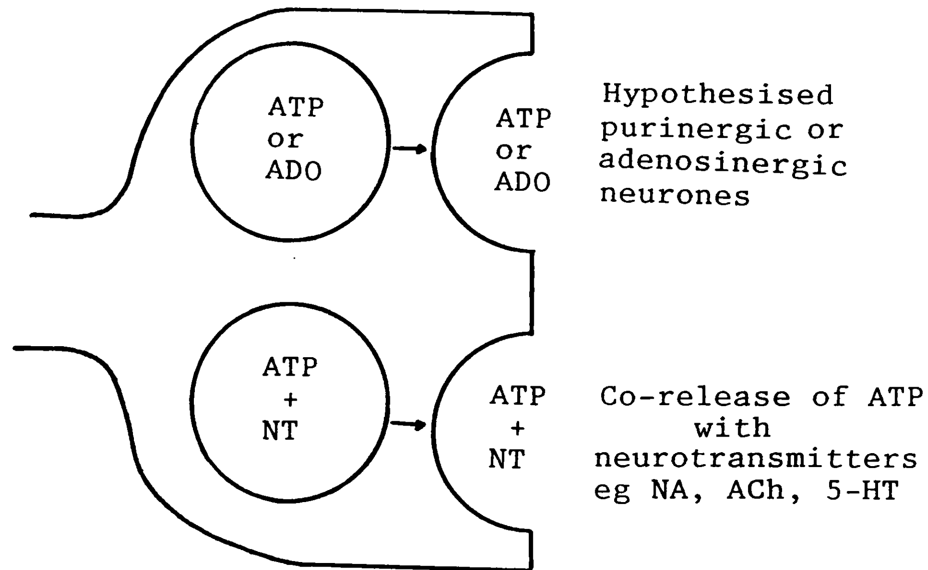
Adenosine is a ubiquitous nucleoside found in all organs and tissues of the body. Due to its rapid metabolism and uptake into cells, adenosine functions as a paracrine agent and consequently only very low levels are observed in the circulation. It generally acts on the very cell which

produced it, or at least its close neighbours, and consequently has been described as a 'local hormone' or 'retaliatory metabolite' (Arch & Newsholme, 1978a,b; Newby, 1984, 1990). For many years adenosine's capacity to modulate cellular function by means of extracellular receptors has been greatly appreciated. These receptor mediated effects furthermore can be blocked directly at the receptor level by xanthine compounds such as caffeine or theophylline.

The extent of adenosine's effects will thus be determined by its tissue levels, in particular that in the extracellular space. These will in turn be controlled by the balance between uptake and release processes as well as its intra- and extracellular metabolism. Extracellular adenosine can be produced by two distinct and separate routes (figure 1.4). Firstly, as a direct link to neurotransmission, it can be produced as an endpoint of the extracellular purine nucleotide phosphohydrolase pathway by the degradation of ATP which is co-released with excitatory neurotransmitters. Adenosine formed in this manner serves to inhibit the release of further transmitter and hence functions as a neuromodulator. Secondly, it is produced from within the cell following metabolic insults which lower the energy charge and result in a rise of cytosolic AMP (Jonzon & Fredholm, 1985; McIlwain & Poll, 1986). The adenosine thus produced upon leaving the cell can elicit protective properties such as vasodilation of the surrounding vasculature.

Throughout the body, adenosine appears to provokes a wide

EXTRACELLULAR ROUTE



INTRACELLULAR ROUTE

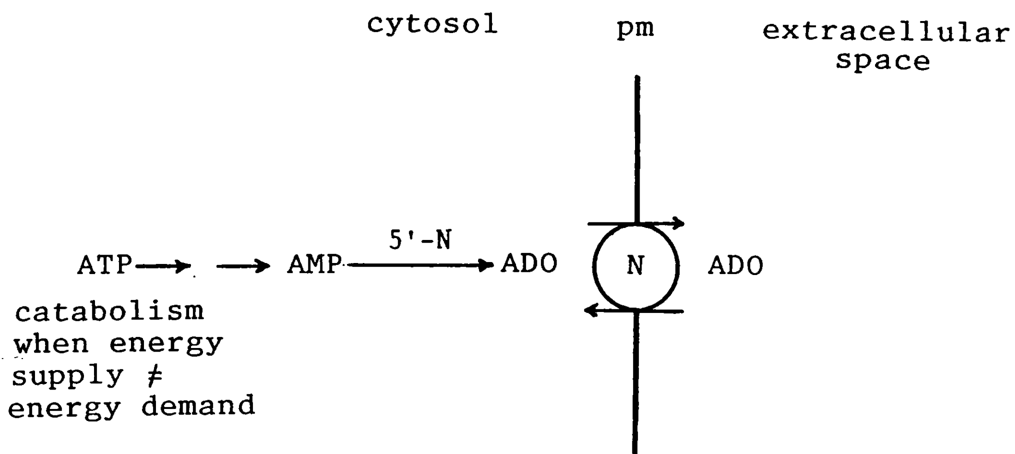


Figure 1.4 Possible routes of extracellular adenosine

Adenosine can be produced directly into the extracellular space following degradation of exocytotically released nucleotides. These can arise from the hypothesised purinergic or adenosinergic neurones, or as a result of co-release with excitatory neurotransmitters. Intracellular adenosine is produced within the cytosol during periods of depleted cellular energy charge. The adenosine so formed leaves the cell via the nucleoside transporter.

Abbreviations: ADO, adenosine; NA, noradrenaline; ACh, acetylcholine; 5-HT, 5-hydroxytryptamine; pm, plasma membrane; 5'-N, 5'-nucleotidase; N, nucleoside transporter.

variety of physiological and behaviourial effects. Many of these can be attributed to its inhibitory action and seems to arise from its ability to suppress spontaneous neuronal firing. This results in the inhibition of both synaptic transmission and excitatory neurotransmitter release (Phillis & Kostopoulos, 1975; Harms *et al.*, 1979; Fredholme & Hedquist, 1980; Hollins & Stone, 1980; Fredholme & Dunwiddie, 1988). These events appears to be mediated by adenosine A1 receptors and probably result from either a direct blockage of presynaptic N-type calcium channels and/or indirectly by opening presynaptic potassium channels (Michaelis *et al.*, 1988; Gross *et al.*, 1989) resulting in hyperpolarization of the membrane.

Adenosine acts as a central depressant (Williams, 1984) eliciting many responses including inhibition of breathing, reduction of heart rate, lowering blood pressure and even acting as a sedative (Dunwiddie & Worth, 1982; Mendelson *et al.*, 1983; Radulovacki *et al.*, 1983, 1984; Snyder, 1985). In fact studies performed by Radulovacki *et al.* (1982) demonstrated that adenosine A1 receptor agonists induced sedation which closely resembled that of natural sleep. Adenosine also shows anti-seizure properties (Snyder, 1985) which has lead to the belief that it may possibly even function as an endogenous anticonvulsant (Dragunow *et al.*, 1985; Dragunow & Robertson, 1987; Dragunow & Faull, 1988; Dragunow, 1988). Its involvement in pain suppression has been postulated (De Lander & Hopkins, 1987; Salter & Henry, 1987; Sawynok *et al.*, 1989), possibly by mediating some of

the effects of analgesics. Exogenously applied opiates are capable of inducing a release of adenosine from brain slices, synaptosomes and even the cortical surface *in vivo.* In fact they are able to decrease neuronal firing which to some extent can be prevented by xanthines again suggesting adenosine's involvement.

Another major physiological event mediated by adenosine is that of its role in the local regulation of blood flow. In most vascular beds, adenosine acts as a vasodilator (Berne, *et al.*, 1981) by means of its actions on vascular adenosine A2 receptors. The original hypothesis proposed by Berne (1964) that intracellular adenosine levels in the heart rise when the oxygen supply is insufficient to meet its demand (as in the case of ischaemia and hypoxia) has also been confirmed in the brain (Berne *et al.*, 1974; Rubio *et al.*, 1975; Winn *et al.*, 1980, 1981; Zetterstrom *et al.*, 1982; Van Wylen *et al.*, 1986; White & Hoehn, 1991). In fact any situation which leads to a reduction in the energy charge of the cell results in the intracellular production of adenosine as a net result of ATP catabolism (Atkinson, 1968; Itoh, 1981b; Itoh *et al.*, 1986; Worku & Newby, 1983). Since the adenylate kinase reaction is essentially an equilibrium reaction, a small depletion of cytosolic ATP would result in a large increase in AMP. Consequently adenosine would be produced as a result of the AMP hydrolysis catalysed by 5'-nucleotidase. The adenosine so formed is then able to leave the cell by the nucleoside transporter and exert its vasodilatory action on the surrounding vasculature. This

would increase the local blood flow and attempt to restore the homeostasis of the tissue. Extracellular ATP is also capable of inducing vasodilation by its action on purinergic P2 receptors. This effect is however indirect and slower than the adenosine response. This can be reasoned since it is mediated by prostacyclin and endothelium-derived relaxing factor released by the action of ATP on endothelial P2Y receptors (Olsson & Pearson, 1990).

1.9.1 EXTRACELLULAR PRODUCTION OF ADENOSINE

The direct production of adenosine within the extracellular space functions not only to produce the nucleoside for its role in neuromodulation, but also to prevent excess purine loss from occurring from the cell. Extracellular ATP can arise due to leakage from broken cells, but more importantly as a result of exocytotic release. The hypothesised purinergic neurones (Burnstock, 1972; 1976) where ATP is the sole transmitter could provide one possible route of extracellular adenine nucleotides, although this has not been conclusively proven. More recently, the direct release of adenosine from postulated adenosinergic neurones has been suggested (Meghji, 1991). Purines can be released in a calcium dependant manner from hippocampal slices (Jonzon & Fredholm, 1985) or from synaptosomes isolated from the cortex (Kuroda & McIlwain, 1974) thus supporting this idea. However release can also occur independently of calcium (Jonzon & Fredholm, 1985) and probably represents adenosine leaving the cell by the nucleoside transporter rather than by

electrically stimulated exocytotic release. ATP does however function as a co-transmitter and consequently fulfils the necessary criteria required to be classed as such (Phillis & Wu, 1981; White, 1988). Firstly, it is both synthesised in the nerve cell and stored within synaptic vesicles (Lagercrantz, 1976; Morel & Meunier, 1981). Upon stimulation it is released in a calcium dependant manner (Silinsky, 1975; Richardson & Brown, 1987; Richardson *et al.*, 1987) along with transmitter substances such as acetylcholine (Zimmerman & Whittaker, 1974; Nagy *et al.*, 1976; Giompres *et al.*, 1981; Richardson & Brown, 1987), noradrenaline (Geffen & Livett, 1971; Lagercrantz, 1971; Westfall *et al.*, 1990) and 5-hydroxytryptamine (Da Prada & Pletscher, 1968). In fact using immunoaffinity purified cholinergic terminals it has been shown conclusively that ATP is co-released with acetylcholine (Richardson & Brown, 1987; Richardson *et al.*, 1987). However, there appear to be different kinetics of release (Stone, 1981) and the ratios of the two vary with respect to the method of stimulation (Morel & Meunier, 1981). These results suggest that although co-release occurs, they are probably released by different mechanisms. Finally there is an effective mechanism for inactivating the released nucleotide by means of its rapid metabolism followed by high affinity uptake processes. Extracellular ATP is completely degraded to adenosine by a purine nucleotide phosphohydrolyse pathway (Pearson *et al.*, 1980; Maire *et al.*, 1984; Grondal & Zimmerman, 1986; 1988; Nagy, 1986; Richardson & Brown, 1987; Richardson *et al.*, 1987; Terrian *et al.*, 1989; Torres

et al., 1990). The ectonucleotidases involved are located on the postsynaptic membrane, with a majority of ecto 5'-nucleotidase on glial cells and myelin (Kreutzberg & Barron, 1978; Kreutzberg *et al.*, 1978; 1986; Heyman *et al.*, 1984; Cammer *et al.*, 1980). All the enzymes have high affinities for their substrates and have been studied independently in immunoaffinity purified striatal cholinergic terminal preparations (James & Richardson, 1993) and in cultured astrocytes (Lai & Wong, 1991a). ATP in the synaptic cleft which can reach millimolar concentrations (Richardson & Brown, 1987; Richardson *et al.*, 1987), becomes rapidly metabolised to AMP, resulting in its accumulation (Richardson & Brown, 1987). This indicates that the 5'-nucleotidase reaction is probably the rate limiting step in adenosine production. This observation seems reasonable since ATP and ADP inhibit ecto 5'-nucleotidase, which would only become active once the nucleotides were degraded. Although Richardson (1983) concluded that no ecto 5'-nucleotidase was present on pre-synaptic cholinergic terminals, and cytochemical studies indicated that most is located on glia (Kreutzberg *et al.*, 1978; 1986), later work (James & Richardson, 1993) have studied the complete extracellular pathway including that of ecto 5'-nucleotidase on the purified striatal cholinergic synapse. Schoen *et al.* (1988) however reported that anti 5'-nucleotidase antibodies recognised the enzyme of glial origin and never that population located on neurones. Whether structural differences exist or its tight packing on neurones hinders

antibody recognition remains to be proven.

The consequence of extracellular adenosine production produced by this pathway is to inhibit the release of further neurotransmitter such as acetylcholine, noradrenaline, dopamine and 5-hydroxytryptamine which has been demonstrated in rat brain preparations (Harms *et al.*, 1979; Fredholm & Dunwiddie, 1988). Although ATP can exert its own effect by means of its action on P2 purinergic receptors, the inhibition of transmitter release is not attributed to the action of this nucleotide, but to that of adenosine. Richardson *et al.* (1987) using cholinergic terminals from the striatum demonstrated that incubation with 1mM ATP resulted in its rapid metabolism, causing a 36% inhibition of acetylcholine release. Using the non-hydrolysable compound α,β-methylene ADP, no effect was observed since adenosine could not be produced from this compound. Furthermore, the inhibitory action resulting from ATP incubation with these terminals could be blocked by adenosine deaminase, by pretreatment with anti-ecto 5'-nucleotidase antiserum or by direct blockage of the adenosine receptor by theophylline. Conversely, dipyridamole potentiated the effect by blocking adenosine uptake. From recent evidence (Richardson & James, 1991; Brown *et al.*, 1990) it appears that the inhibitory action of adenosine on acetylcholine release is mediated by the adenosine A1 receptor sub-type. There do however appear to be variations in the pathway amongst brain regions in that the inhibitory effect is seen in cholinergic terminals from the striatum but not those isolated from the cortex.

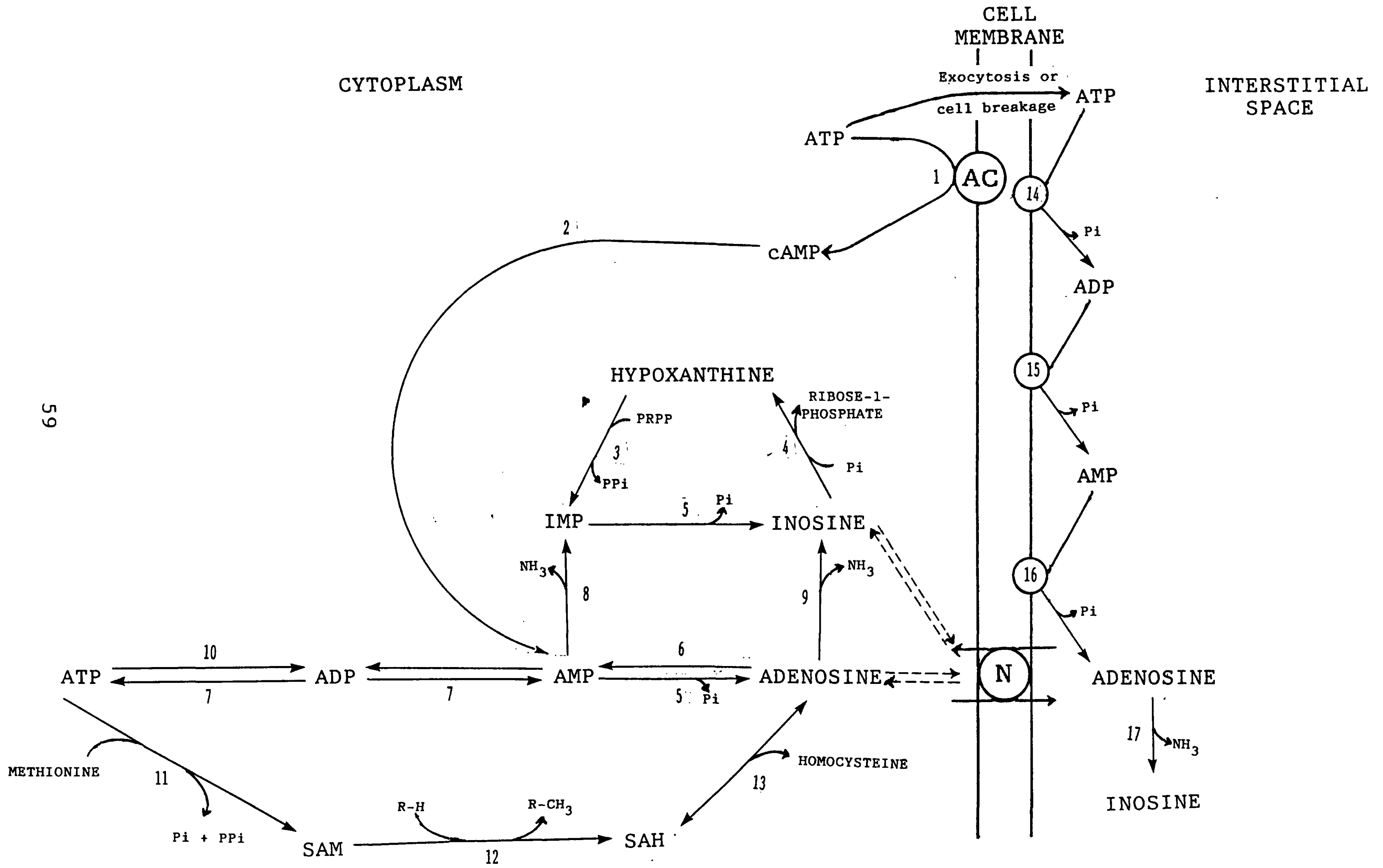
Cortical cholinergic neurones appear to lack the complete ectonucleotidase pathway as well as presynaptic adenosine receptors (Richardson *et al.*, 1987). In the cortex, acetylcholine appears to modulate its own release in contrast to an adenosine mediated process (Marchi *et al.*, 1983).

1.9.2 INTRACELLULAR PRODUCTION OF ADENOSINE

Adenosine can be produced by two main ways in the cytosol of the cell (figure 1.5). Firstly by the hydrolysis of S-adenosyl-L-homocysteine (SAH) which is produced from S-adenosyl-L-methionine in the course of transmethylation reactions. The enzyme S-adenosyl-L-homocysteine hydrolase has been shown to produce a large proportion of adenosine in the well oxygenated heart (Lloyd *et al.*, 1988) although no such studies have been carried out in brain tissue. However, adenosine produced by this route appears to be oxygen insensitive in that the flux through the pathway is not accelerated during hypoxia and ischaemia. In these cases adenosine is produced from AMP hydrolysis as a net result of ATP catabolism. Moreover, the SAH hydrolase enzyme appears to favour the synthesis of SAH rather than its hydrolysis (Fox & Kelly, 1978; Schrader, 1983) and thus may be more important in adenosine's removal rather than in its production. However due to further metabolism of the products of the reaction, SAH hydrolysis predominates over synthesis and may provide an important basal level of adenosine under normoxic conditions. Using rat hippocampal

Figure 1.5 Pathways of adenosine metabolism

1. Adenylate cyclase (EC 4.6.1.1)
2. cAMP phosphodiesterase (3.1.4.17)
3. Hypoxanthine phosphoribosyl transferase (EC 2.4.2.8)
4. Purine nucleoside phosphorylase (EC 2.4.2.1)
5. 5'-nucleotidase (EC 3.1.3.5)
6. Adenosine kinase (EC 2.7.1.20)
7. Adenylate kinase (EC 2.7.4.3)
8. AMP deaminase (EC 3.5.4.6)
9. Adenosine deaminase (EC 3.5.4.4)
10. ATP utilizing reactions
11. ATP; L-methionine adenosyltransferase (EC 2.5.1.6)
12. S-adenosyl-L-methionine: R methyltransferase (EC 2.1.1)
13. S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1)
14. Ecto ATPase (EC 3.6.1.3) and extracellular kinases
15. Ecto ADPase (EC 3.6.1.6)
16. Ecto 5'-nucleotidase (EC 3.1.3.5)
17. Exo adenosine deaminase (EC 3.5.4.4)


CELL
MEMBRANE
CYTOPLASM
INTERSTITIAL
SPACE
Exocytosis or
cell breakage
ATP
ATP
AC
1
14
Pi
cAMP
2
ADP
15
Pi
HYPOXANTHINE
RIBOSE-1-
PHOSPHATE
PRPP
3
4
Pi
PPi
AMP
IMP
5
Pi
INOSINE
16
Pi
NH3
8
9
NH3
10
6
ATP
ADP
AMP
ADENOSINE
N
ADENOSINE
7
7
5
Pi
17
NH3
METHIONINE
11
13
HOMOCYSTEINE
INOSINE
Pi + PPi
R-H
R-CH3
SAM
12
SAH

slices, Reddington & Pusch (1983) observed that adenosine was predominantly recycled back into the nucleotide pool rather than directed into SAH synthesis. This observation however can be explained by the limited availability of L-homocysteine to the pathway in that addition of this amino acid accelerated SAH production (Schrader, 1983; McIlwain & Poll, 1986).

The more physiologically relevant production of adenosine comes from that of AMP hydrolysis by the action of 5'-nucleotidase. This family of enzymes will be discussed in detail in section 1.10 due to their major importance in the production of adenosine. Although many non-specific phosphatases are also present in the cell, their activities are very low at physiological pH and are thus not quantitatively important. Intracellular AMP hydrolysis appears to be directly linked to the energy status of the cell and the activity of 5'-nucleotidase also responds accordingly. To date at least four forms of 5'-nucleotidase are known to exist, three soluble forms and a membrane bound ectoenzyme. The ectoenzyme is unable to use cytosolic AMP, it is entirely concerned with extracellular adenosine production. This results from released nucleotides and thus can be seen as a direct link to neurotransmission. The cytosolic forms however directly link adenosine production to the energy status of the cell in that a reduction in energy status initiates adenosine production which consequently exits the cell to elicit its physiological responses via the extracellular receptors. In fact studies

performed on cultured heart cells in the presence of adenosine transport inhibitors conclusively show that intracellular adenosine accumulates under these conditions (Meghji *et al.*, 1985, 1988a,b; Altschuld *et al.*, 1987).

1.9.3 ADENOSINE DEACTIVATION AND UPTAKE

Adenosine is removed primarily from the extracellular space by a high affinity uptake process. This functions not only to terminate the physiological effects, but also to salvage the nucleoside hence preventing excess purine loss from the cell. Uptake is mediated by a bidirectional nucleoside carrier protein in the membrane, the rate of which is regulated by the adenosine concentration gradient. In addition, transport across the membrane can also occur by simple diffusion as demonstrated by its non-saturability, lack of competition by other substrates for the carrier or by lack of blockade by nucleoside transporter inhibitors (Clanachan *et al.*, 1987). The importance of uptake can be reinforced by observations that inhibitors of the transporter by agents such as nitrobenzylthioinosine or dipyridamole produce effects common to those of exogenously applied adenosine (Meghji, 1991).

Following uptake, adenosine is either phosphorylated by adenosine kinase and recycled back into the adenine nucleotide pool or deaminated by adenosine deaminase. Adenosine kinase exhibits a Km for adenosine in the range <0.2-10μM whilst that of the deaminase between 7 and 57μM (Pull & McIlwain, 1974; Arch & Newsholme, 1978; Phillips &

Newsholme, 1979; Yamada *et al.*, 1980; Brosh *et al.*, 1990). The route taken however depends on the cytosolic adenosine concentration which in turn will be governed by the rate of its formation and transport processes. The kinase reaction is more prevalent at low adenosine concentrations and proportionately decreases with further increase in the concentration of this nucleoside (Fisher & Newsholme, 1984). At adenosine concentrations up to 17µM the phosphorylation reaction appears to predominate in a variety of neuronal preparations (Barberis *et al.*, 1981; Reddington & Pusch, 1983; Wolinsky & Patterson, 1985). Taking into account the low Km of the kinase in conjunction with reduced rates of phosphorylation at higher adenosine concentrations it appears likely that under these conditions deamination is probably a major means of its removal. However the importance of adenosine deaminase in terminating physiological effects of the nucleoside have been demonstrated by Radulovacki *et al.* (1983) by showing that inhibition of the enzyme by deoxycoformycin produced adenosine-like effects. The deactivation processes so far discussed occur within the cytosol following uptake. However there is also evidence that a population of adenosine deaminase is also localized in the extracellular compartment (Andy & Kornfeld, 1982; Hellewell & Pearson, 1983; Maire *et al.*, 1984; Meghji *et al.*, 1988). This may be free in solution or possibly attached to the membrane by an adenosine deaminase binding protein (Andy & Kornfeld, 1982; Schrader *et al.*, 1983,1984; Franco *et al.*, 1986). The presence of an exo adenosine deaminase would

permit adenosine to become deactivated without prior uptake. The inosine so formed could be salvaged by uptake through the same nucleoside transporter.

1.10 5'-NUCLEOTIDASE

5'-nucleotidase (EC 3.1.3.5) catalyses the dephosphorylation of purine and pyrimidine nucleoside 5'-monophosphates to their corresponding nucleosides. There appear to be at least four distinct forms based on their subcellular distribution, substrate preferences and modulation by adenine nucleotides (reviewed by Zimmerman, 1992). One form is anchored to the plasma membrane and the three remaining forms are soluble. Two of the soluble activities appear to be truly cytosolic enzymes. They are similar in many respects but differ in their substrate preferences, modes of regulation and probably perform quite different roles within the cell. In addition, another soluble form appears to exist with properties similar to that of the ectoenzyme. It is unclear however whether this form is a truly cytosolic enzyme, whether it originates from the extracellular compartment or is only present as a consequence of cleavage of the ectoenzyme from either the plasma membrane or from within an intracellular pool. Table 1.2 shows a summary of the chemical and physical properties of the different forms known to date, each of which will be discussed separately.

Table 1.2 Comparison of the various forms of 5'-nucleotidase

	Plasma membrane ectoenzyme	High Km AMP-specific cytosolic enzyme	Low Km AMP-specific soluble enzyme	IMP-specific cytosolic enzyme
Preferred substrate	AMP	AMP	AMP	IMP
Km	5-40μM	1-5mM	7-20μM	0.5-1.4mM
Subunit size	60-80kDa	40kDa	60-80kDa	51-58kDa
Structure	Homodimer	Homotetramer	Homodimer or tetramer	Homotetramer
pH optimum	7.0-8.0	6.5-7.0	7.0-8.0	6.5
Magnesium ions	Not required	Required	Not required	Required
Phosphate	No inhibition	Inhibition	No inhibition	Inhibition
ATP	Ki 1.5-4.4μM	No effect	Ki 60-100μM	Activates in mM range with ATP>ADP
ADP	Ki 0.08μM	Activates in the mM range	Ki 2-15μM	
α,β-methylene ADP	Potent inhibition	No inhibition	Potent inhibition	No inhibition
Anti-ectoenzyme antibody	Potent inhibition	No inhibition	Potent inhibition	No inhibition

Km values quoted are those for the preferred substrate. For reference see text.

1.10.1 ECTO 5'-NUCLEOTIDASE

The most abundant and widely studied form of 5'-nucleotidase is the membrane bound ectoenzyme. It is attached to the plasma membrane by means of a phosphatidylinositol glycan (PIG) linkage at its C-terminus. Evidence for such a membrane attachment comes not only from the fact that detergent solubilized purified enzyme contains equimolar amounts of myo-inositol (Bailyes *et al.*, 1990; Klemens *et al.*, 1990), but more importantly due to its susceptibility to partial cleavage from the plasma membrane by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) (see Zimmerman, 1992). The physiological relevance of this type of membrane linkage however is not yet fully understood, but may be responsible for facilitating release of the enzyme by endogenous PIPLC (Fox *et al.*, 1987; Davitz *et al.*, 1989). Moreover, Klip *et al.* (1988) have further supported this idea by suggesting that cleavage, at least from skeletal muscle membranes, may be mediated by an endogenous insulin-activated PIPLC. The role of the anchor has also been postulated to be involved in the tight packing of membrane proteins within the plasma membrane. This in part offers one possible explanation as to the reason why only partial release from the membrane by PIPLC is seen (presumably due to the steric hindrance of the lipase action by neighbouring proteins), whilst the enzyme purified following detergent solubilization and reconstituted into phospholipid vesicles can be totally cleaved (Stochaj *et al.*, 1989). This is not however conclusive evidence and alternative suggestions have been

made as to a population of the ectoenzyme possessing either a chemically modified anchor (Rosenberry *et al.*, 1990) or as being embedded in the membrane by transmembrane segments (Zachowski *et al.*, 1981; Dieckhoff *et al.*, 1984; Klemens *et al.*, 1990).

Ecto 5'-nucleotidase activity is widely distributed throughout the body, but has an uneven distribution within the brain (Hess & Hess, 1986; Eisenman & Hawkes, 1989; Mazurkiewicz & Saggerson, 1989b). In the peripheral nervous system it is associated with Schwaan cells and myelin (Grondal *et al.*, 1988), and in the central nervous system mainly at the surface of Glia (Schoen *et al.*, 1987), myelin (Cammer *et al.*, 1980) as well as neuronal surfaces for example cholinergic nerve terminals from rat striatum (Richardson *et al.*, 1987).

Since the active site protrudes to the outside of the cell, it functions to dephosphorylate extracellular 5'-AMP to adenosine (Stanley *et al.*, 1980; Baron *et al.*, 1986; Bailyes *et al.*, 1990). In nervous tissue this is of particular importance since ATP which is co-released with excitatory neurotransmitters (Burnstock, 1986) or released directly from the hypothesised purinergic neurones (Burnstock, 1972) must be degraded to adenosine prior to its re-uptake (and hence prevent excessive purine loss from the cell), or before it can elicit any of its extracellular physiological effects. Implications have also been made about the role of the ectoenzyme in cell-cell interactions and perhaps even transmembrane signalling. The ectoenzyme

from some neuronal sources carries the HNK-1 sugar epitope which is common with other surface located proteins involved in cell-cell and cell-matrix interactions (Vogel *et al.*, 1991a,b). In addition the two extracellular matrix glycoproteins laminin and fibronectin are also capable of interacting with and modulating the function of the ectoenzyme (Dieckhoff *et al.*, 1986; Olmo *et al.*, 1992). It is the ability of these extracellular proteins to interact with cellular receptors and consequently inducing changes in the behaviour of the cell which provokes the exciting idea that ecto 5'-nucleotidase may additionally function as a receptor for these proteins as well as functioning as an enzyme.

Ecto 5'-nucleotidase exclusively hydrolyses nucleoside 5'-monophosphates, shows no activity towards their 2' and 3' counterparts, and has little or no activity towards 2-deoxyribose compounds. AMP is the preferred substrate with a Km in the low micromolar region (typically 5-40μM), and shows a broad pH optimum in the range pH 7-8. ADP and ATP are potent competitive inhibitors with Ki values in the low micromolar range, and that of their non-hydrolysable α,β-methylene derivatives in the nanomolar range. Considering these properties, even if the ectoenzyme was able to use cytosolic AMP, it would be inhibited by cytosolic nucleotides eg. 5mM ATP under normoxic conditions. Moreover, at times when intracellular adenosine is being produced as in the case of ischaemia or hypoxia, the enzyme would be more inhibited due to the rise in cytosolic ADP. In contrast with certain

soluble forms of 5'-nucleotidase, no inhibition is seen with phosphate, and magnesium ions are not required for enzyme activity, but do inhibit however in the millimolar range.

Ecto 5'-nucleotidase has been purified from several sources and its molecular and biochemical properties studied (see Zimmerman, 1992 for review). The subunit size falls within the range 60-80 kDa as judged by SDS-PAGE, again depending on the source and seems to exist as a dimer in its native conformation linked by interchain disulphide bridges. In fact these bridges are essential to enzyme activity and thiol reagents have been shown to inactivate the enzyme (Worku *et al.*, 1984; Fini *et al.*, 1985). More recently, ecto 5'-nucleotidase has been sequenced and cloned from rat liver, human placenta and the brain of the electric ray (Misumi *et al.*, 1990a,b; Volknandt *et al.*, 1991). From their cDNA sequences, several deductions have been made about the mature enzyme. All these enzymes are expected to consist of 548 amino acids giving a non-glycosylated subunit weight of 61 kDa. The C-terminus contains a stretch of charged and hydrophobic amino acids which during processing are presumably cleaved and exchanged for the PIG which is attached at Ser 523 (Ogata *et al.*, 1990; Misumi *et al.*, 1990). At the amino acid level, the liver and placental enzymes exhibit nearly 90% identity, whereas the electric ray enzyme shows only 61% identity with either of the mammalian forms. However, before any real conclusions can be made about the species variation of the enzyme, more sequence information from other sources is necessary. Fini *et al.*

(1990) demonstrated that ecto 5'-nucleotidase purified from chicken gizzard is a zinc-containing metalloprotein having two moles of zinc ions bound per mole of protein. Interestingly, the electric ray enzyme, although not directly shown to contain zinc, contains the necessary cysteine residues to provide potential zinc binding sites. By the use of group-specific reagents and inactivation studies, Worku *et al.* (1984) suggested that the enzyme belongs to a group of histidine phosphatases.

Predictions made from the cDNA sequences indicate that the human placental and electric ray enzymes are expected to possess four N-linked glycosylation sites and that of the rat liver to contain five. This agrees favourably with previous estimates from analysis of the purified enzymes of five potential sites for the liver enzyme (Van den Bosch *et al.*, 1986; Wada *et al.*, 1986) and four sites from human chorionic cells (Burgemeister *et al.*, 1990). As yet, no O-linked sites have been implicated. The oligosaccharide component of the rat liver enzyme appears to be a processed form of the high mannose or the complex type glycosylation (Wada *et al.*, 1986; Baron & Luzio, 1987). In contrast, Van den Bosch et al. (1986) indicated that a population of the enzyme isolated from a rat hepatoma cell line retains one or two unprocessed carbohydrate chains. The mature ectoenzyme contains sialic acid residues (Harb *et al.*, 1983; Wada *et al.*, 1986, 1987; Van den Bosch *et al.*, 1988; Buschette-Brambrink & Gutensohn, 1989; Vogel *et al.*, 1991b). In fact due to different sialic acid contents, up to 13 isoforms have been shown to exist by

the use of 2D electrophoresis. This high number of isoforms can however be dramatically reduced by pretreatment with neuraminidase (Wada *et al.*, 1987; Buschette-Brambrink & Gutensohn, 1989; Vogel *et al.*, 1991b).

Although ecto 5'-nucleotidase is located on the plasma membrane, up to 50% of the enzyme can reside in membrane-bound pools within the cell. These include the endocytic and the lysosomal pools (Maguire & Luzio, 1985; Wada *et al.*, 1987; Tanaka *et al.*, 1989) and also transcytotic vesicles (Mullock *et al.*, 1983). In fact studies of cultured hepatocytes (Stanley *et al.*, 1980), fibroblasts (Widnell *et al.*, 1982) or rat hepatoma cells (Van den Bosch *et al.*, 1988) indicate that ecto 5'-nucleotidase is in constant cycling between the cell surface and the intracellular pool. It is not certain however whether this phenomenon is only observed in cultured cells. 5'-nucleotidase located in lysosomes seems to be the same as the ectoenzyme. A large proportion is bound to the lysosomal membrane (75%) and the remaining 25% within the lumen (Maguire & Luzio, 1985; Wada *et al.*, 1987; Tanaka *et al.*, 1989). Whereas the population in the lysosomal membrane can be largely cleaved by PIPLC, the soluble form has been shown by phase separation experiments to lack the PIG, raising questions again as to the presence of an endogenous PIPLC even within the lysosome (Zimmerman, 1992).

1.10.2 IMP-SPECIFIC SOLUBLE ENZYME

The existence of an IMP-specific cytosolic 5'-nucleotidase has

been appreciated for many years. It shows a wide tissue distribution and consequently has been characterized from a variety of sources. Within the nervous system, it has been detected and studied in bovine (Montero & Fes, 1982; Mallol & Bozal, 1983), pig (Itoh & Yamada, 1991) and rat brain (Newby *et al.*, 1987; Bontemps *et al.*, 1989; Itoh & Yamada, 1991), although the bovine enzyme appears somewhat atypical and probably represents the soluble low Km enzyme (see section 1.10.4).

Generally, purified preparations appear as a single band of 51-58 kDa following SDS-PAGE and a 205-210 kDa peak upon gel filtration (Naito & Tsushima, 1976; Spychala *et al.*, 1988; Spychala *et al.*, 1989; Tsushima, 1986). A larger variant has however been recently reported in a pig lung preparation (Itoh & Yamada, 1990). In all cases a homo-oligomeric structure has been assumed, being comprised of four identical subunits.

The enzyme is capable of dephosphorylating a number of purine and pyrimidine 5'-monophosphates but shows some 30-fold preference for IMP over AMP. When using IMP, hyperbolic kinetics are displayed with a Km in the range 0.5-1.4mM. Conversely when using AMP, a sigmoidal plot is obtained (h=1.5-1.8) with an $S_{0.5}$ value ranging from 10 to 30mM (Itoh, 1981a; Itoh & Yamada, 1990; Tsushima, 1986: Itoh & Oka, 1985; Itoh *et al.*, 1986). However, a slight degree of sigmoidicity even with IMP as substrate has been reported by Van den Berghe *et al.* (1977) using rat liver cytosol which was further confirmed by Worku and Newby (1982).

The enzyme is activated by high ionic strength, ADP and

more potently, by ATP. When using IMP, activation is accomplished by increasing the maximal velocity whilst reducing the Km (0.08-0.2mM). A more striking observation however is seen with AMP as substrate in that activators reduce the sigmoidicity of the plot without altering the Vmax. In fact once fully activated, hyperbolic kinetics are obeyed showing a Km in the 4-14mM region (Itoh, 1981a; Itoh & Yamada, 1990; Tsushima, 1986; Itoh & Oka, 1985; Itoh *et al.*, 1986). In addition, stimulation of the IMP-specific enzyme has also been demonstrated in a variety of tissues with glycerate 2,3-bisphosphate (Bontemps *et al.*, 1988; Bontemps *et al.*, 1989; Itoh & Yamada, 1990; Tozzi *et al.*, 1991) as well as with diadenosine polyphosphates (Pinto *et al.*, 1986; Itoh & Yamada, 1990). Millimolar levels of phosphate are inhibitory (independent of substrate used) and the same authors showed this effect to be due to an increase in the sigmoidicity of the kinetics as shown by an increase in the Hill coefficient to a value of greater than 2.

In contrast to the surface-located ectoenzyme, the cytosolic IMP-specific enzyme shows an absolute requirement for magnesium ions, a pH optimum of 6.5 and lack of inhibition by anti-ecto-5'-nucleotidase antibodies (Worku & Newby, 1983). In addition, α,β-methylene ADP fails to inhibit the cytosolic activity. In fact activation by this compound has been observed in a preparation of the enzyme from pig lung (Itoh & Yamada, 1990).

In uricotelic animals, the enzyme appears responsible for catalysing the first step in uric acid formation from IMP, an

important function in the elimination of nitrogen from proteins and amino acids (Tsushima, 1986). In addition, it is likely to contribute to a protective role following a metabolic shock to the cell at least in some tissues, and has been shown to become activated following an imbalance in the energy charge of the cell (Itoh *et al.*, 1986; Worku & Newby, 1983; Itoh *et al.*, 1986). Worku & Newby (1983) further concluded that its activity was sufficient to account for adenosine production in rat polymorphonuclear leucocytes, a cell type which lacks the AMP-specific enzyme. Moreover the rate of adenosine formation could be decreased by inhibiting the enzyme with 5'-deoxy-5'-isobutylthio derivatives of adenosine and inosine (Skladanowski *et al.*, 1989). In rat cardiac tissue the activity has been reported insufficient alone to account for ischaemia induced adenosine production (Meghji *et al.*, 1988a) as with the ischaemic brain (Newby *et al.*, 1987). It is known however that rat heart also contains the AMP-specific cytosolic enzyme and both are likely to contribute to adenosine production under these conditions but to different extents. ATP levels in normal as well as in hypoxic or mildly ischaemic cells are expected to lie within the millimolar range, concentrations of which are more than sufficient to maintain the enzyme in its more active state. Under these conditions therefore it would respond to increases in AMP concentration in a proportional manner up into the millimolar range. However due to its high preference for IMP as substrate, the presence of IMP would effectively inhibit AMP hydrolysis. Moreover, during metabolic insults it would be expected to become more inhibited

due mainly to a rise in phosphate levels (Itoh, 1981b; Itoh *et al.*, 1986). Nevertheless, even in the presence of relatively high levels of phosphate the activity was still higher at a low energy charge than that of the control level. It is probably more likely under physiological conditions therefore that a change in AMP concentration is the more important regulating factor since a drop in energy charge is accompanied by an increase in cytosolic AMP and a much smaller drop in the total ADP plus ATP concentrations. Although it seems likely therefore to contribute to adenosine production under ischaemic conditions, it is more likely that in combination with AMP-deaminase (which is modulated in a similar fashion with respect to phosphate and ATP) the less biologically active product inosine would be formed during ATP catabolism and thus reduce excessive physiological effects caused by adenosine.

1.10.3 HIGH Km AMP-SPECIFIC SOLUBLE 5'-NUCLEOTIDASE

To date this variety of soluble 5'-nucleotidase only appears to have been studied in the hearts of the pigeon (Gibson & Drummond, 1972; Newby, 1988; Skladanowski & Newby, 1990), rabbit (Collinson *et al.*, 1987; Yamazaki *et al.*, 1991) and the rat (Tuoung *et al.*, 1988). Previous lack of detection may however be attributed to the labile nature of the enzyme in the absence of relatively large amounts of glycerol. Purification to homogeneity has been accomplished for the rabbit heart enzyme recently by Yamazaki and co-workers (1991) who additionally demonstrated its mobility on SDS-PAGE gels as a 40 kDa polypeptide. Estimates of native molecular weight by

gel filtration yielded values of 150 kDa for the partially purified pigeon enzyme (Skladanowski & Newby, 1990), again suggesting a tetrameric structure. Initial sequence data shows no homology with that of the ectoenzyme (see Zimmerman, 1992) and anti ecto 5'-nucleotide antibodies were ineffective at inhibiting this activity (Collinson *et al.*, 1987) indicating distinct species.

The general properties appear similar to that of the IMP-specific enzyme. It is capable of hydrolysing a wide range of nucleoside 5'-monophosphates but shows some 15-20 fold more activity towards AMP than IMP and displays a Km for AMP in the 1-5mM range. In the absence of activators however, sigmoidal kinetics are observed for AMP and IMP which upon activation change to hyperbolic. Unlike the IMP-specific variety, ATP is unable to cause activation; but whereas ADP stimulates in a similar manner by reducing the sigmoidal nature of the plot, it additionally increases the maximal velocity. No inhibition is observed with α,β-methylene ADP (Newby *et al.*, 1987; Newby, 1988; Skladanowski & Newby, 1990) and little or none with the 5'-deoxy-5'-isobutylthio derivatives of adenosine and inosine (Newby, 1988) thus providing further evidence for its distinct nature from other 5'-nucleotidases. Rat heart additionally contains the IMP-specific enzyme and the two can be separated on columns of phosphocellulose (Truong *et al.*, 1988; Newby, 1988). In common with the IMP-specific enzyme, an acidic pH optimum is observed, magnesium ions are required for activity, and phosphate inhibits although less effectively (Itoh *et al.*, 1986; Newby, 1988; Truong *et al.*, 1988). High magnesium ion

concentration as well as high ionic strength have been reported to be inhibitory (Skladanowski & Newby, 1990). The kinetic properties suggest that during ATP catabolism the enzyme would be activated to a greater extent than the IMP-specific form. Activation would then enable it to generate an increase in the rate of adenosine formation in response to an increase in cytosolic AMP. In pigeon heart which is almost completely devoid of ecto 5'-nucleotidase, adenosine production almost certainly would be mediated by this enzyme (Meghji *et al.*, 1988a; Newby, 1988). Assays under conditions which mimic those of ischaemic tissue, Newby (1988) concluded that it was the enzyme responsible for ischaemia-induced adenosine formation. In agreement, Yamazaki and colleagues (1991) reached the same conclusions, but further implied that ADP was probably the key regulator *in vivo*. With the knowledge of the lability of this enzyme, it remains to be seen whether its presence can be detected in brain or in tissues other than those active in adenosine formation.

One form of the enzyme has been purified from the post microsomal supernatant of bovine liver (Zekri *et al.*, 1988). General properties indicate that this soluble form is of the high Km AMP-specific type, although it shows other characteristics more common to the low Km enzyme. It migrated as two bands on SDS-PAGE showing molecular weights of 65 and 57kDa, and eluted as a 140kDa protein on gel filtration. The authors concluded a heterodimeric structure. Antibodies raised against the ectoenzyme were capable of inhibiting the enzyme, although only the 65kDa band cross reacted on a western blot.

General kinetics and modulation of activity by adenine nucleotides were however consistent with the high Km enzyme. In contrast, it showed affinity towards concanavalin A indicating a glycoprotein nature. Whereas wheat germ agglutinin inhibits the ectoenzyme (Harb *et al.*, 1983) it was ineffective towards this soluble type indicating differences in the structure of the carbohydrate chains.

1.10.4 LOW Km AMP-SPECIFIC SOLUBLE 5'-NUCLEOTIDASE

Recent studies have focused on another form of soluble 5'-nucleotidase which, as its name implies, shows a Km in the low micromolar range. It has been purified and studied from a variety of sources including human placenta (Madrid-Marina & Fox, 1986; Klemens *et al.*, 1990), rat liver (Spychala *et al.*, 1989; Fritzson *et al.*, 1986), rat and bovine brain (Fredholm & Lindgren, 1983; Orford *et al.*, 1991b; Lai & Wong, 1991b; Montero & Fes, 1982; Mallol & Bozal, 1982; Vogel *et al.*, 1992), rat kidney (Piec & Le Hir, 1991; Le Hir & Dubach, 1988), Torpedo electric organ (Vogel *et al.*, 1992) and cultured human T and B lymphoblasts (Spychala *et al.*, 1989). In fact the enzyme studied from snake venoms may also represent this form of soluble 5'-nucleotidase (Drummond & Yamamoto, 1971; Iwanaga & Suzuki, 1978). General properties are very similar to that of the ectoenzyme. It exhibits a Km in the low micromolar range (typically 7-20μM) for AMP, the preferred substrate, shows potent inhibition by ADP, ATP and α,β-methylene ADP as well as showing specific binding to the lectin concanavalin A. It appears to be a metalloprotein demonstrated indirectly by

its reversibility of EDTA inhibition by magnesium. In addition direct evidence for a tight association of zinc has been demonstrated for the seminal plasma enzyme (Fini *et al.*, 1990). In most cases, the soluble enzyme migrates with a similar subunit molecular weight as that of the ectoenzyme upon SDS-PAGE. The human placental enzyme can be totally deglycosylated by treatment with N-glycosidase F, revealing a core protein size of 56kDa. Estimates of native molecular weight judged by gel filtration chromatography indicate that the soluble enzyme can exists both as a tetramer or a dimer in contrast to the ectoenzyme which is exclusively dimeric (see Zimmerman, 1992). The soluble enzyme is strongly inhibited by ATP and ADP showing Ki values of 60-100μM and 2-15μM respectively (Madrid-Marina & Fox, 1986; Lai & Wong, 1991b). Whereas ADP inhibition is of the mixed type, ATP inhibition is complex. At low ATP concentrations (0-0.8mM) inhibition is of a competitive nature, whilst at higher concentrations (0.8-5mM) it appears to be of a mixed type. These results indicate that inhibition may occur at two sites, with low ATP concentrations being competitive at the catalytic site, and higher ATP concentrations causing inhibition at both the catalytic and a second distinct site (Le Hir & Dubach, 1988). With the kinetic characteristics thus shown and assuming a cytosolic location, it would be expected that the enzyme would probably be inactive under normal conditions. However during transient ischaemia ATP levels would reduce with a parallel increase in AMP, resulting in adenosine production, perhaps by this enzyme (Hagberg *et al.*, 1987). It co-exists with the high Km AMP-specific enzyme in

tissues such as rat liver, human placenta and cultured human T and B lymphoblasts but in varying proportions (Spychala *et al.*, 1989). With the presence of more than one soluble 5'-nucleotidase within the cell the regulation of adenosine production would be defined as a balance between the separate activities, each of which appear to be regulated in a different manner.

The fact that such strong similarities to the ectoenzyme exists, has prompted several comparative studies (Spychala *et al.*, 1989; Klemens *et al.*, 1990, Thompson, 1991; Lai & Wong, 1991b). Several lines of evidence indicate that this soluble enzyme may have originated from the ectoenzyme. Firstly, Piec & Le Hir (1991) using Triton X-114 phase partitioning experiments demonstrated that its hydrophobicity could be reduced by pre-treatment with PIPLC. Secondly, the human placental soluble enzyme contains the same amount of myo-inositol as the ectoenzyme (Klemens *et al.*, 1990). Thirdly, amino acid sequence and composition of the N-terminal regions of the soluble enzyme corresponds very closely to that of the ectoenzyme (Klemens *et al.*, 1990). In addition, antibodies raised against the ectoenzyme cross react with and inhibit the soluble form (Stochaj *et al.*, 1989; Piec & Le Hir, 1991; Vogel *et al.*, 1992; Thompson, 1991). In contrast to the ectoenzyme, the soluble form responds differently to treatment with glycosidases (Lai & Wong, 1991b). Evidence exists that many PIG anchored membrane proteins are also found in a soluble form, for example N-CAM, Thy 1 or the FC III protein of neutrophils (Low, 1990). One idea is that endogenous

phospholipase C (PLC) might release the ectoenzyme under normal physiological conditions or following homogenization of the excised tissue. Piec & Le Hir (1991) concluded that the soluble 5'-nucleotidase detected in the post microsomal supernatant of rat kidney represented cleaved ectoenzyme. Moreover, the amount recovered in this fraction was dependant on the number of homogenization strokes employed, an observation which was seen earlier by Fritzon *et al.* (1986). Generally, only low levels (1.5-6.8%) are recovered in this fraction (Fritzson *et al.*, 1986; Piec & Le Hir, 1991; Vogel *et al.*, 1992). However even after 15 strokes of the homogenizer pestle, only 6.8% was recovered in this fraction, an amount significantly lower than that seen in other tissues by other authors; 25% (Thompson *et al.*, 1987), 30% (Lai & Wong, 1991b) and even as much as 95% (Montero & Fes, 1982). The mechanical forces of homogenization may indeed increase the yield to some extent, but cannot explain the amounts seen in all cases. If the ancestry of this soluble form was from the plasma membrane, it would be likely that release would occur by means of endogenous phospholipases. This has been indicated by the use of an antibody which recognises the 1,2-(cyclic) monophosphate on the inositol ring (Hooper *et al.*, 1991), the chemical group so formed when PLC cleaves an intact PIG anchor. This antiserum cross reacts with the soluble enzyme, but not with detergent-solubilized ectoenzyme (Vogel *et al.*, 1992). Furthermore, mild acid treatment to hydrolyse the cyclic monophosphate diester bond eliminates this antibody cross reactivity without destroying recognition by the general anti

5'-nucleotidase antiserum. Although blood plasma contains phosphlipase D (Low & Prasad, 1988) which can also cause cleavage of the ectoenzyme, it is unlikely that this enzyme is responsible. Upon cleavage of the PIG anchor, PLD causes hydrolysis of the inositol phosphate bond and thus the inositol 1,2-(cyclic) monophosphate group is not formed. Consequently no reaction is observed with the antiserum to this epitope following this method of cleavage of the ectoenzyme (Hooper & Turner, 1989). It can be concluded therefore that if release is mediated by phospholipases, PLC would be responsible. It is also possible that a soluble pool of ectoenzyme exists, either within the cytosol, or within intracellular vesicles (Stanley *et al.*, 1980; Widnell *et al.*, 1982) and soluble membrane fragments can be produced during fractionation (Fritzson *et al.*, 1986; Meghji *et al.*, 1988). However, not all the soluble enzyme detected appears to have been PIG anchored and it is possible that constitutive secretion may occur rather than cleavage. If cleavage does occur *in vivo* it raises the question as to the physiological relevance of the process.

1.11 HYPO- AND HYPERTHYROIDISM

Thyroid hormones work on nearly every cell of the body and seem necessary for their appropriate functioning. Generally they stimulate the basal metabolic rate and are essential for normal maturation processes of the developing brain. Any perturbation in circulating thyroid hormone levels leads to the clinical conditions of hypo- and hyperthyroidism.

Hypothyroidism or myxoedema usually arises from an

autoimmunity whereby the immune system proceeds to destroy the cells of the thyroid gland. However an iodine deficiency also accounts for some cases especially in developing countries. Consequently abnormally low levels of thyroid hormones are observed in the circulation. In children, thyroid insufficiency can retard growth and lead to the onset of cretinism if left untreated. The hypothyroid patient has a lowered basal metabolic rate. This can lead to weight gain despite normal feeding, an enhanced susceptibility to overdose of medication and a general feeling of fatigue and weakness. In fact untreated myxoedema can lead to coma. Generally however a decrease in brain excitability is apparent resulting in an impaired memory, depression, anxiety, cerebellar ataxia and increased thresholds to light and sound. In severe cases these disruptions in mental processes can lead to dementia. Patients however respond well to thyroid hormone replacement therapy and live normal lives.

Hyperthyroidism or thyrotoxicosis results from an overactivity of the thyroid gland leading to an over secretion of thyroid hormones. This condition is especially prevalent in young females, and like hypothyroidism is associated with an immune defect. Antibodies known as thyroid-stimulating immunoglobulins (TSIGs) are produced which bind to and stimulate the TSH receptor thus increasing thyroid hormone output. Common symptoms are generally opposite to those seen in hypothyroidism. The basal metabolic rate is increased and the patient feels hot and looses weight. In many cases a goitre is present and protuberance of the eyes is apparent.

In addition these symptoms are associated with extreme nervousness, agitation, restlessness, anxiety, emotional instability and a poor concentration span. This condition can however be adequately treated by drug therapy or a partial thyroidectomy.

1.12 OBJECTIVES OF THE PROJECT

The objectives of this project were two fold. Firstly, to provide direct immunological evidence for the up-regulation of the pertussis toxin-sensitive G-proteins in the hypothyroid rat brain thus confirming the findings of Mazurkiewicz & Saggerson (1989b). Consequently, it was decided to look for changes in both receptor function and G-protein abundance in the hyperthyroid brain and correlate changes observed with those seen in hypothyroidism. Secondly to look for changes in soluble 5'-nucleotidase in 6 anatomically distinct regions of the hypothyroid rat brain and correlate these changes with those seen in the ectoenzyme as reported by Mazurkiewicz & Saggerson (1989b). During this study, a novel form of the soluble enzyme was detected. It was therefore necessary to purify this enzyme to homogeneity and characterize its kinetics and regulatory properties.

CHAPTER TWO

METHODS AND MATERIALS

2.1 MATERIALS

All reagents used were of the highest grade available and were purchased from; Sigma Chemical Company, Poole, Dorset, U.K. and BDH Ltd., Poole, Dorset, U.K.

6-n-propylthiouracil (PTU), triiodothyronine (T_3), Ficoll, fatty acid-poor bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), ascorbic acid, inosine-5'-monophosphate (IMP), guanosine-5'-monophosphate (GMP), cytidine-5'-monophosphate (CMP), uridine-5'-monophosphate (UMP), xanthosine-5'-monophosphate (XMP), Thymidine-5'-monophosphate (TMP), 2'-deoxyadenosine-5'-monophosphate (dAMP), 2'-deoxyinosine-5'-monophosphate (dIMP), 2'-deoxyuridine-5'-monophosphate (dUMP), 2'-deoxycytidine-5'-monophosphate (dCMP), 2'-deoxyguanosine-5'-monophosphate (dGMP), adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate, adenosine, inosine, SDS-PAGE prestained molecular weight markers, standard molecular weight markers for gel filtration, anti-sheep IgG (affinity purified), chloramine T, tyrosine, gelatin (60 bloom from bovine skin), Tween 20, 4-chloro-1-naphthol, silver nitrate, formaldehyde, protein A (attached to acrylic beads), ammonium borate, β-glycerophosphate, zinc sulphate, 0.3N barium hydroxide solution, NADH, 5,5' dithiobis-(2-nitrobenzoic acid) (DTNB), Triton X-100, lubrol PX, acetylthiocholine, methyl α-D-mannopyranoside, Freund's adjuvant (complete and incomplete), Dowex 1x8-200 (chloride form), concanavalin A sepharose 4B and 5'-AMP sepharose 4B were all from Sigma chemical company, Poole, Dorset, U.K.

Ammonium molybdate, EDTA (disodium), acrylamide, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethylethylene-diamine (TEMED), ammonium persulphate, glycine, β-mercaptoethanol, bromophenol blue and perchloric acid were all from BDH Ltd., Poole, Dorset, U.K.

Pyruvate, (-)-N^6-(R-phenyl-isopropyl)-adenosine (PIA), adenosine-5'-monophosphate, guanosine-5'-triphoshate, guanylyl-imidodiphosphate (GppNHp) and adenosine deaminase (calf intestine) were all purchased from Boehringer Mannheim, Lewes, East Sussex, U.K.

Sodium [^{125}I] iodide, [2-^{3}H] AMP, [G-^{3}H] PIA and 1,3-[^{3}H] dipropyl-8-cyclopentylxanthine ([^{3}H] DPCPX) were all from Amersham International PLC, Little Chalfont, Bucks., U.K.

Empty glass C10/10 columns, Mono Q HR5/5 and Superose 12 HR10/30 were purchased from Phamacia Ltd., Central Milton Keynes, Bucks, U.K. Folin-Ciocalteu reagent and trichloroacetic acid (TCA) were from FSA laboratory supplies, Loughborough, Leics., U.K. Hydrogen peroxide and ammonium sulphate (especially low in heavy metals) were purchased from Fisons Scientific Apparatus, Loughborough, Leics., U.K. In addition, Horseradish peroxidase-conjugated anti-rabbit IgG was from ICN Biomedicals (High Wycombe, Bucks., U.K.), polyethylenimine cellulose F thin layer chromatography sheets were from Aldrich (Gillingham, Dorset, U.K.), nitrocellulose sheets from Schleicher and Schuell (Dassel) and Ecoscint A was from National Diagnostics (Manville, New Jersey, USA).

2.2 ANIMALS

These were male Sprague-Dawley rats either bred at University College London or from Charles River. All animals had constant access to drinking water and to Rat and Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.) which contained (w/w) 21% digestible crude protein, 4% digestible crude oil and 39% starches and sugars. The light/dark cycle was 13 hours/11 hours with light from 06:00 to 19:00. Rats to be made hypothyroid were selected at age 4 weeks (80-90g body weight) and then fed on an iodine-deficient version of the No. 3 Breeding diet and drank water containing 0.01%(w/v) 6-n-propylthiouracil (PTU) (Chohan *et al.*, 1984; Saggerson and Carpenter, 1986). The animals were killed 4 weeks after the start of this treatment (140-170g body weight). Euthyroid age-matched controls fed on the normal diet weighed 260-280g at the time of death. Rats to be made hyperthyroid were selected at 7-8 weeks of age and injected subcutaneously with triiodothyronine (T_3) (1mg/kg body weight) dissolved in 10mM NaOH, 0.03%(w/v) bovine serum albumin (Sugden *et al.*, 1983). The T_3 injection was repeated 24 and 48 hours after the initial injection and the rats were then killed 24 hours after this final administration. During this procedure body weights changed from 229 ± 2 to 225 ± 2g. Euthyroid controls were injected with the NaOH/BSA vehicle and showed body weight changes from 225 ± 3 to 247 ± 3g over this period.

2.3 PREPARATION OF SYNAPTOSOMAL, MYELIN AND CYTOSOLIC FRACTIONS FROM RAT BRAIN

The method of preparing relatively pure subcellular fractions

of synaptosomes, myelin and the soluble fraction from rat brain homogenates was performed by the method of Booth and Clark (1978).

Brains were rapidly removed, dissected into distinct brain regions as necessary and placed in ice cold isolation medium (0.32M sucrose, 10mM Tris-HCl, pH 7.4, 1mM EDTA). After weighing the tissue, it was chopped into small pieces, blood and cell debris being washed off by decanting the supernatant and adding fresh isolation medium to the chopped tissue. This washing proceedure was repeated several times. The tissue was then homogenized in a glass Potter-type homogenizer using a teflon pestle (6 up and down strokes at 500rpm, radial clearance 0.2mm) and diluted to 15%(w/v) homogenate with isolation medium. Initial centrifugation at 1,500g for 3 minutes was performed in a sorvall RC5-B refrigerated centrifuge fitted with an SS34 rotor. The supernatant was harvested and recentrifuged in the same rotor for 10 minutes at 18,000g to produce the crude synaptosomal, myelin and mitochondrial pellet. The resulting pellet was resuspended in isolation medium to a final volume of 5ml and mixed well with 25ml of 12% Ficoll/sucrose medium (12%(w/w) Ficoll, 0.32M sucrose, 10mM Tris-HCl, pH 7.4, 50μM EDTA) giving a final concentration of 10% Ficoll. 5ml aliquots were pipetted into 6 separate 13ml polycarbonate centrifuge tubes to form the bottom layer of a three step Ficoll/sucrose gradient. The samples were then overlayed with 4ml of 7% Ficoll (7%(w/w) Ficoll, 0.32M sucrose, 10mM Tris-HCl, pH 7.4, 50μM EDTA) followed by 4ml of isolation medium. The gradients were

centrifuged at 110,000g for 1 hour in a Beckman L8 high speed centrifuge fitted with an SW41 Ti swing out rotor. The myelin and synaptosomes banded at the first and second interfaces respectively, while the mitochondria formed a pellet at the bottom of the tubes. The myelin band was removed, resuspended in 50mM Tris-HCl, pH 7.4 and centrifuged for 1 hour at 105,000g in a Beckman L8 high speed centrifuge fitted with a 70.1 Ti rotor. The resulting pellet was respended in 50mM Tris-HCl, pH 7.4 and stored at -70°C in 0.2ml aliquots. The synaptosomal band was removed and lysis initiated by resuspension in 50ml of 5mM Tris-HCl, pH 8.0 . Following sonication for 30 seconds, the suspension was left on ice for 1 hour. The resulting synatic membranes were harvested by centrifugation at 105,000g in the same Beckman centrifuge as described before. The pellet formed was resuspended in 50mM Tris-HCl, pH 7.4 and stored at -70°C in 0.2ml aliquots.

The cytosolic fraction was prepared by re-centrifuging the supernatant from the 18,000g spin at 105,000g for 1 hour in the same high speed centrifuge fitted with a 70.1 Ti rotor. It was subsequently split into 1ml aliquots and stored at -70°C.

2.4 ESTIMATION OF PROTEIN

2.4.1 LOWRY PROTEIN ASSAY

Protein concentration was predominantly measured by the method of Lowry *et al.*, (1951).

Samples and bovine serum albumin standards were made up to 0.2ml with 0.1M NaOH. 2ml of 0.05%(w/v) sodium potassium tartrate, 0.01%(w/v) copper II sulphate, 2% (w/v) sodium

carbonate, 0.1M NaOH was added to each tube, mixed well and allowed to stand at room temperature for 5-10 minutes. 50µl of undiluted Folin-Ciocalteu reagent was then added to each sample and standard, mixed immediately and colour production was allowed to develop over a 40 minute period at room temperature. The absorbance at 660nm was then measured against a reagent blank on a Unicam SP8-100 spectrophotometer. Protein concentrations of the samples was estimated from the standard curve obtained after subjecting the standard values to linear regression analysis.

2.4.2 PETERSON PROTEIN ASSAY

When protein concentration was particulary low especially during enzyme purification, it was estimated by a modification of the method of Peterson (1977). This modification of the Lowry method provides greater sensitivity and by the inclusion of SDS in the reagent, potential interference from non-ionic detergents eliminated.

Samples and standards contained in a total volume of 50µl were mixed with 1ml of 0.025%(w/v) copper II sulphate, 0.05%(w/v) sodium potassium tartrate, 2.5%(w/v) sodium carbonate, 2.5%(w/v) SDS, 0.2M NaOH and incubated at room temperature for 10 minutes. 0.1ml of undiluted Folin-Ciocalteu reagent was then added to each sample and standard, mixed well and allowed to further incubate for 40 minutes at room temperature. The absorbance at 750nm was then measured against a reagent blank and protein concentration estimated from the standard curve obtained.

2.5 PHOSPHATE DETERMINATION

Liberated inorganic phosphate in terminated 5'-nucleotidase assay supernatents was measured by the method of Chen *et al.* (1956).

Samples and standards of disodium hydrogen orthophosphate contained in a total volume of 0.5ml were mixed with 0.5ml of 0.6M sulphuric acid, 0.5%(w/v) ammonium molybdate, 2%(w/v) ascorbic acid and incubated at 45°C for 30 minutes. The absorbance at 820nm was then measured against a reagent blank and phosphate concentrations of the unknowns were estimated from the standard curve obtained.

2.6 LIQUID SCINTILLATION COUNTING

Samples were counted in 5ml of Ecoscint A in disposable plastic vial inserts in a Packard CA1500 liquid scintillation counter. Vials were always counted for a minimum of 10 minutes/vial or until 40,000 cpm had registered.

2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) was carried out essentially by the method of Laemmli (1970).

Glass gel cassettes (14cm x 13cm x 1.5mm) were assembled and clamped in an upright position. The bottom 5mm of the cassettes were then sealed using a quick setting gel and allowed to set prior to the casting of the separating gel. The separating gel mix consisted of 10%(w/v) acrylamide, 0.27%(w/v) N,N'-bis-methylene acrylamide, 0.375M Tris-HCl, pH 8.8 and

0.1%(w/v) SDS. Just prior to casting, the polymerisation was initiated by the addition of N,N',N'-tetramethylethylenediamine (TEMED) to a final concentration of 0.1%(v/v) and ammonium persulphate to a final concentration of 0.1%(w/v). The mixture was quickly stirred and poured into the glass cassette leaving sufficient room at the top for the stacking gel. By overlaying the top of the gel with butanol saturated water, it was possible to form a perfectly flat gel top thus ensuring proteins would enter the gel at a perfect right angle. The gel was then left to set for a minimum of one hour. The water/butanol was washed off the gel top and the stacking gel applied. This consisted of 4.5%(w/v) acrylamide, 0.12%(w/v) N,N'-bis-methylene acrylamide, 0.125M Tris-HCl, pH 6.8 and 0.1%(w/v) SDS, which was polymerised exactly as for the separating gel. After applying the stacking gel, a well forming comb containing 10 well spaces was inserted and again the apparatus was left for a minimum of one hour to allow the gel to set. The comb was consequently removed and the gel was allowed to stand overnight to allow the cross linkages to flatten out thus improving the sharpness of the resolved bands. The gel slabs were mounted in a conventional vertical gel apparatus and both electrode chambers were filled with running buffer (50mM Tris-HCl, pH 8.3, 0.38M glycine and 0.1%(w/v) SDS.

Samples to undergo SDS-PAGE were added to sample buffer to give final concentrations of; 0.125M Tris-HCl, pH 6.8, 2%(w/v) SDS, 5%(v/v) β-mercaptoethanol, 10%(v/v) glycerol and 0.001%(w/v) bromophenol blue as the tracking dye. After boiling the preparations for 30 minutes they were cooled and

loaded into the wells of the gel. Prestained molecular weight markers were treated in the same way as the samples and consisted of subunits of; α_2-macroglobulin (180kDa), β-galactosidase (116kDa), fructose-6-phosphate kinase (84kDa), pyruvate kinase (58kDa), fumarase (48.5kDa), lactate dehydrogenase (36.5kDa) and triosephosphate isomerase (26.6kDa).

Electrophoresis was carried out at maximum voltage (450V) with a constant current of 40mA/gel until the samples entered the separating gel, after which the current was increased to 50mA/gel.

2.8 WESTERN BLOTTING

The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed by the method of Towbin *et al.* (1979) in the following manner.

Two sheets of filterpaper were wetted with transfer buffer (25mM Tris-HCl, pH 8.3, 192mM glycine, 20%(v/v) methanol) and layered on a Scotch-Brite pad which was supported on a rigid plastic grid. A sheet of nitrocellulose (0.45μm pore size) was then wetted with the same buffer and layered on top of this. The gel to be blotted was placed onto the nitrocellulose sheet and all the air bubbles removed from the interface. After trimming the nitrocellulose to the same size as the gel, two further pre-wetted pieces of filter paper were overlayed followed by a second Scotch-Brite pad. The top plastic grid was placed in position and the whole assembly secured together with elastic bands. This ensured that the gel was both firmly

and evenly pressed against the nitrocellulose sheet. The assembly was placed in a conventional blotting chamber containing transfer buffer with the gel facing the anode. A 50V supply was connected between the plates for one hour to ensure maximum transfer of the resolved proteins to the nitrocellulose support.

2.9 IODINATION OF IMMUNOGLOBULINS

The preparation of Iodine-125-labelled IgG of high specific activity was carried out essentially by the method of Greenwood *et al.*, (1963).

Disposable columns were prepared from plastic pasteur pipettes by cutting off the top of the bulb and inserting a plug of glass wool at the tip. The anion exchange matrix was prepared by mixing Dowex 1x8-200 (chloride form) with distilled water to form a slurry. The column was packed with the slurry to a height of 9-10cm, giving a bed volume of about 1ml. The column was equilibrated with a minimum of 8 column volumes of 0.1M sodium phosphate buffer, pH 7.5 containing 0.2%(w/v) bovine serum albumin (BSA) as a carrier to prevent non-specific adsorption of protein.

Prior to use, the stock solution of sodium [^{125}I] iodide was diluted to 1mCi/ml with distilled water.

In small disposable plastic vials, 20µl of antibody was mixed with 100µl of 0.1M sodium phosphate buffer, pH 7.5 and 100µl of the diluted sodium [^{125}I] iodide solution (100µCi). The reaction was initiated by the addition of 10µl of 18mM chloramine T prepared in phosphate buffer and was allowed to

proceed for 120 seconds. To terminate the reaction 50μl of 2.2mM tyrosine prepared in phosphate buffer was added and the mixture was applied to the column. The iodinated protein was eluted from the column with 2ml of phosphate buffer whilst the iodinated tyrosine complex was retained.

The column eluate containing the iodinated protein was always checked to ensure that the detectable label was indeed conjugated to protein and not just in free solution. This was done by measuring the ratio of bound to free iodine in the prepared sample. 10μl aliquots of the iodinated protein were diluted to 1ml with phosphate buffer. A 10μl aliquot of this diluted form was added to 200μl of BSA solution (100mg/ml BSA in phosphate buffer) and mixed well. The proteins were then precipitated by the addition of 1ml of ice cold 10%(w/v) trichloroacetic acid. The precipitated proteins were pelleted by centrifugation at 7,000g_{av} for 5 minutes in an eppendorf bench centrifuge and the supernatant was carefully removed without disturbing the pellet. The radioactivity of both the supernatant and the pellet was determined on a Nuclear Enterprises NE 1600 gamma counter. The percentage of conjugated iodine was calculated from the ratio of the counts in the pellet to the total counts of the pellet plus the supernatent, multiplied by 100. This figure was always in the range 95-99% if the iodination process was successful. The specific activity of the iodinated product was usually in the range 30-40 million cpm/ml for immunoglobulins.

2.10 STAINING PROCEDURES

2.10.1 IMMUNOLOGICAL STAINING OF WESTERN BLOTS

Proteins were resolved by SDS-PAGE as described in section 2.7 and electrophoretically transferred to nitrocellulose sheets as described in section 2.8.

Prior to probing with primary antisera, it was first necessary to 'block' unoccupied sites on the nitrocellulose with an irrelevant protein. This was achieved by immersing the sheets in 20mM Tris-HCl, pH 7.4, 500mM NaCl (TBS) containing 3%(w/v) gelatin at 37°C for a minimum of 90 minutes. The gelatin was poured off and replaced typically with a 1:100 dilution of the primary antiserum in 1%(w/v) gelatin in TBS, and left to incubate overnight at 25°C. The primary antiserum was then removed and the blots extensively washed with water followed by two 15 minute washes with TBS containing 0.5ml/l Tween 20 (TTBS). After a further two x15 minute washes with TBS, a 1:50 dilution of second antibody in 1%(w/v) gelatin in TBS was added and allowed to incubate at 25°C for 2-3 hours. The second antibody consisted of horseradish peroxidase-conjugated anti-rabbit IgG which had been previously iodinated as described in section 2.9. The second antibody was poured off and the previous washing cycle repeated. The blots were developed with 2.8mM 4-chloro-1-naphtol, 0.5%(v/v) hydrogen peroxide in TBS as a substrate for the peroxidase to make cross reacting bands visible.

2.10.2 SILVER STAINING OF POLYACRYLAMIDE GELS

Protein bands on resolved SDS-PAGE gels were visualised by the highly sensitive neutral silver staining technique described by Harlow and Lane (1988).

Following electrophoresis, gels were fixed in a solution of 30%(v/v) ethanol, 10%(v/v) acetic acid for 3 hours followed by two 30 minute washes in 30%(v/v) ethanol. After a further three 10 minute washes with distilled water, gels were incubated in the dark for 30 minutes in a solution of 0.1%(w/v) silver nitrate. The gels were then extensively re-washed with water and developed with a solution of 2.5%(w/v) sodium carbonate, 0.2%(v/v) formaldehyde until the protein bands began to darken. The reaction was terminated before excessive background colour developed with a solution of 1%(v/v) acetic acid followed by several washes with distilled water. The method is highly sensitive, visualising protein in the region of 1-10ng protein/band.

2.11 QUANTITATION OF G-PROTEIN α-SUBUNITS IN SYNAPTOSOMAL MEMBRANES

Synaptosomal membranes were prepared from euthyroid, hypothyroid and hyperthyroid rats as described in section 2.3. Primary antisera used were a generous gift from Dr. G. Milligan, University of Glasgow. The polyclonal antisera raised in New Zealand White rabbits were directed against glutaraldehyde conjugates of keyhole-limpet haemocyanin and synthetic peptides representing sections of various G-proteins. Antiserum SG2 was raised against the C-terminal decapeptides of the α-subunit of transducin (KENLKDCGLF) and recognised both

$G_i1\alpha$ and $G_i2\alpha$. Antiserum I1C was raised against a peptide (LDRIAQPNYI) and represents an internal sequence of $G_i1\alpha$. The antiserum OC1 was raised against an internal sequence of $G_0\alpha$ (ANNLRGCGLY).

When immunoblotting to detect $G_i1\alpha$ or $G_0\alpha$ alone (using antisera I1C and OC1 respectively), electrophoresis was performed (using 100µg of protein for $G_i\alpha$ detection and 10µg of protein for $G_0\alpha$ detection) in gels containing 10%(w/v) acrylamide and 0.27%(w/v) N,N' bis-methylene acrylamide. When resolution and simultaneous detection of the $G_i1\alpha$ and $G_i2\alpha$ doublet was required (using antiserum SG2) the gel system was modified as described by Milligan *et al.* (1987) and Mitchell *et al.*, (1989). These gels contained 12.5%(w/v) acrylamide and 0.063%(w/v) N,N' bis-methylene acrylamide. Following SDS-PAGE under these conditions, the resolved proteins were transferred to nitrocellulose (see section 2.8) and subjected to the immunological staining proceedure described in section 2.10.1. The resulting blots were air dryed and radioactivity in excised cross reacting bands were quantified by gamma counting. Equivalent sized unstained regions of the blots were excised and counted for radioactivity to determine background, which was subtracted from all values. Individual blots were normalized relative to each other by comparison against a standard sample of cerebral cortex from normal rats which was run on every gel.

The quantitation method was linear with respect to amount of membrane protein resolved (figure 2.1).

2.12 ENZYME ASSAYS

2.12.1 5'-NUCLEOTIDASE (EC 3.1.3.5)

Cytosolic 5'-nucleotidase activity was assayed by a modification of the method of Newby *et al.* (1975) and involved measuring the release of adenosine from [2-^{3}H] AMP as a tracer.

The assay was performed in a final volume of 0.25ml in 1.5ml eppendorf tubes at 37°C containing final concentrations of; 200µM AMP, 0.15µCi [2-^{3}H] AMP, 50mM Tris-HCl, pH 7.4 and 20mM β-glycerophosphate. The β-glycerophosphate was present in the assay at a high concentration as a substrate for all the non-specific phosphatases present in the cytosolic fraction. This ensured that the AMP hydrolysis measured was attributed exclusively to the action of 5'-nucleotidase.

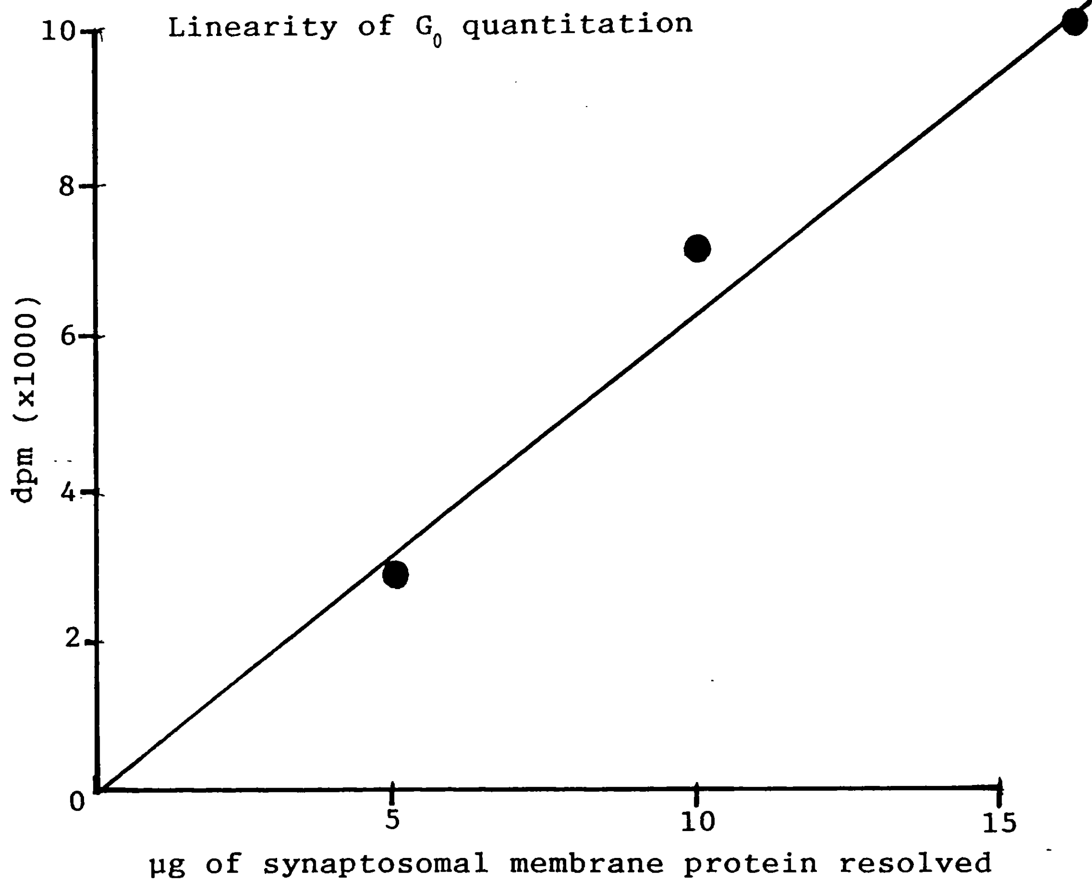
The reaction was initiated by the addition of 100µg of soluble protein and terminated typically after 10 minutes by the addition of 0.2ml of ice cold 0.15M zinc sulphate followed by 0.2ml of 0.15M barium hydroxide. After leaving the tubes on ice for 10 minutes they were centrifuged in an Eppendorf 5412 bench centrifuge for 5 minutes to remove precipitated material. This process preferentially precipitates unreacted nucleotide substrate whilst leaving the produced nucleoside in the supernatant. 100µl of the supernatant was counted for tritium in 5ml of Ecoscint A in a Packard CA1500 liquid scintillation counter.

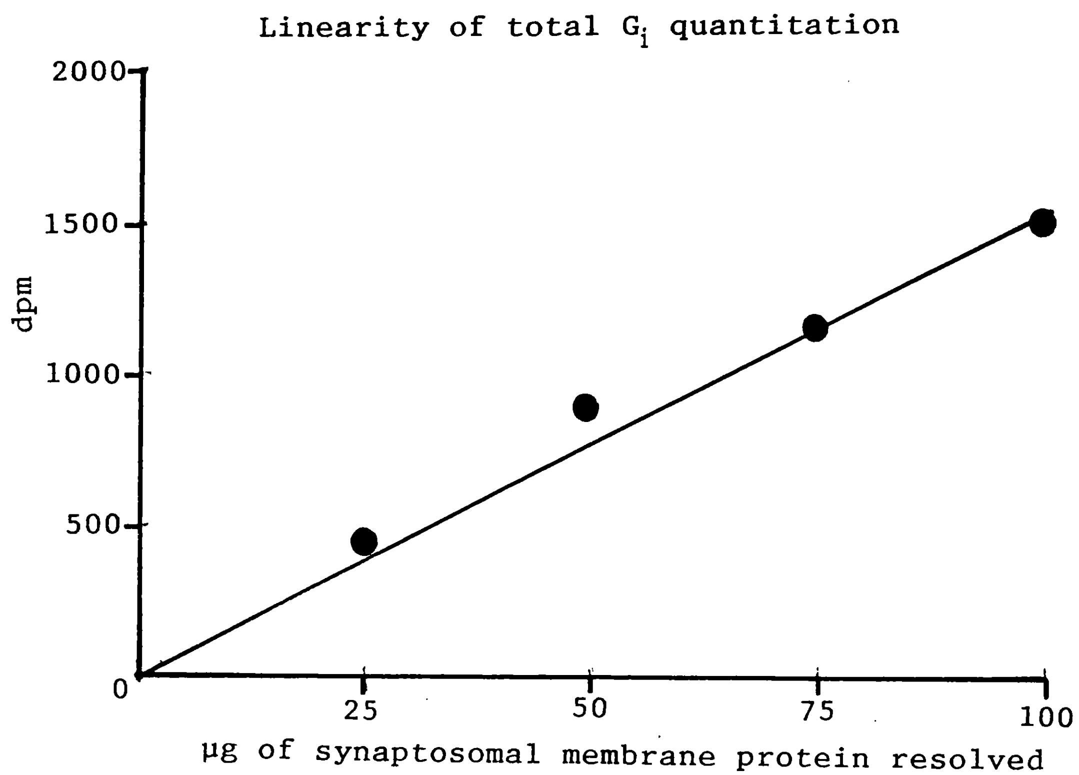
The assay appeared to be linear with respect to both protein content and time (figure 2.2).

Fig. 2.1 **Quantitation of G-protein α subunits with respect to amount of protein resolved**

Varying amounts of synaptosomal membranes from the cerebral cortex of euthyroid rats were resolved by SDS-PAGE. G-protein α subunits were quantitated as decribed in section 2.11 to demonstrate the linearity of the system.

Total $G_i\alpha$ was probed with antiserum SG2 and $G_0\alpha$ with antiserum OC1. Quantitation experiments were always performed using protein levels falling within the region of these curves. The values given represent the means of two separate experiments


Linearity of G_0 quantitation
dpm (x1000)
10
8
6
4
2
0
5
10
15
μg of synaptosomal membrane protein resolved


Linearity of total G_i quantitation
dpm
2000
1500
1000
500
0
25
50
75
100
μg of synaptosomal membrane protein resolved

2.12.2 **LACTATE DEHYDROGENASE (EC 1.1.1.27)**

This marker for the cytosol was assayed by the spectrophotometric method of Saggerson (1974).

The assay was performed at 25°C in 3ml plastic cuvettes containing 65mM Tris-HCl, pH 7.4, 0.3mM NADH, 1.3mM pyruvate in a final volume of 3ml. The reaction was initiated by the addition of 20-50µl of tissue extract and the decrease in absorbance at 340nm was followed against a reagent blank (omitting tissue extract) in a Unicam SP8-100 spectrophotometer. Enzyme activity was calculated from the extinction coefficient of 6.22$\mu mol^{-1}ml$ for NADH at 340nm.

2.12.3 **ACETYLCHOLINESTERASE (EC 3.1.1.7)**

Acetylcholinesterase was used as a marker for synaptic membranes and was measured by the spectrophotometric method of Ellman *et al.* (1961).

Enzyme activity was measured by following the formation of 5-thio-2-nitrobenzoic acid at 412nm from thiocholine and 5:5-dithio-2-nitrobenzoate, the thiocholine being produced by the action of acetylcholinesterase. The assay was performed at 25°C in 1ml plastic cuvettes containing 0.2M potassium phosphate buffer, pH 8.0, 10mM dithiobisnitrobenzoic acid (DTNB), 10%(v/v) Triton X-100, 10-50µl tissue extract in a final volume of 1ml. The reaction was initiated by the addition of 10µl 75mM acetylthiocholine iodide and the increase in absorbance at 412nm was monitored on a Unicam SP8-100 spectrophotometer against a reagent blank (omitting tissue

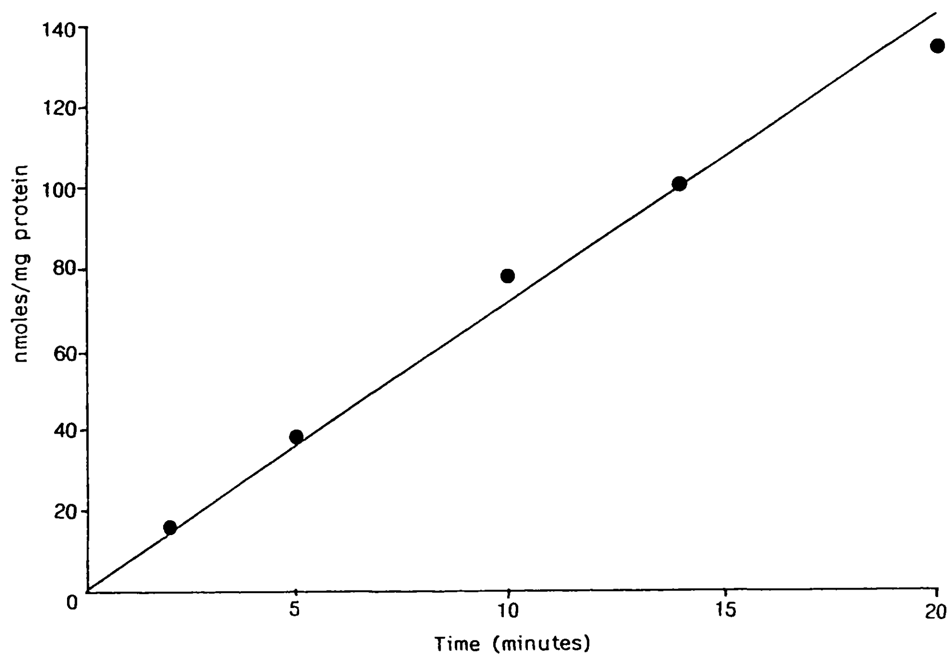
Fig. 2.2 **Time course and variation of 5'-nucleotidase activity as a function of protein concentration in rat brain cytosol**

The cytosolic fraction from rat brain was assayed for 5'-nucleotidase activity as described in section 2.12.1. Assays were performed at different times as indicated using 50μg soluble protein. Assays were also performed for 10 minutes using different concentrations of soluble protein. Protein concentration was determined as described in section 2.4.1.

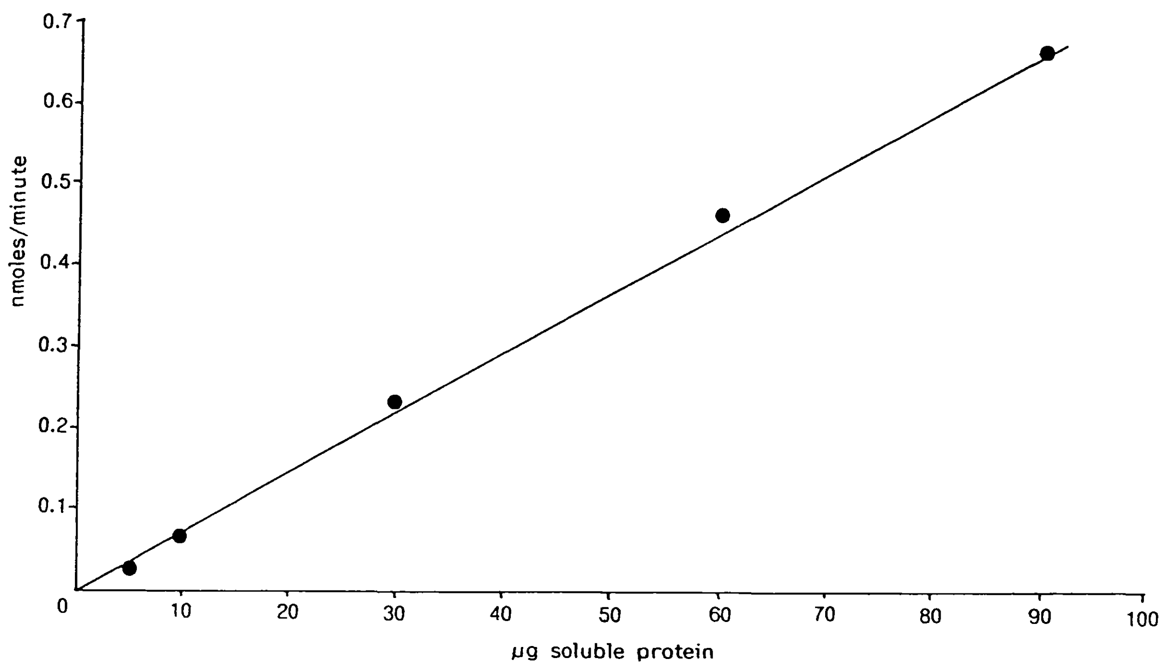
All assays were performed using 15mM AMP as substrate.

The values given represent the means of two separate experiments, each assayed in duplicate

5'-nucleotidase activity in cytosol as a function of time



5'-nucleotidase activity in cytosol as a function of soluble protein added



extract).

Enzyme activity was calculated from the extinction coefficient of 13.6$\mu mol^{-1}ml$ for 5-thio-2-nitrobenzoate at 412nm.

2.13 ANALYSIS OF NUCLEOTIDES AND NUCLEOSIDES BY THIN LAYER CHROMATOGRAPHY

Nucleosides and their phosphates in terminated 5'-nucleotidase assay supernatants were analysed by the thin layer chromatographic system of Neuhard *et al.* (1965).

Assays of 5'-nucleotidase were performed as described in section 2.12.1 except that these were terminated by adding perchloric acid to a final concentration of 5%(v/v). The tubes were placed on ice for 10 minutes after which they were centrifuged for 2 minutes in an Eppendorf 5412 bench centrifuge to remove precipitated proteins. The supernatants were neutralised by adding concentrated potassium carbonate solution, then further centrifuged as before to remove precipitated potassium chlorate. 10µl aliquots containing 10-20 nmoles of nucleosides and their phosphates were spotted onto polyethylenimine cellulose F coated plastic sheets which were impregnated with an indicator that fluoresced at 254nm. 10-20 nmoles of standards of AMP, IMP, adenosine and inosine were also applied to separate tracks of the plates and developed under saturating conditions in a Whatman filter paper lined tank in 3.3M ammonium borate buffer pH 7.0 which had been previously equilibrated overnight with the same buffer. Development was allowed to proceed for approximately 2 hours after which the solvent front had reached 10cm from the top of the plates. After drying the plates under a stream of warm

air, spots were located under ultraviolet light, scraped off and counted for tritium in 10ml of Ecoscint A. This system resolved AMP (R_f=0.5) and adenosine (R_f=0.4) but did not discriminate between IMP and inosine (R_f=0.64).

2.14 RADIOLIGAND BINDING ASSAYS

2.14.1 DISPLACEMENT OF BOUND [^{3}H] DPCPX BY PIA

These assays were performed essentially by the method of Lohse *et al.* (1984) and further described by Mazurkiewicz and Saggerson (1989).

Synaptosomal membranes from the cerebral cortex were prepared as described in section 2.3 and diluted to a concentration of 1mg/ml with 50mM Tris-HCl, pH 7.4. Endogenous adenosine was removed from the system by preincubating the membranes for 20 minutes at 37°C with 3U/ml of adenosine deaminase. Binding assays were performed in a total volume of 0.4ml containing 100µg treated membranes, 50mM Tris-HCl, pH 7.4, 3nM [^{3}H] DPCPX, varying concentrations of unlabelled PIA (10^{-4}-10^{-10}M in a logarithmic fashion) in the presence and absence of 100µM GTP. Binding was allowed to proceed at 37°C for 30 minutes after which the membranes were separated from unbound radioactivity by vacuum filtration through a Whatman GF/B filter, using a Brandel cell harvester. The filters were washed twice with 3ml of ice cold 50mM Tris-HCl, pH 7.4, removed from the apparatus and counted for tritium in 10ml of Ecoscint A.

2.14.2 MODIFICATION OF [^{3}H] PIA BINDING BY GppNHp

The effect of this non hydrolysable GTP analogue on the binding of PIA was studied by the method of Green and Johnson (1991). Binding assays were performed in essentially the same way as for the DPCPX displacement studies as described in section 2.14.1. Pretreated synaptosomal membranes from the cerebral cortex (100µg) were incubated in a final volume of 0.4ml containing 50mM Tris-HCl, pH 7.4, 0.5nM [^{3}H] PIA and varying amounts of unlabelled GppNHp (0-1mM). Estimations of non-specific binding were performed exactly as above but with the addition of unlabelled PIA to a final concentration 100µM.

2.15 DEVELOPMENT OF A PURIFICATION PROTOCOL FOR SOLUBLE 5'-NUCLEOTIDASE

Soluble 5'-nucleotidase was purified to apparent electrophoretic homogeneity by a combination of concanavalin A Sepharose affinity chromatography, Mono Q anion exchange and AMP Sepharose affinity chromatography. Additional techniques are described in the text for the purpose of molecular sizing and for the separation of an unknown ATP sensitivity conferring factor.

2.15.1 FRACTIONAL AMMONIUM SULPHATE PRECIPITATION

Cytosol was initially brought to 20% ammonium sulphate saturation and left on ice with constant stirring for 1 hour. The resulting precipitate was collected by centrifugation at 16,000xg for 20 minutes in a Sorvall refrigerated centrifuge fitted with an SS34 rotor. The supernatant was then further

increased in ammonium sulphate saturation in increments as required using the same method of mixing and spinning as described above. The purpose was to yield distinct ranges of ammonium sulphate saturation to ascertain the solubility range of the enzyme. The pellets were therefore individually resuspended in 20mM Tris-HCl, pH 8.5, 0.05%(w/v) Lubrol PX and assayed for 5'-nucleotidase activity as described in section 2.12.1.

2.15.2 CONCANAVALIN A SEPHAROSE 4B AFFINITY CHROMATOGRAPHY

Concanavalin A Sepharose 4B (5ml suspension) was pre-washed in buffer A (50mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 20 and 1mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$). The cytosolic fraction was gently mixed with matrix for 30 minutes on ice prior to packing into a glass column (10 x 1cm). Following an extensive wash with buffer A until the absorbance of the eluate read less than 0.01, 5'-nucleotidase was eluted with 100ml of 0.3M α-D-mannopyranoside in buffer A. The eluate was rapidly concentrated to 2-5ml by ultrafiltration and dialysed with two buffer changes at 4°C for 12 hours against 2l of buffer B (20mM Tris-HCl, pH 8.5, 0.1%(v/v) Tween 20.

2.15.3 ION EXCHANGE CHROMATOGRAPHY

Anion exchange chromatography was performed on a Pharmacia fast protein liquid chromatography (FPLC) system using a Mono Q column. It involved the ionic interaction of charged species at pH 8.5 with the quaternary nitrogen functional group of the

matrix. The dialysed protein solution was applied to a 1ml Mono Q HR5/5 column which had been previously equilibrated in buffer B (20mM Tris-HCl, pH 8.5, 0.1%(v/v) Tween 20) at a flow rate of 1ml/min. After unbound proteins were washed off the column (4-5 column volumes), bound proteins were eluted with a linear gradient of buffer B to 200mM sodium chloride in buffer B over 10 column volumes. One ml fractions were collected and assayed for 5'-nucleotidase activity as described in section 2.12.1.

2.15.4 AMP SEPHAROSE 4B AFFINITY CHROMATOGRAPHY

The final affinity step in the purification involved the specific interaction of 5'-nucleotidase with its substrate, AMP, covalently attached to a sepharose 4B matrix. The commercial preparation of the matrix employed attachment of the N^6 amino group of AMP to sepharose 4B via a 6 carbon spacer arm to minimise stearic hindrance of the protein binding with its substrate.

The bound and eluted enzyme from the ion exchange step was dialysed as before against buffer C (50mM Tris-HCl, pH 7.4, 0.1%(v/v) Tween 20) and applied to a 2ml column of 5'-AMP Sepharose 4B at a flow rate of 1.8ml/hour. Unbound proteins were washed from the matrix with buffer C until a steady baseline at 280nm was obtained. Proteins showing a lower affinity for the matrix were then eluted with 0.6M sodium chloride in buffer C which also slowly initiated the desorption of 5'-nucleotidase. By increasing the salt concentration of the eluate to 1M sodium chloride in buffer C, 5'-nucleotidase

could be rapidly eluted from the column.

2.15.5 SUPEROSE 12 GEL FILTRATION CHROMATOGRAPHY

Gel filtration was performed in 1M sodium chloride in buffer C on an FPLC Superose 12 column. Prior to use, the column was calibrated by injecting standards of known molecular weights and measuring their retention times. These consisted of; cytochrome C (12.4kDa), carbonic anhydrase (29kDa), albumin (66kDa), alcohol dehydrogenase (150kDa) and β-amylase (200kDa). The void volume was estimated using blue dextran (molecular weight 2000kDa). A linear plot was then constructed by plotting elution volume divided by void volume on the y-axis versus log molecular weight on the x-axis. By the use of a plot of this type, it was possible to estimate the molecular weight of purified 5'-nucleotidase from its retention time.

2.15.6 PREPARATION OF ATP SENSITIVITY CONFERRING FACTOR

Cytosolic proteins were precipitated between 0-65% ammonium sulphate saturation as described in section 2.15.1. Following centrifugation, the pellet was redissolved in 20mM Tris-HCl, pH 8.5, 0.1%(v/v) Tween 20 (or 0.05%(w/v) Lubrol PX), and dialysed for 12 hours against 2l of the same buffer. Mono Q ion exchange was then performed exactly as described in section 2.15.3 and the proteins excluded from the column collected. The resulting protein solution was concentrated to 2-5ml by ultrafiltration and dialysed for 12 hours against 2l of 50mM Tris-HCl, pH 7.4, 0.1%(v/v) Tween 20. The factor was finally stripped from the enzyme by performing concanavalin A affinity

chromatography as described in section 2.15.2. The 5'-nucleotidase was absorbed onto the matrix and eluted as previously described (section 2.15.2), whilst the factor remained in the unbound proteins which were excluded from the matrix. They were subsequently concentrated to approximately 5ml by ultrafiltration using a membrane having a cut-off of 30k molecular weight,and dialysed overnight against 2L of 50mM Tris-HCl, pH 7.4

2.16 POLYCLONAL ANTIBODY PRODUCTION

An attempt was made to raise a polyclonal antiserum directed towards the purified soluble 5'-nucleotidase. The purified protein (50μg) in a total volume of 0.1ml was mixed well with an equal volume of Freund's complete adjuvant until a smooth emulsion was formed. It was consequently administered in four intramuscular injections into a Dutch cross rabbit. Three booster injections of the same dose were administered in Freund's incomplete adjuvant by a subcutaneous route over an eight week period. Blood was collected from the ear marginal vein 10 days following the injection and allowed to clot prior to collecting the serum after centrifugation.

2.17 ANTIBODY INHIBITION AND PRECIPITATION STUDIES

A polyclonal antiserum directed towards purified rat liver ecto-5'-nucleotidase was raised in sheep and was a generous gift from Dr. A. Newby, Department of Cardiology, University of Wales. 0.3μg of purified soluble 5'-nucleotidase was incubated at 37°C for two hours with varying amounts of

antiserum in a total volume of 0.2ml. The antibody-antigen complex was then further incubated at room temperature with shaking for 30 minutes with 10mg of protein A (immobilized on acrylic beads). Following centrifugation for 3 minutes at 7,000 g_{av}, the supernatant was carefully removed and assayed for 5'-nucleotidase activity as described in section 2.12.1. For inhibition studies, the protein A step was ommited, and the antibody-antigen complex mixed well by vortexing prior to direct assay for 5'-nucleotidase assay.

2.18 STATISTICAL METHODS

Statistical significance was assessed by Student's t test for unpaired samples. Throughout the work, the following labels are used; A, $P<0.05$, B, $P<0.02$, C, $P<0.01$ and D, $P<0.001$. Values quoted represent the mean data ± SEM. If Error bars are not visible in a figure, they lie within the symbol.

CHAPTER THREE

RESULTS AND DISCUSSION

G-PROTEINS

3.1 EFFECT OF HYPOTHYROIDISM ON THE ABUNDANCE OF THE α-SUBUNITS OF G_i1, G_i2 AND G_o IN SYNAPTOSOMAL MEMBRANES FROM SEVERAL REGIONS OF THE RAT BRAIN

Altered cell signalling in pathological and perturbed physiological states may play a key role in cellular dysfunction under these conditions. These changes may arise by alteration of any of the individual components of the signal transduction pathway, ie. the receptor, the G-protein or the effector molecule. Whereas altered receptor function has been studied for many years, changes in G-protein abundance has only been performed relatively recently owing to the availability of specific G-protein antisera. Although several studies have provided indirect evidence for altered G-protein abundance in the CNS, no such direct quantitation has been performed to confirm these important preliminary studies until recently. Mazurkiewicz & Saggerson (1989a) provided good initial evidence to support this conjecture with the observation that Forskolin-stimulated adenylyl cyclase in synaptosomal membranes isolated from hypothyroid rats was inhibited by 100μM GTP in the presence of 100mM NaCl, an observation not seen with control membranes. GTP binding to the inhibitory G-protein G_i serves to initiate its dissociation resulting in the inhibition of cyclase by the active α_i so formed. The fact that GTP mediates this effect more readily in the hypothyroid membranes indicates that an increase in G_i may be apparent. In the same study, they also observed an enhancement of cyclase inhibition by the adenosine A1 agonist PIA in hypothyroid membranes, despite no change in receptor number or affinity of the receptor for

the agonist. These results suggested that post receptor regulation was apparent, possibly by the up-regulation of G_i. It was therefore decided to further this study by quantitating these G-protein α-subunits in hypothyroid membranes. The use of specific antisera has been used for the quantitation of G-protein α-subunits by several authors (see Milligan, 1990). These antisera are raised against unique peptide sequences of the respective α-subunit in preference to the entire G-protein as the former yield more specific antisera thereby making the interpreptation more valid.

In synaptosomal membranes isolated from hypothyroid rats, a general increase in the abundance of the α-subunits of G_i1, G_i2 and G_0 were observed throughout the six regions of the brain tested. Antiserum I1C was raised against the peptide sequence LDRIAQPNYI and is monospecific for $G_i1\alpha$. Using this antiserum, $G_i1\alpha$ was seen to be the least variable of all the α-subunits tested only showing a significant increase in the striatum and cerebral cortex. Increases were seen in the cerebellum and hippocampus, although they were not statistically significant. In contrast, no change was observed in the abundance of this α-subunit in the medulla oblongata and a non-significant decrease was seen in the hypothalamus (Table 3.1 and Figure 3.1).

Quantitation of $G_i2\alpha$ was attempted with a specific antiserum raised against the sequence LERIAQSDYI. This represented an internal sequence of $G_i2\alpha$ corresponding to amino acids 160-169 (Mitchell *et al.*, 1989). However,

Table 3.1 Effect of hypothyroidism on the abundance of Gi1α in synaptosomal membranes from six brain regions

Synaptosomal membranes (96μg) were resolved in 10% SDS gels as described in section 2.7. Following transfer to nitrocellulose sheets, Gi1α was probed with antiserum I1C and detection with ^{125}I-labelled second antibody (as described in section 2.10.1). The number of individual preparations are indicated in parentheses. C indicates $p<0.01$.

Figure 3.1 Immunoblot of representative preparations of euthyroid and hypothyroid synaptosomal membranes visualising Gi1α

Individual lanes represent synaptosomal membranes isolated from various brain regions as indicated. The resolved proteins (100μg) were probed with antiserum I1C which specifically recognises Gi1α and visualised with horseradish peroxidase-conjugated second antibody.

Lane 1, prestained molecular weight markers

Lanes 2 and 3, striatum

Lanes 4 and 5, cerebral cortex

Lanes 6 and 7, cerebellum

Lanes 8 and 9, hippocampus

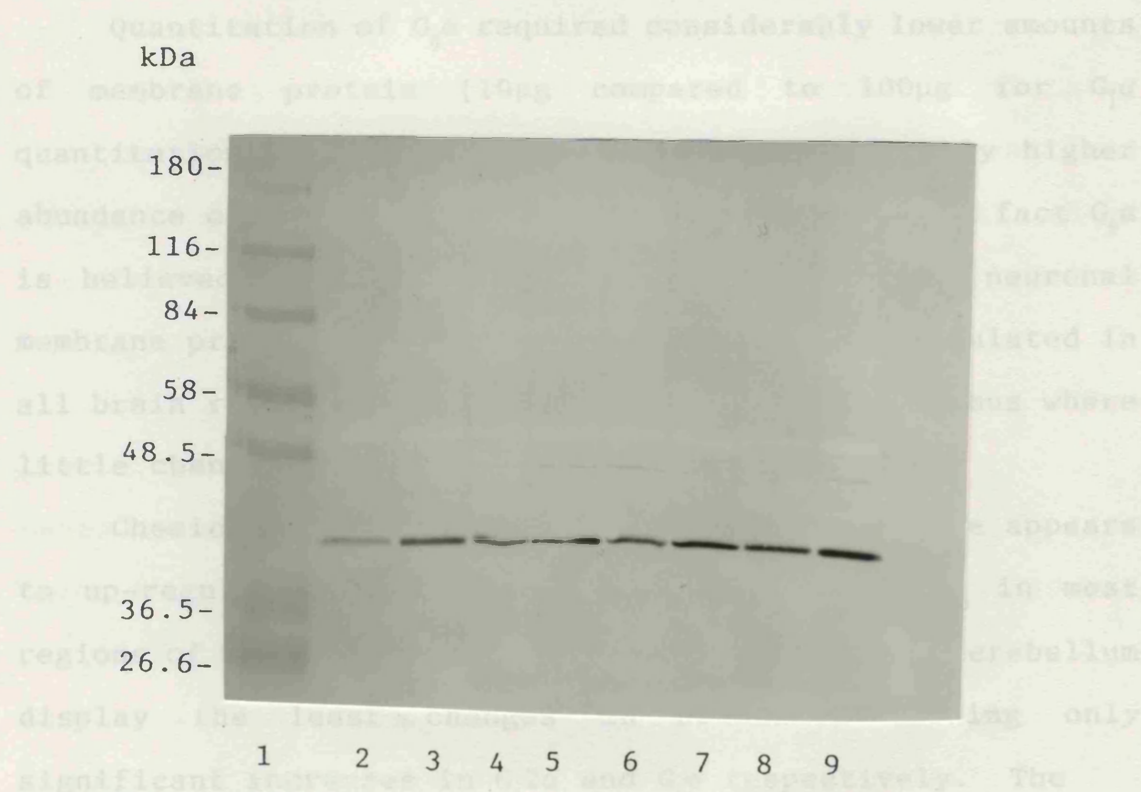
Lanes 2,4,6 and 8 represent euthyroid membranes

Lanes 3,5,7 and 9 represent hypothyroid membranes

Table 3.1

Brain region	Gi1α (dpm/100µg of membrane protein)	
	Euthyroid	Hypothyroid
Striatum	241 ± 55 (5)	648 ± 96 (5)[c]
Cerebral cortex	255 ± 20 (4)	876 ± 165 (5)[c]
Cerebellum	262 ± 68 (5)	483 ± 89 (5)
Medulla oblongata	283 ± 34 (5)	276 ± 34 (5)
Hypothalamus	372 ± 89 (4)	186 ± 27 (4)
Hippocampus	379 ± 75 (5)	710 ± 144 (4)

Figure 3.1



quantitation using this antiserum was unsuccessful in that cross-reacting bands were barely visible, reflected by radioactive counts approximating background levels. The use of antiserum SG2 which recognises both $G_i1\alpha$ and $G_i2\alpha$ was therefore used in preference. Since both α-subunits cross react with this antiserum and display very similar subunit molecular weights, the gel conditions used were modified thereby enabling adequate resolution of the $G_i1\alpha$ and $G_i2\alpha$ doublet prior to staining and excision of the lower $G_i2\alpha$ band (figure 3.2). Quantitation of $G_i2\alpha$ using this system indicated that this α-subunit displayed the largest increases in abundance compared to the other α-subunits tested. A significant increase of approximately 3-4 fold was observed in all brain regions except the cerebellum, which although showing an increase was not statistically significant.

Quantitation of $G_0\alpha$ required considerably lower amounts of membrane protein (10µg compared to 100µg for $G_i\alpha$ quantitation), which is attributed to considerably higher abundance of this G-protein in neuronal tissue. In fact $G_0\alpha$ is believed to constitute up to 1-2% of total neuronal membrane protein. In hypothyroidism, $G_0\alpha$ is up-regulated in all brain regions by 2-3 fold except the hypothalamus where little change is seen (Table 3.3 and Figure 3.3).

Chemical induction of hypothyroidism therefore appears to up-regulate the α-subunits of G_i1, G_i2 and G_0 in most regions of the brain tested. The hypothalamus and cerebellum display the least changes in abundance showing only significant increases in $G_i2\alpha$ and $G_0\alpha$ respectively. The

Table 3.2 Effect of hypothyroidism on the abundance of Gi2α in synaptosomal membranes from six brain regions

Synaptosomal membranes (96µg) were resolved in 12.5% SDS gels (low bis-acrylamide) as described in section 2.11. Following transfer to nitrocellulose sheets, Gi1α and Gi2α was probed with antiserum I1C and detection with ^{125}I-labelled second antibody (as described in section 2.10.1). The lower Gi2α band was consequently excised from the nitrocellulose and counted for radioactivity. The number of individual preparations are indicated in parentheses. A, C and D indicate $P < 0.05$, 0.01 and 0.001 respectively.

Figure 3.2 Immunoblot of representative preparations of euthyroid and hypothyroid synaptosomal membranes visualising Gi1α and Gi2α

Individual lanes represent synaptosomal membranes isolated from various brain regions as indicated. The resolved proteins (60µg) were probed with antiserum SG2 which recognises Gi1α and Gi2α and visualised with horseradish peroxidase-conjugated second antibody.

Lane 1, prestained molecular weight markers

Lanes 2 and 3, hippocampus

Lanes 4 to 9, separate preparations from the cerebral cortex

Lanes 2,4,6 and 8 represent euthyroid membranes

Lanes 3,5,7 and 9 represent hypothyroid membranes

Table 3.2

Brain region	Gi2α (dpm/100μg of membrane protein) Euthyroid	Hypothyroid
Striatum	186 ± 18 (6)	558 ± 134 (3)[A]
Cerebral cortex	294 ± 52 (6)	796 ± 111 (5)[C]
Cerebellum	186 ± 66 (6)	413 ± 122 (6)
Medulla oblongata	138 ± 37 (6)	550 ± 78 (3)[D]
Hypothalamus	227 ± 22 (6)	599 ± 85 (5)[C]
Hippocampus	208 ± 52 (6)	814 ± 178 (5)[C]

Figure 3.2

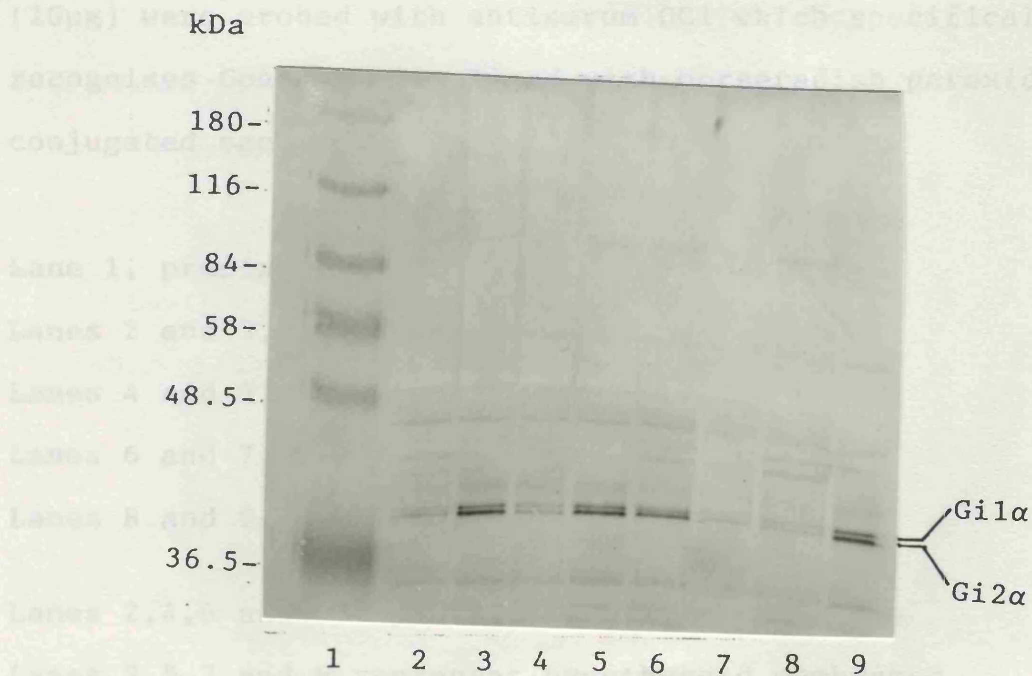


Table 3.3 <u>Effect of hypothyroidism on the abundance of Goα in synaptosomal membranes from six brain regions</u>

Synaptosomal membranes (10µg) were resolved in 10% SDS gels as described in section 2.7. Following transfer to nitrocellulose sheets, Goα was probed with antiserum OC1 and detected with ^{125}I-labelled second antibody (as described in section 2.10.1). The number of individual preparations are indicated in parentheses. B, C and D represent P<0.02, 0.01 and 0.001 respectively.

Figure 3.3 <u>Immunoblot of representative preparations of euthyroid and hypothyroid synaptosomal membranes visualising Goα</u>

Individual lanes represent synaptosomal membranes isolated from various brain regions as indicated. The resolved proteins (10µg) were probed with antiserum OC1 which specifically recognises Goα and visualised with horseradish peroxidase-conjugated second antibody.

Lane 1, prestained molecular weight markers

Lanes 2 and 3, cerebral cortex

Lanes 4 and 5, cerebellum

Lanes 6 and 7, hippocampus

Lanes 8 and 9, striatum

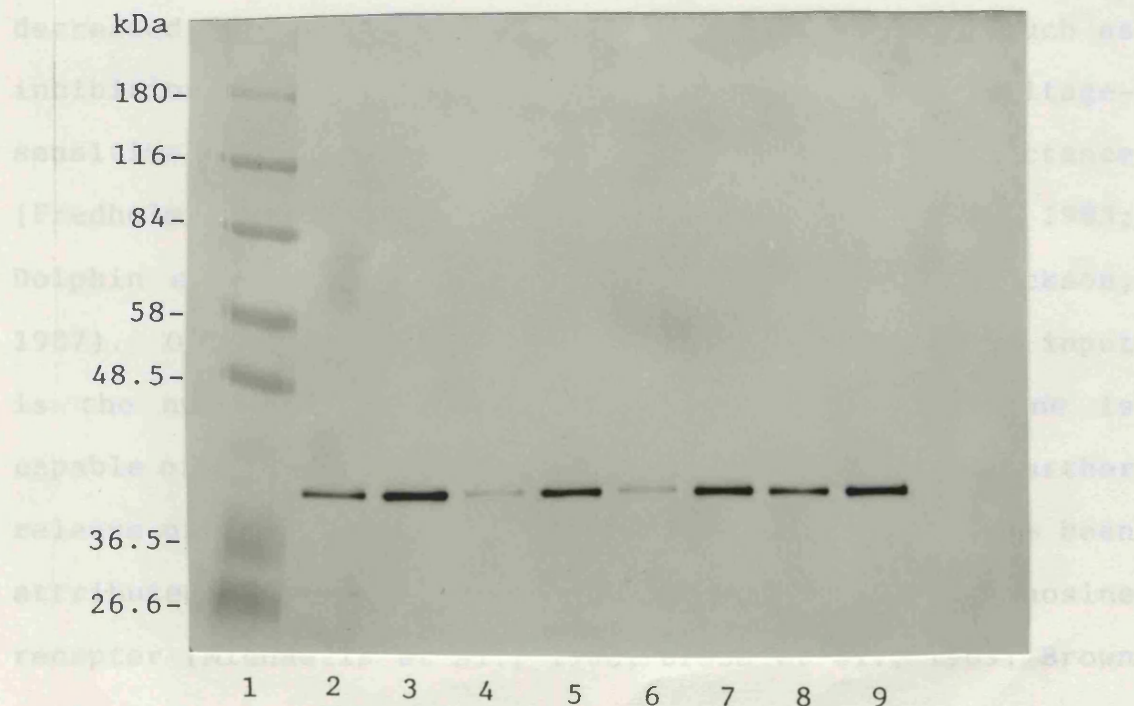
Lanes 2,4,6 and 8 represent euthyroid membranes

Lanes 3,5,7 and 9 represent hypothyroid membranes

Table 3.3

Brain region	Gioα (dpm/100μg of membrane protein) Euthyroid	Hypothyroid
Striatum	3696 ± 422 (5)	9979 ± 1795 (5)[C]
Cerebral cortex	5808 ± 633 (5)	10613 ± 1478 (5)[B]
Cerebellum	3115 ± 844 (5)	8712 ± 317 (5)[D]
Medulla oblongata	1320 ± 211 (5)	2746 ± 422 (5)[B]
Hypothalamus	2006 ± 422 (5)	2429 ± 844 (5)
Hippocampus	3538 ± 264 (5)	8078 ± 1320 (5)[B]

Figure 3.3



greatest increases were observed in the striatum and cerebral cortex where all the α-subunits were up-regulated. The model used in this study represented adult hypothyroidism as chemical induction was commenced once processes in the 'early period' of brain development were essentially complete. These early events include myelination and expression of enzymes of neurotransmitter synthesis. It is frequently reported that the adult brain is insensitive to thyroid hormone, despite its significant intracellular content (Heninger & Albright, 1975; Obregon *et al*, 1978) and high affinity receptors for the same (Oppenheimer *et al.*, 1974; Valcana & Timiras, 1978; Kolodny *et al.*, 1985). Extensive clinical literature indicates that adult hypothyroid patients have a general decrease in brain excitability (see section 1.11). It may therefore be possible that the up-regulation of $G_i1\alpha$, $G_i2\alpha$ and $G_0\alpha$ seen in this study may serve to strengthen these inhibitory signal inputs leading to decreased neurotransmitter release through pathways such as inhibition of adenylyl cyclase, inhibition of voltage-sensitive calcium channels or increased potassium conductance (Fredholm & Dunwiddie, 1988; Proctor & Dunwiddie, 1983; Dolphin *et al.*, Scott & Dolphin, 1987; Trussell & Jackson, 1987). One such compound able to exert an inhibitory input is the nucleoside adenosine. Extracellular adenosine is capable of modulating neuronal activity by inhibiting further release of neurotransmitter. This inhibitory event has been attributed to adenosine's action at the A1 type adenosine receptor (Michaelis *et al.*, 1988; Gross *et al.*, 1989; Brown

et al., 1990; Richardson & James, 1991) which couples to adenylyl cyclase via G_i. Extracellular production of adenosine arises as a result of ATP degradation by an extracellular purine nucleotide phosphohydrolase pathway. The last enzyme in the pathway is ecto 5'-nucleotidase which catalyses the formation of adenosine from AMP. The activity of this enzyme has been reported to substantially increase in the hypothyroid brain (Mazurkiewicz & Saggerson, 1989b). The increased amounts of adenosine produced under these conditions would therfore be capable of interacting with the A1 adenosine receptors thereby strengthening the inhibitory input by this pathway. There are however many biological compounds capable of exerting such an inhibitory role for example neurotransmitters such as acetylcholine which is capable of inhibiting its own release (Marchi *et al.*, 1983). It is likely that the hypothyroid state enhances more than one of the inhibitory pathway simultaneously, although many more studies would be required to strengthen this hypothesis.

3.2 EFFECT OF HYPERTHYROIDISM ON THE ABUNDANCE OF $G_i1\alpha$, $G_i2\alpha$ and $G_o\alpha$ IN SYNAPTOSOMAL MEMBRANES ISOLATED FROM THREE REGIONS OF THE RAT BRAIN

These experiments were conducted to further the studies which demonstrated an increase in G-protein α-subunit abundance in the hypothyroid brain (Orford *et al.*, 1991a). As previously stated in section 3.1, chemical induction of hypothyroidism was accompanied by significant increases in $G_i1\alpha$ and $G_i2\alpha$ in the cerebral cortex, and variable but non-significant increases in the cerebellum. In contrast, no

changes were observed in the medulla oblongata. Therefore, these three brain regions were chosen for this study due to their different responses to changes in G-protein abundance in the hypothyroid state. Using antiserum SG2 which recognises both $G_i1\alpha$ and $G_i2\alpha$, a significant decrease in both these α-subunits were observed in the cerebral cortex of 54% and 44% respectively (Tables 3.4 and 3.5). This was apparent after only three days of T3 administration in comparison to the four week period used in the hypothyroid model to detect the increase in G-protein abundance. The decrease in $G_i1\alpha$ in the cortex was further confirmed using antiserum I1C which is mono-specific for $G_i1\alpha$ (Table 3.6). In the cerebellum, a significant decrease of $G_i1\alpha$ was observed when probed with antiserum SG2 following excision of the upper $G_i1\alpha$ band of the doublet. However, this significant decrease could not be confirmed using antiserum I1C. This may however reflect the lower relative changes of this α-subunit observed in this brain region or possibly due to slightly different selectivities of the antisera towards polypeptides in the 41kDa region. In common with the hypothyroid model, the medulla oblongata appeared to be unresponsive to thyroid hormone in modulating G-protein abundance as shown by little or no change in either of the studies performed. In contrast to the hypothyroid study, no significant changes in $G_0\alpha$ abundance were observed when synaptosomal membranes were probed with antiserum O1C (Table 3.7).

Results presented for euthyroid membranes in this study show minor discrepancies compared to the same measurements

The arbitrary units were defined as the ratio of radioactive counts in the excised band relative to that of a standard preparation of cerebral cortex which was run on every gel as an internal standard.

Table 3.4

Brain region	Gi1α (arbitrary units/ 100μg of membrane protein)	
	Euthyroid	Hyperthyroid
Cerebral cortex	1.21 ± 0.24	0.56 ± 0.06[B]
Cerebellum	0.86 ± 0.18	0.44 ± 0.07[A]
Medulla oblongata	0.69 ± 0.18	0.49 ± 0.08

Table 3.5

Brain region	Gi2α (arbitrary units/ 100μg of membrane protein)	
	Euthyroid	Hyperthyroid
Cerebral cortex	1.24 ± 0.17	0.70 ± 0.06[C]
Cerebellum	1.08 ± 0.17	0.72 ± 0.12
Medulla oblongata	0.91 ± 0.22	0.69 ± 0.17

Tables 3.4 and 3.5 <u>Effects of hyperthyoidism on the abundance of Gi1α and Gi2α in synaptosomal membranes from three brain regions</u>

Synaptosomal membranes (100μg) were resolved in 12.5% SDS gels (low bis-acrylamide) as described in section 2.11. Following transfer to nitrocellulose sheets, Gi1α and Gi2α were probed with antiserum SG2 and detection with ^{125}I-labelled second antibody (as described in section 2.10.1). The upper (Gi1α) and lower (Gi2α) bands were consequently excised from the sheet and counted for radioactivity.

The values expressed are means ± SEM for 9 and 8 separate control and T_3-treated preparations respectively each made from the pooled brain tissue from 6 rats. A, B and C represent P,0.05, 0.02 and 0.01 respectively.

Table 3.6

Brain region	Gi1α (arbitrary units/ 100μg of membrane protein)	
	Euthyroid	Hyperthyroid
Cerebral cortex	0.94 ± 0.11	0.64 ± 0.07[A]
Cerebellum	0.86 ± 0.18	0.61 ± 0.07
Medulla oblongata	0.65 ± 0.07	0.44 ± 0.12

Table 3.7

Brain region	Goα (arbitrary units/ 100μg of membrane protein)	
	Euthyroid	Hyperthyroid
Cerebral cortex	11.1 ± 0.7	8.8 ± 1.1
Cerebellum	8.3 ± 0.5	7.6 ± 0.9
Medulla oblongata	6.5 ± 0.7	4.4 ± 1.2

Tables 3.6 and 3.7 <u>Effects of hyperthyoidism on the abundance of Gi1α and Goα in synaptosomal membranes from three brain regions</u>

Synaptosomal membranes (100μg for $G_i1\alpha$ and 10μg for $G_0\alpha$ determinations) were resolved in 10% gels as described in section 2.11. Following transfer to nitrocellulose sheets, $G_i1\alpha$ and $G_0\alpha$ were probed with antiserum I1C or OC1 respectively and detection with ^{125}I-labelled second antibody (as described in section 2.10.1).

The values expressed are means ± SEM for 9 and 8 separate control and T_3-treated preparations respectively each made from the pooled brain tissue from 6 rats. A indicates $P<0.05$.

made in the hypothyroid study. In contrast to the hypothyroid experiments, slightly lower relative abundancies in the cerebellum and medulla oblongata were found compared to the cerebral cortex. In addition, no significant differences were detected between the brain regions with respect to $G_i2\alpha$ in contrast to that measured in the hypothyroid study where lower amounts were observed in the cerebellum and medulla oblongata compared to the cortex. Finally, the previous study indicated that lower amounts of $G_0\alpha$ were observed in the cerebellum and medulla oblongata compared to the cerebral cortex. This difference was not however observed in this later series of experiments. The reason for these discrepancies are unclear, but may be attributed to rats obtained from different sources, and a three year period separating the two studies. Such down-regulation of these inhibitory G-proteins in the hyperthyroid state may serve to reduce the inhibitory input to certain neurones, resulting in hyper-neuronal activity. Further experiments should be directed towards measuring abundance changes of the stimulatory G-protein G_s, which may be up-regulated to strengthen the stimulatory inputs in addition to the down-regulation of the inhibitory pathway. This may provide a possible explanation for some of the symptoms observed in hyperthyroid subjects, especially those associated with increased brain excitability (see section 1.11).

It is unclear which of the receptor-mediated transignalling pathways are affected by changes in these

inhibitory G-proteins. Mazurkiewicz & Saggerson (1989a) provided evidence for alteration in the adenylyl cyclase system in the hypothyroid state and altered signalling of the adenosine A1-mediated pathway. It was therefore necessary to look for such changes in synaptosomal membranes isolated from hyperthyroid rats. Radioligand binding studies were performed on synaptosomal membranes from the cerebral cortex as this brain region showed the greatest change in G-protein abundance in hyperthyroidism. Initial investigations were directed towards the effect of 100μM GTP to modify the displacement of the adenosine antagonist [^{3}H]-DPCPX by unlabelled L-PIA. The presence of GTP serves to shift the displacement curve to the right as the affinity of the receptor for the agonist but not antagonist is reduced. This reduction in affinity is brought about on binding of GTP to the G-protein, thereby activating it (see section 1.3). Using such a technique, Mazurkiewicz & Saggerson (1989a) provided evidence that 100μM GTP is more effective at lowering the affinity of PIA for the adenosine A1 receptor in synaptosomal membranes isolated from hypothyroid animals. This was apparent from the observation that GTP increased the Hill coefficient from 0.32 to 0.98 in hypothyroid membranes compared to an increase from 0.48 to 0.75 in membranes isolated from euthyroid rats. This difference was not observed in the present study where 100μM GTP increased the Hill coefficients by the same magnitude in synaptosomal membranes isolated from both euthyroid and hyperthyroid rats (figures 3.4 & 3.5). Although an increase in the Hill

Fig 3.4 & 3.5 <u>Displacement of [^{3}H]-DPCPX by PIA in euthyroid and hyperthyroid synaptic membranes</u>

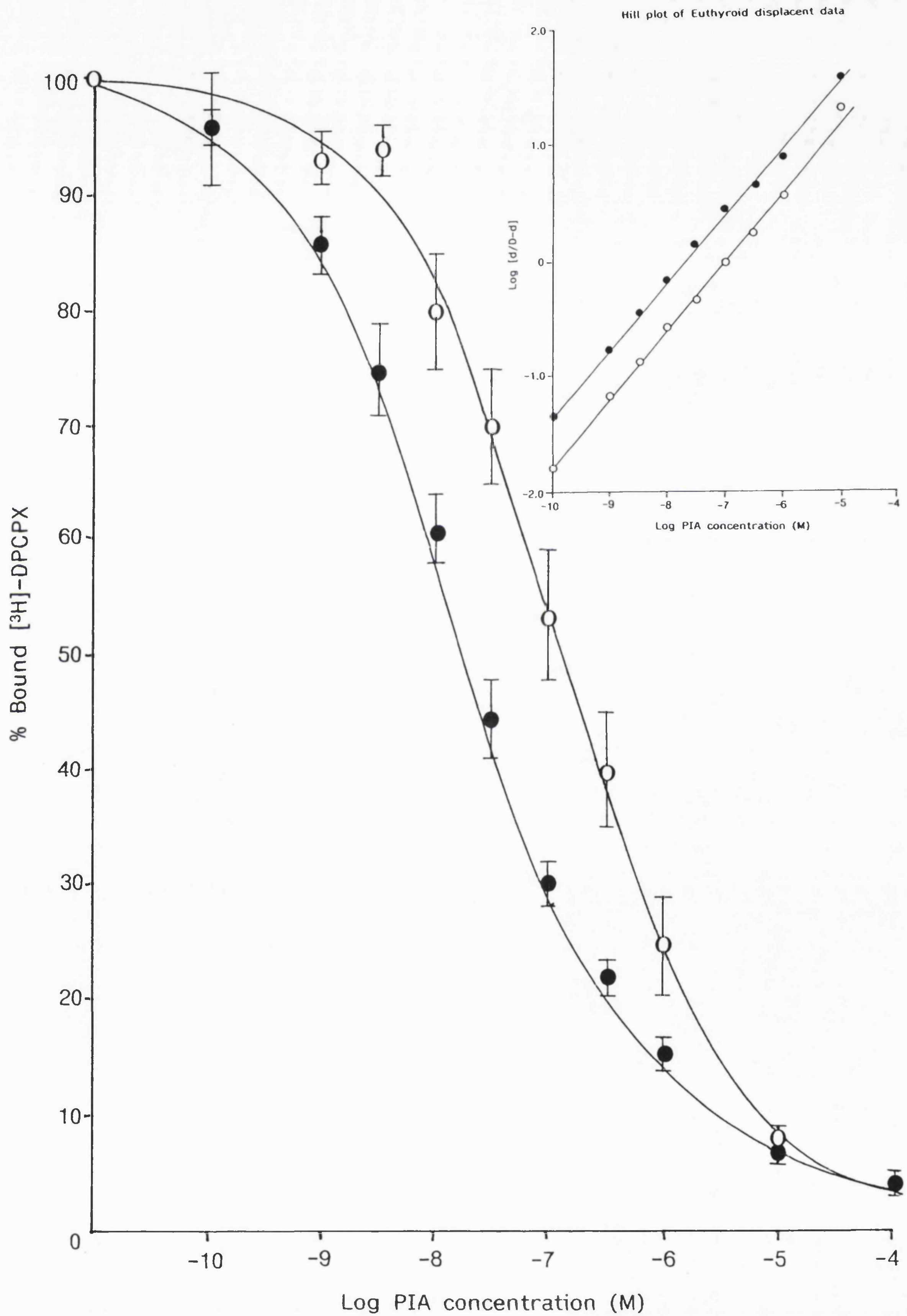
100µg of purified synaptic membranes isolated from the cortex of euthyroid (fig 3.4) and hyperthyroid (fig 3.5) were incubated with 3nM [^{3}H]-DPCPX in 50mM Tris-HCl buffer, pH 7.4 and the indicated concentrations of unlabelled PIA as described in section 2.14.2. Antagonist displacement curves were generated in the presence (○) and absence (●) of 100µM GTP and analysed by Hill plots (insets).

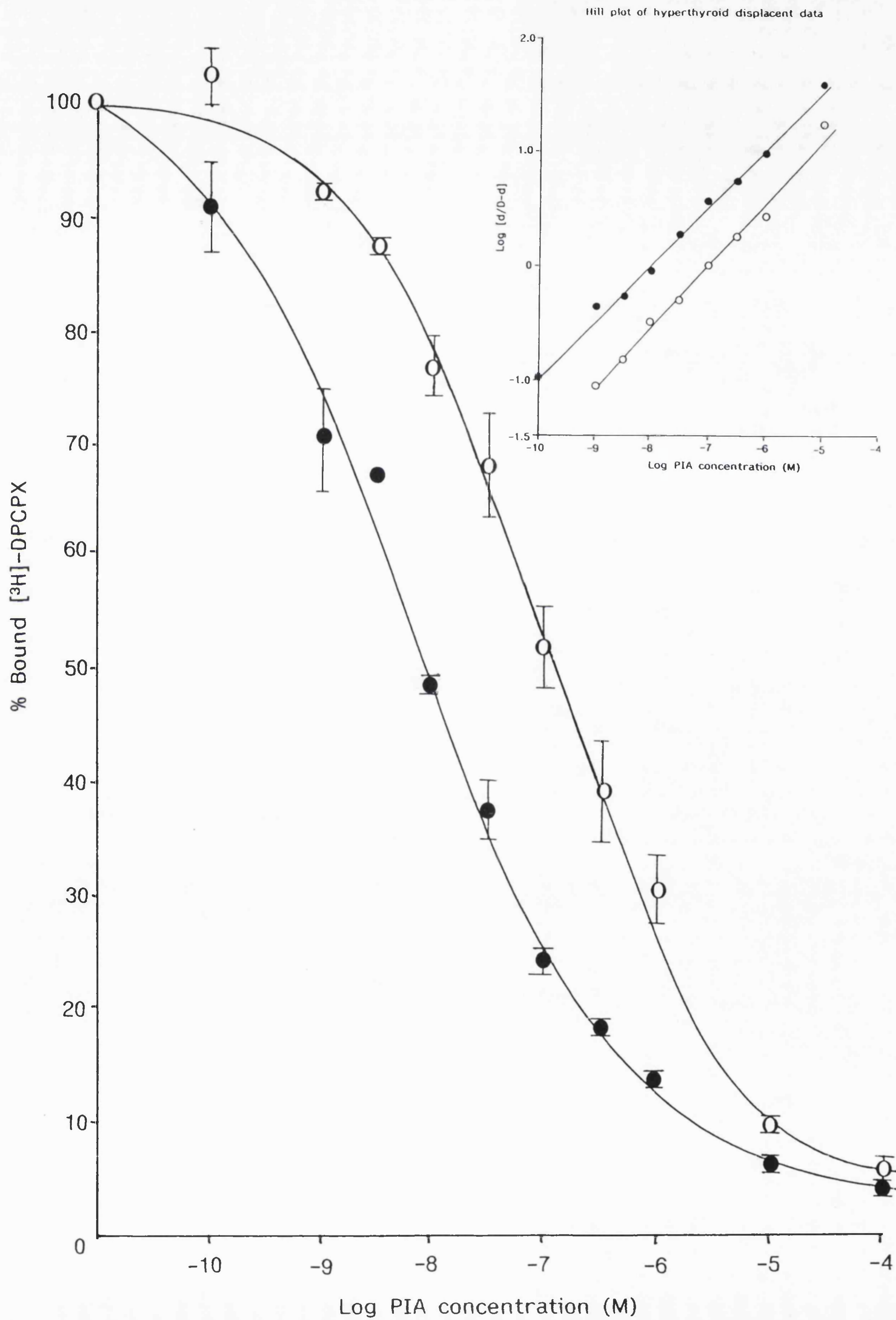
Controls (n=4): h=0.575, Γ=0.999 (-GTP)

h=0.604, Γ=0.999 (+GTP)

Hyperthyroids (n=3): h=0.499, Γ=0.997 (-GTP)

h=0.557, Γ=0.995 (+GTP)


Displacement of [3H]-DPCPX by PIA in euthyroid cortex synaptic membranes
Hill plot of Euthyroid displacent data
Log [d/D-d]
Log PIA concentration (M)
% Bound [3H]-DPCPX
Log PIA concentration (M)



Displacement of [³H]-DPCPX by PIA in hyperthyroid cortex synaptic membranes
Hill plot of hyperthyroid displacent data
2.0
1.0
0
-1.0
-1.5
Log [d/D-d]
-10
-9
-8
-7
-6
-5
-4
Log PIA concentration (M)
100
90
80
70
60
50
40
30
20
10
0
% Bound [³H]-DPCPX
-10
-9
-8
-7
-6
-5
-4
Log PIA concentration (M)

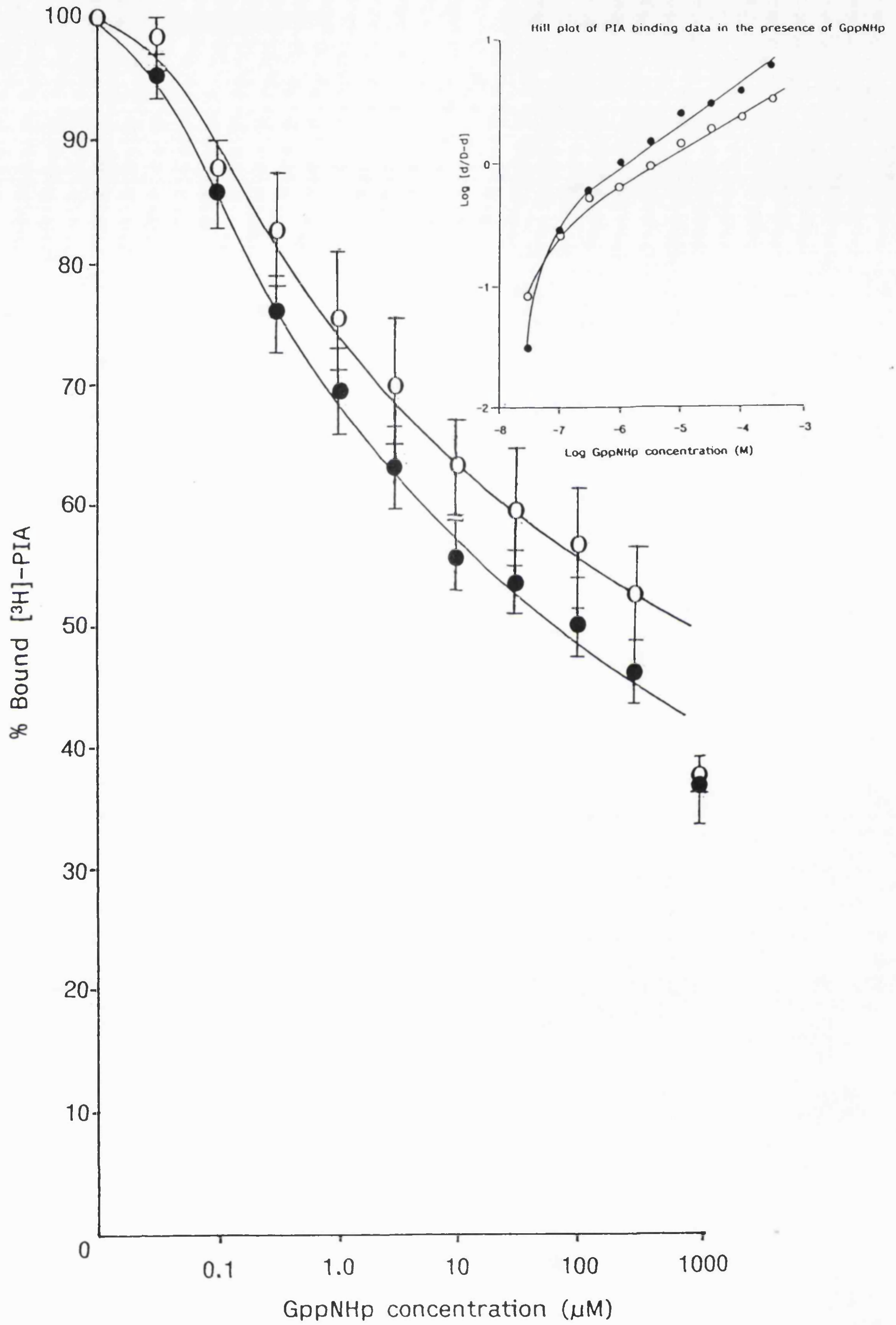
coefficients was apparent in the presence of GTP, it was not elevated to the same extent as those reported by Mazurkiewicz & Saggerson (1989a). Adenosine receptors generally exist as a mixture of high and low affinity states of approximately 50:50% (Goodman *et al.*, 1982; Mazurkiewicz & Saggerson, 1989a) although a higher ratio has been reported of 69:31% of high and low affinity states respectively (Lohse *et al.*, 1984). The high affinity component is usually lost upon addition of GTP to membranes in the presence of agonist. This loss of high affinity binding is attributed to activation of the associated G-protein on binding GTP, thereby dissociating from the agonist-receptor complex. In the present study a two state model is evident by a low value of Hill coefficient, although no computer analysis was performed to emphasize the point. As these slope factors were not raised by any significant amount, it appears that 100μM GTP fails to eliminate the high affinity component. This low responsivness of the membranes to GTP in the presence of adenosine A1 receptor agonist implies no differences in this particular receptor mediated pathway in the hyperthyroid brain. This observation was further confirmed in a separate experiment which studied the effect of GppNHp (0.03-1000μM) to decrease the binding of the adenosine agonist [^{3}H]-PIA. This method was previously used by Green & Johnson (1991) to detect altered function of G_i in diabetic adipocyte membranes. As previously stated, binding of GTP to the G-protein associated with the agonist-receptor complex initiates its dissociation and lessens the affinity of the

Fig 3.6 <u>Effect of GppNHp on binding of [^{3}H]-PIA to euthyroid and hyperthyroid synaptic membranes</u>

100μg of purified synaptic membranes isolated from the cortex of euthyroid (●) and hyperthyroid (○) rats were incubated with 0.5nM [^{3}H]-PIA in 50mM Tris-HCl buffer, pH 7.4 and the indicated concentrations of GppNHp as described in section 2.14.2. Displacement curves were generated which were subject to Hill analysis (inset).

Controls (n=6): h=0.276, Γ=0.992, IC_{50}=1.0μM

Hyperthyroids (n=6): h=0.308, Γ=0.989, IC_{50}=0.5μM


Effect of GppNHp on binding of [3H]-PIA to euthyroid
and hyperthyroid cortex synaptic membranes
% Bound [3H]-PIA
GppNHp concentration (μM)
Hill plot of PIA binding data in the presence of GppNHp
Log [d/D-d]
Log GppNHp concentration (M)

receptor for the agonist. Therefore by titrating the membranes with increasing amounts of GTP or a stable analogue, it should be possible to reduce agonist binding. This is demonstrated in figure 3.6, although no changes were observed in the hyperthyroid membranes with respect to the control. These results suggest that no significant change is observed indicative of altered G-protein effects at the adenosine A1-receptor in the cortex following T3 treatment. This does not however rule out the possiblity of signalling pathways initiated by other receptors being altered following a brief increase in circulating thyroid hormone.

CHAPTER FOUR

RESULTS AND DISCUSSION

5'-NUCLEOTIDASE

4.1 SOLUBLE 5'-NUCLEOTIDASE ACTIVITIES IN RAT BRAIN CYTOSOL

It is generally appreciated that at least four distinct isoforms of 5'-nucleotidase exist (see Zimmerman, 1992 for review). One form is an ectoenzyme which is believed to facilitate production of extracellular adenosine. It displays a low Km (µM range) for its preferred substrate AMP and is potently inhibited by adenine nucleotides. Two of the other isoforms are cytosolic and are believed to mediate intracellular adenosine formation following a decrease in the energy charge of the cell. Whereas they differ in their preferences for AMP or IMP as substrate, both display Km's in the mM range and are activated by adenine nucleotides. Many studies have been performed to date on the different forms of the enzyme in a variety of tissues, although studies on neuronal tissue appears to have somewhat lagged behind. Mazurkiewicz & Saggerson (1989b) studied the ectoenzyme in 6 anatomical regions of the rat brain, and provided evidence for its increased activity in the hypothyroid state. Since no such measurements were made with respect to the soluble activity, it was decided to embark on such a study. However, initial assays performed under the conditions routinely used for measurement of the cytosolic activity failed to show activation by ATP. In fact assays conducted in the presence of 5mM ATP demonstrated that the soluble activity appeared to be inhibited, a finding more in common with that of the ectoenzyme.

Initial experiments were therefore directed towards the effect of ATP on the activity of 5'-nucleotidase in the post

microsomal supernatant of rat brain. In such crude preparations, many enzymes are present which are capable of utilizing ATP and for this reason 5'-nucleotidase assays were performed using short incubation times, with high AMP concentrations (15mM) in order to minimise dilution of the [^{3}H]-AMP substrate pool by degraded ATP. Furthermore, 20mM β-glycerophosphate was present in all assays to saturate any non-specific phosphatase activity thereby minimising the degradation of AMP by this route. These assays were performed at pH 6.5 as this condition has been routinely used for assay of the ATP-activated cytosolic 5'-nucleotidase activity. Magnesium was also present at a concentration of 5mM , again because the cytosolic enzymes have been reported to show an absolute requirement for this divalent cation. The effect of ATP on the soluble 5'-nucleotidase activity was complex and indicated the presence of at least two activities (figure 4.1). One activity appeared to be inhibited by low concentrations of ATP (0.1-0.5mM) and the second activated or 'ATP-tolerant' at higher ATP concentrations (1.5-2mM). At higher still levels of ATP (5mM), it could therefore be reasoned that the inhibitory component would be eliminated and only the 'ATP-tolerant' activity would remain. The same experiment was repeated at pH 8.0 as this condition is commonly used for assays of the ectoenzyme (Newby *et al.*, 1974; Mazurkiewicz & Saggerson, 1989b). Again the same observation was seen, although slightly higher amounts of ATP were needed to elicit the effect. From these results it was

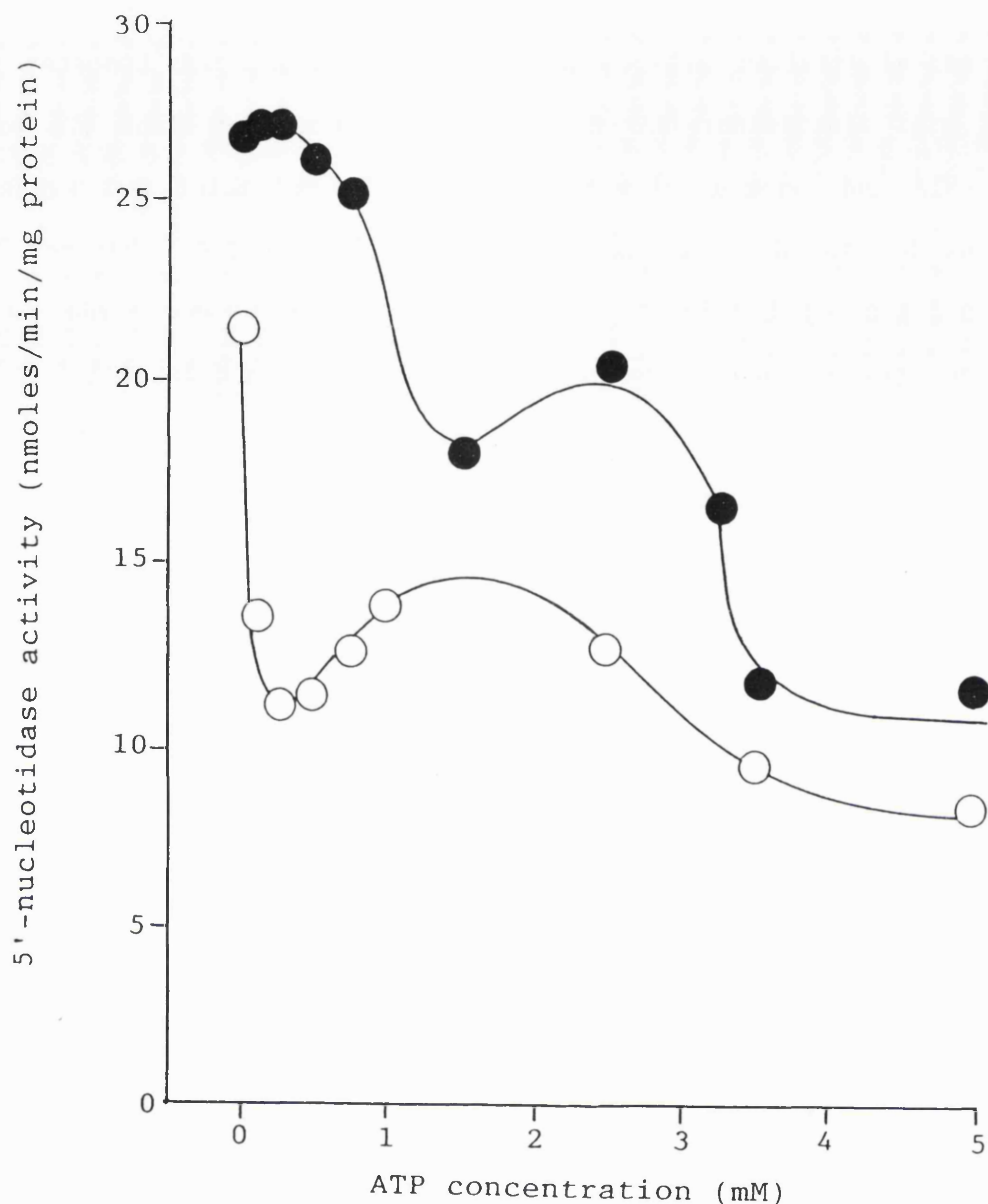


Fig 4.1 <u>Effect of ATP concentration on brain cytosol 5'-nucleotidase activity</u>

Soluble fractions (105,000 g supernatants) were obtained from whole brains. Assays were performed at two different pH values with 15mM AMP as described in section 2.12.1. The values are means of two separate experiments each assayed in duplicate.

○, pH 6.5

●, pH 8.0

therefore reasoned that measurements of 5'-nucleotidase activity in the absence of ATP would predominantly measure the ATP-inhibitable form, whilst assays containing 5mM ATP would selectively measure the 'ATP-tolerant' variety. Although this assumption was made, the use of an antibody to inhibit one of the enzymes would have alleviated any possible residual activity which may have still been present. Unfortunately, at the time of the study no such antibody was available.

In common with the membrane bound ectoenzyme, one form of soluble activity was inhibited by ATP as discussed above. The kinetics of this soluble form were therefore compared to the ectoenzyme at pH 8.0 in the absence of ATP (figure 4.2). There appeared to be no significant difference in the Km values between the soluble form (Km=28μM) and membrane bound ectoenzyme (Km=13μM) both isolated from the cerebral cortex. However, it was unlikely that the soluble activity was due to membrane fragments on the basis of marker enzyme assays. The synaptic membrane marker enzyme, acetylcholinesterase measured in the cytosolic fraction showed a specific activity of 6.8 ± 0.6 (n=3) nmoles/min/mg compared to 414.0 ± 5.5 (n=3) nmoles/min/mg measured in synaptic membranes. This calculates to be some 1.6% contamination of membranes in the soluble fraction. Incidentally, a value of 5% contamination has previously been reported (Mazurkiewicz & Saggerson, 1989b). Moreover, lactate dehydrogenase (cytosolic marker enzyme) yielded a 12% contamination by cytosol of the synaptic membrane fraction (3102.9 ± 90.5 nmoles/min/mg, n=3 and 369.1 ± 16.8 nmoles/min/mg, n=3 respectively). These results however do not rule out the possibility that the soluble activity may represent the ectoenzyme released from the plasma membrane by endogenous PIPLC.

The crude cytosolic fractions are likely to contain

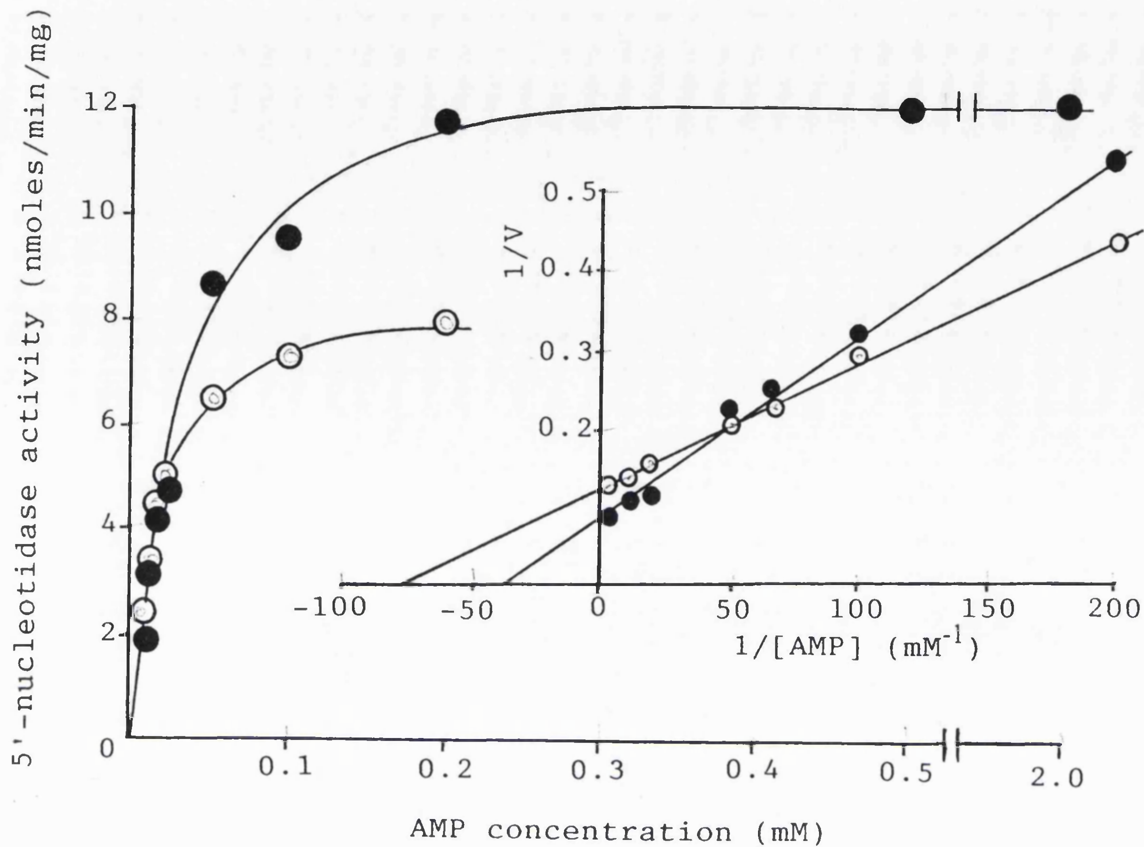


Fig 4.2 Effect of AMP concentration on soluble and membrane bound 5'-nucleotidase activity

Soluble fractions (105,000 g supernatant) and purified synaptic membranes were obtained from cerebral corex. Assays were performed at pH 8.0 in the absence of ATP as described in section 2.12.1. The values are from a single experiment . Km values for AMP were determined from the inset Lineweaver-Burk plots.

●, soluble fraction: Km=28μM

○, synaptic membranes: Km=13μM

other nucleotide and nucleoside metabolising enzymes in addition to 5'-nucleotidase. For example in the presence of high levels of ATP, nucleoside formation may be initiated by AMP deaminase converting AMP to IMP prior to its dephosphorylation by the IMP-specific cytosolic 5'-nucleotidase. This route would consequently result in the formation of inosine rather than adenosine. To rule out this possibility, assays were therefore performed in the presence of 5mM ATP and the terminated reaction supernatents were analaysed by thin layer chromatography (figure 4.3). The results indicated that approximately 70% of the AMP was not recovered in the AMP spot even at time zero. It is likely that endogenous adenylate kinase was responsible for this observation since it has been reported that perchloric acid is insufficient to completely inactivate its activity, even at 4°C (Williamson & Corkey, 1969). However, of the AMP recovered, 94% was degraded to adenosine, inosine and IMP. Of this, 83% of the reaction products was detected as adenosine, with the remaining 17% appearing as inosine plus IMP. Thus under these conditions, most of the labelled AMP was probably converted to ADP or ATP by adenylate kinase. It was concluded however that a majority of nucleoside formation was attributed to that of adenosine rather than inosine. Future experiments should therefore contain Ap4A to inhibit the adenylate kinase reaction.

The activities of the 'ATP-inhibitable' and 'ATP-tolerant' forms were measured in six anatomically distinct regions of the rat brain isolated from control and hypothyroid animals. This perturbed metabolic state was chosen because previously Mazurkiewicz & Saggerson (1989b) observed increases in the ectoenzyme under these conditions. Interestingly, the distribution of the ectoenzyme measured by these authors correlates very well with the soluble 5'-nucleotidase activity measured in this study in the absence of ATP ($\Gamma=0.95$, $P<0.01$). In the hypothyroid state however, this correlation broke down. Whereas the ectoenzyme was seen

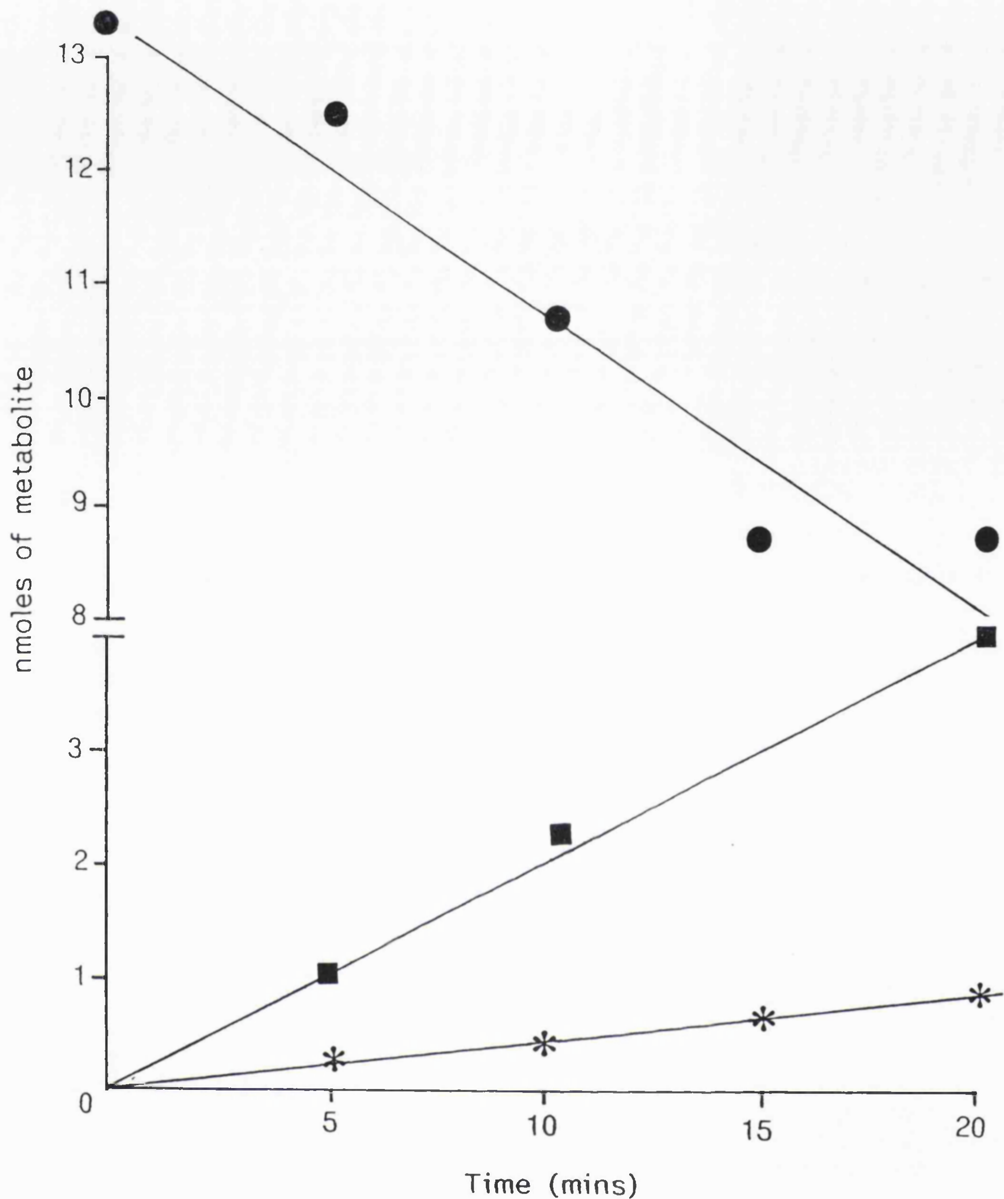


Fig 4.3 Thin layer chromatographic analysis of soluble 5'-nucleotidase assay products

Soluble fractions (105,000 g supernatants) were assayed at pH 8.0 at various time points as described in section 2.12.1 with 15mM AMP and 5mM ATP. ^{3}H-labelled products were separated as described in section 2.13, scraped off the plate and counted for tritium. The values are means of two separate experiments each assayed in duplicate.

●, AMP

■, Adenosine

✳, Inosine + IMP

Brain region	Euthyroid [ATP] = 0 (A)	Euthyroid [ATP] = 5mM (B)	Euthyroid Ratio A/B	Hypothyroid [ATP] = 0 (A)	Hypothyroid [ATP] = 5mM (B)	Hypothyroid Ratio A/B
Cerebellum	23.8 ± 1.5 (4)	9.1 ± 1.0 (4)	2.6	20.1 ± 3.8 (4)	12.1 ± 0.9 (3)	1.7
Cerebral cortex	11.1 ± 0.4 (4)	9.7 ± 1.0 (5)	1.1	11.8 ± 0.9 (3)	6.0 ± 1.4 (3)	2.0
Hippocampus	24.6 ± 3.6 (3)	27.3 ± 5.2 (5)	0.9	11.3 ± 0.8 (3)[A]	9.5 ± 1.2 (3)[B]	1.2
Hypothalamus	34.3 ± 2.8 (3)	16.4 ± 2.6 (5)	2.1	13.5 ± 1.9 (4)[C]	29.1 ± 2.5 (3)[B]	0.5
Striatum	27.0 ± 1.0 (4)	6.9 ± 1.7 (4)	3.9	13.9 ± 0.6 (4)[D]	25.5 ± 1.9 (3)[D]	0.6
Medulla oblongata	56.8 ± 2.4 (4)	10.3 ± 0.5 (5)	5.5	59.3 ± 6.0 (3)	9.7 ± 1.2 (3)	6.1

Table 4.1 Soluble 5'-nucleotidase activity in six brain regions from euthyroid and hypothyroid rats

5'-nucleotidase was assayed at pH 8.0 with 15mM AMP in the presence of the indicated concentrations of ATP. The values (means ± SEM of the number of independant measurements indicated in parentheses) are expressed as nanomoles per minute per milligram of protein.

A, B, C and D represents $P<0.05$, 0.02, 0.01 and 0.001 respectively

to increase in the cerebellum, striatum and cerebral cortex no such increases were observed with respect to the ATP inhibitable soluble form. In fact, decreases were observed in this activity in the hippocampus, hypothalamus and striatum. These results further suggest that this soluble activity is distinct from that of the ectoenzyme. Table 4.1 provides further evidence for the distinct nature of the two soluble 5'-nucleotidase activities. Assays in the presence of 5mM ATP show different distributions of 5'-nucleotidase to that measured in its absence. Furthermore, changes in this 'ATP-tolerant' activity induced by hypothyroidism do not mirror changes in the ATP sensitive form. Whereas significant decreases are observed with the inhibitable enzyme in the hippocampus, hypothalamus and striatum, only the hippocampus shows this decrease in the hypothyroid state with the tolerant activity. In fact, this activity increases in the hypothyroid state in the hypothalamus and striatum.

In summary, there appear to be at least two distinct soluble 5'-nucleotidase activities present in the post-microsomal supernatant of rat brain, which show different distributions between brain regions. Moreover, changes in their activities seen in the hypothyroid state also appear to differ. It seems likely therefore that these two activities are differentially regulated *in vivo*, although specific physiological roles for them have yet to be conclusively assigned. Evidence exists that an ATP-activated soluble 5'-nucleotidase is responsible for ischaemia-induced adenosine formation (Newby, 1988; Yamazaki *et al.*, 1991).

Although the presence of this enzyme has not been studied in brain to date, the 'ATP-tolerant' activity reported here may fall into this category. It can also be rationalised that a low Km ATP-inhibitable 5'-nucleotidase could also contribute towards a neuroprotective role under these conditions. By removing ATP inhibition induced by extensive cellular ATP catabolism, it could be expected to result in the activation of this type of the enzyme. However, it has been reported that Ki values for ATP for this soluble enzyme lie within the nM-μM range (Mallol & Bozal, 1983; Madrid-Marina & Fox, 1986; Lai & Wong, 1991a). This then raises the question as to whether this enzyme would be active at physiological ATP concentrations. It is possible however that other compounds also regulate this enzyme *in vivo* to either activate it, or attenuate the ATP inhibitory effects. A likely candidate is magnesium ion and the interplay of this cation with ATP may further regulate the enzyme (see later).

4.2 INITIAL FRACTIONATION STUDIES OF SOLUBLE 5'-NUCLEOTIDASE FROM RAT BRAIN CYTOSOL

With the presence of a soluble 5'-nucleotidase which resembled the ectoenzyme more closely than either of the cytosolic forms, it was decided to fractionate the enzyme and attempt its purification. It was initially noted that the 105,000g supernatant of rat brain contained approximately 30% of the total 5'-nucleotidase activity measured in brain homogenate. This value is identical to previous reports of 30% for the low Km soluble enzyme in both rat (Lai & Wong, 1991b) and bovine brain (Vogel *et al.*, 1992).

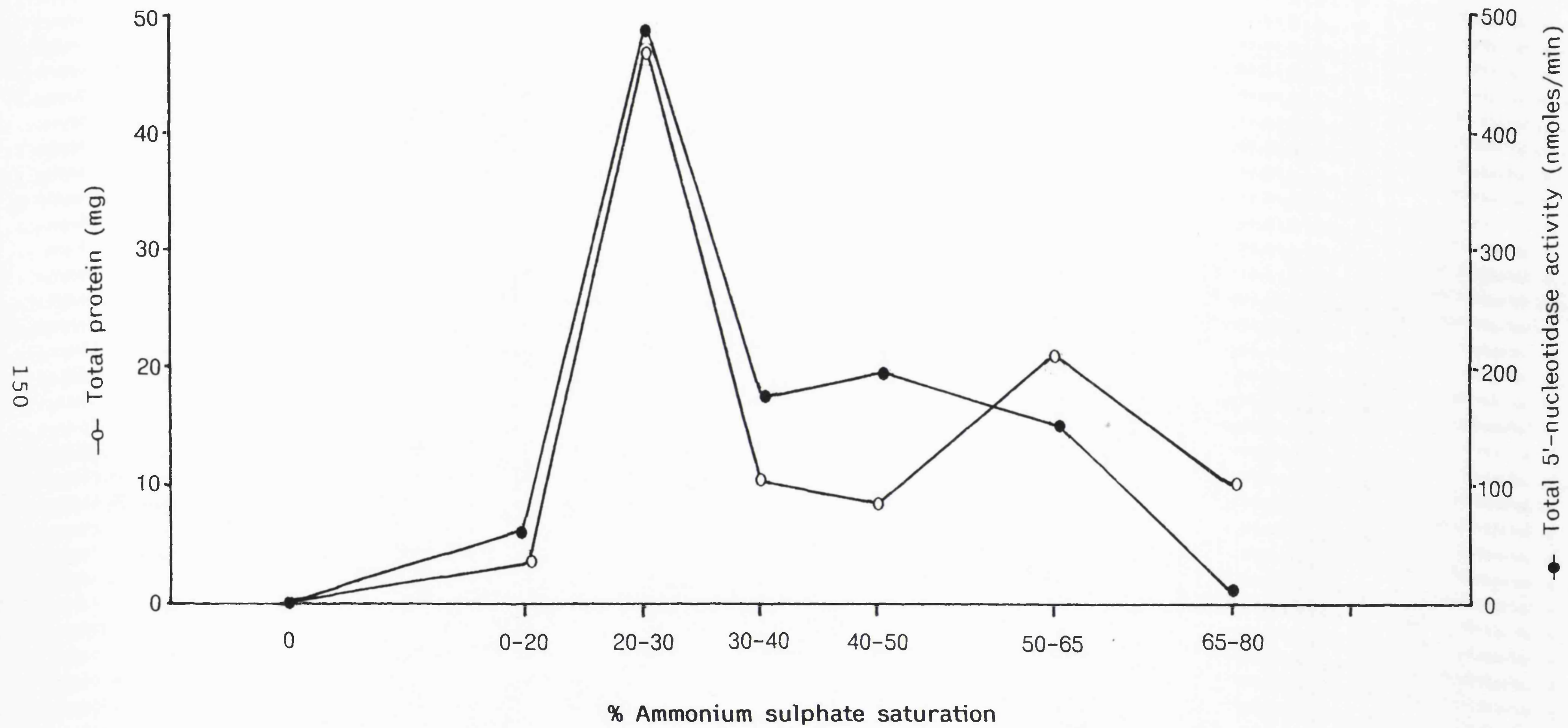
It was reasoned that fractionation of cytosol with ammonium sulphate would selectively 'salt out' the desired enzyme to increase its purity and provide a useful means of buffer exchange for subsequent chromatographic steps. Initial fraction studies (figure 4.4) indicated that a large proportion of the activity was seen to precipitate between 20 and 30% saturation, although a significant amount was also observed at the higher end of the fractionation range (40-65%). This result suggested that two forms of the enzyme may exist with differing solubilities. Due to the relatively low resolution of this technique to separate two proteins, the cytosolic fraction was brought to 65% saturation with ammonium sulphate because it was reasoned that a high resolution chromatographic technique would separate the two forms more efficiently. Although fractionation of this kind was used for initial studies, it was impractical for purification of the enzyme. This conclusion was made on examination of enzyme yield following dialysis of the resuspended ammonium sulphate fraction. The 0-65% ammonium sulphate precipitate resisted complete resuspension, even in the presence of non-ionic detergents such as Tween 20 or Lubrol PX. Moreover, following dialysis, up to 50% of the enzyme activity was irreversibly lost. The use of ammonium sulphate precipitation as a purification step was therefore abandoned. The recovered activity following dialysis was however retained in order to test the idea that two populations of 5'-nucleotidase activity were present as indicated by the ammonium sulphate fractionation experiment.

Figure 4.4 Ammonium sulphate fractionation of soluble 5'-nucleotidase

Cytosolic proteins were precipitated with the desired saturation range of ammonium sulphate as described in section 2.15.1 and assayed for 5'-nucleotidase activity as described in section 2.12.1. Results are means of two separate experiments each assayed in duplicate.

●, Total 5'-nucleotidase activity (nmoles/min)

○, Total protein (mg)


Ammonium sulphate fractionation of cytosol
50
40
30
20
10
0
—o— Total protein (mg)
500
400
300
200
100
0
—●— Total 5'-nucleotidase activity (nmoles/min)
0
0-20
20-30
30-40
40-50
50-65
65-80
% Ammonium sulphate saturation

The dialysed fraction was therefore applied to a Mono Q column equilibrated in 50mM Tris-HCl, pH 8.5. In the absence of surfactants multiple active fractions were observed upon ion exchange chromatographic separation and indicated the presence of enzyme aggregates (figure 4.5). The relevance of this aggregation is unclear, but it may be attributed to hydrophobic patches on the surface of the enzyme, or possibly due to the presence of a PIG anchor. However, there is considerable evidence that the low Km soluble enzyme is derived from the ectoenzyme by cleavage of the PIG anchor (Hooper *et al.*, 1991; Vogel *et al.*, 1992). If this is the case for the soluble brain enzyme, the presence of an intact PIG anchor would be unlikely to account for this slight degree of hydrophobicity observed. Incidently this phenomenon was also reported by Montero & Fes (1982) with studies of soluble 5'-nucleotidase from bovine cerebral cortex. However, by including non-ionic detergents in the separation buffers the aggregation effect could be reversed. As figure 4.6 shows, two populations of activity were observed, one which bound to the ion exchanger, and the other which was excluded. With 5 separate cytosolic preparations 42 ± 1% activity appeared in the unbound fraction and the remaining 58 ± 1% which bound and could be subsequently eluted. The idea that the two activities could result from incomplete disaggregation of the enzyme under these conditions was ruled out by repeating the experiments in the presence of higher amounts of detergent. In fact inclusion of Lubrol PX up to 1%(v/v) in all chromatography buffers did

Figure 4.5 Mono Q anion exchange chromatography of cytosolic proteins in the absence of detergent

2mg of cytosolic proteins were applied to a mono Q column at pH 7.4 in the absence of detergent as described in section 2.15.3. Following 5'-nucleotidase assay multiple active fractions were observed, indicating the formation of aggregates of 5'-nucleotidase upon chromatographic separation.

Figure 4.6 Mono Q anion exchange chromatography of cytosolic proteins in the presence of detergent

Cytosolic proteins were precipated with ammonium sulphate between the range 0-65% saturation as described in section 2.15.1. Following extensive dialysis, 10mg of the enzyme preparation was applied to a mono Q column in the presence of 0.1%(v/v) Tween 20 at pH 8.5 (see section 2.15.3). Assays of 5'-nucleotidase activity revealed the presence of two populations of enzyme activity. Further additions of Tween 20 up to 1%(v/v) still resulted in two populations of activity. Furthermore, application of the unbound fraction to the column under the same conditions still resulted in its exclusion from the exchanger.

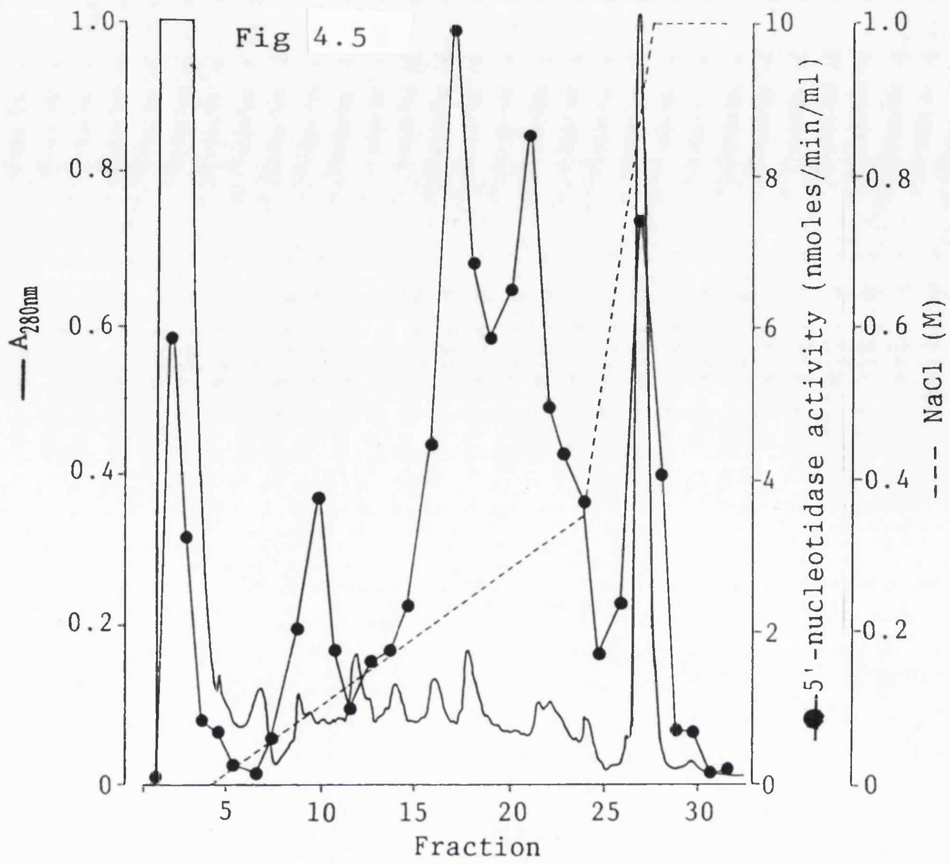


Fig 4.5
— A280nm
●— 5'-nucleotidase activity (nmoles/min/ml)
--- NaCl (M)
Fraction
0
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
5
15
20
25
30

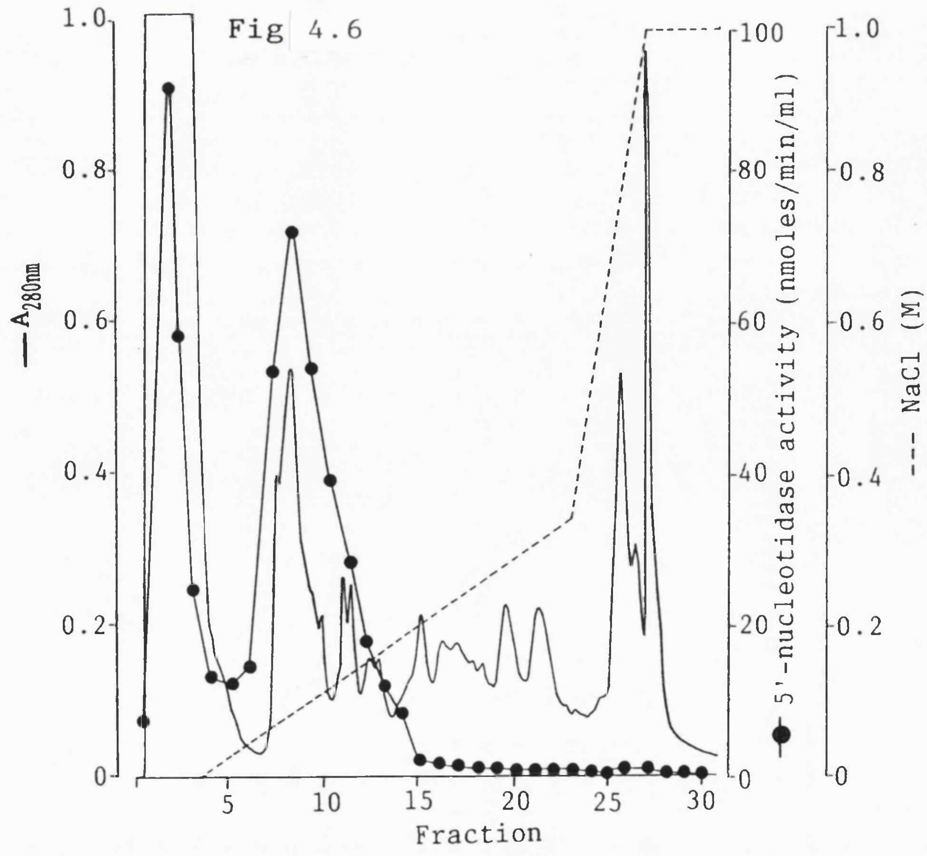


Fig 4.6
— A280nm
● 5'-nucleotidase activity (nmoles/min/ml)
--- NaCl (M)
Fraction
0
0.2
0.4
0.6
0.8
1.0
20
40
60
80
100
5
10
15
25
30

not eliminate the phenomenon. Another explanation for the presence of the unbound enzyme activity could result from overloading of the column with excessive amounts of protein. Again this possibility was ruled out by loading low amounts of protein and hence preventing column saturation. In addition, it was noted that the unbound population of 5'-nucleotidase was still excluded from mono Q when pooled and reapplied to the column under identical conditions. It was therefore concluded that the two populations of 5'-nucleotidase activity were separate entities which could be resolved by Mono Q ion exchange under these conditions. A similar observation was also seen when the ammonium sulphate precipitate or crude cytosol was chromatographed directly over AMP-sepharose. Again two populations of activity were apparent, one which bound to the affinity matrix, and the other which was excluded (results not shown). Incidently, during the purification of 5'-nucleotidase from bovine brain, Montero & Fes (1982) also observed a significant amount of activity which was excluded from DEAE sepharose. This pool was quite separate from that which bound and was consequently purified. These authors however did not study this population of unbound activity further. With the presence of potentially two isoforms of 5'-nucleotidase within the same subcellular compartment, a study was undertaken to compare their properties. Although slight differences in their Km for AMP were observed in a single representative experiment (29μM and 78μM for the bound and unbound forms respectively) a more striking difference was observed with

respect to ATP inhibition. Characterization experiments revealed that both forms were inhibited by ATP, but to different extents. This ruled out the idea that the two forms separated may have been the ATP-tolerant and ATP-inhibitable forms previously described. Furthermore, the ATP-tolerant form appears to be relatively unstable following fractionation unless stored in a buffer containing 25% glycerol and 6mM AMP (Yamazaki *et al.*, 1991). Since these stabilizing agents were not present in the brain preparations it seems unlikey that this activity would survive storage. Assays for enzyme activity were performed using 200μM AMP as substrate compared to the initial characterization studies described above (figure 4.1) where 15mM AMP was used. The ATP-activated cytosolic 5'-nucleotidase has a Km much higher than the substrate concentrations used in these experiments and consequently may not be detected under the conditions of these assays even if it did survive storage. Moreover, it was the ATP-inhibitable enzyme which was of interest and assay conditions were consequently chosen to selectively detect this species. Comparative assays at pH 8.0 in the presence of 10mM magnesium chloride with varying amounts of ATP showed that the bound form had an IC_{50} of approximately 1.5mM whereas the unbound variety had an IC_{50} of less than 0.2mM (figure 4.7). Furthermore, this striking difference in ATP sensitivity was confirmed in a later series of experiments in the absence of magnesium where the ATP was in an unchelated state (section 4.6). Assays under the same conditions with ADP as the inhibitor revealed that changes

Figure 4.7 Differences in sensitivity of 'bound' and 'unbound' forms of 5'-nucleotidase to inhibition by ATP and ADP

'Bound' and 'unbound' forms of soluble 5'-nucleotidase were prepared by mono Q ion exchange chromatography of ammonium sulphate-precipitated cytosolic proteins (see sections 2.15.1 and 2.15.3). The two populations were assayed at pH 8.0 with 200µM AMP. In addition, assays contained 10mM $MgCl_2$ and 20mM β-glycerophosphate. The values are the means ± SEM of 3-4 separate experiments each assayed in duplicate.

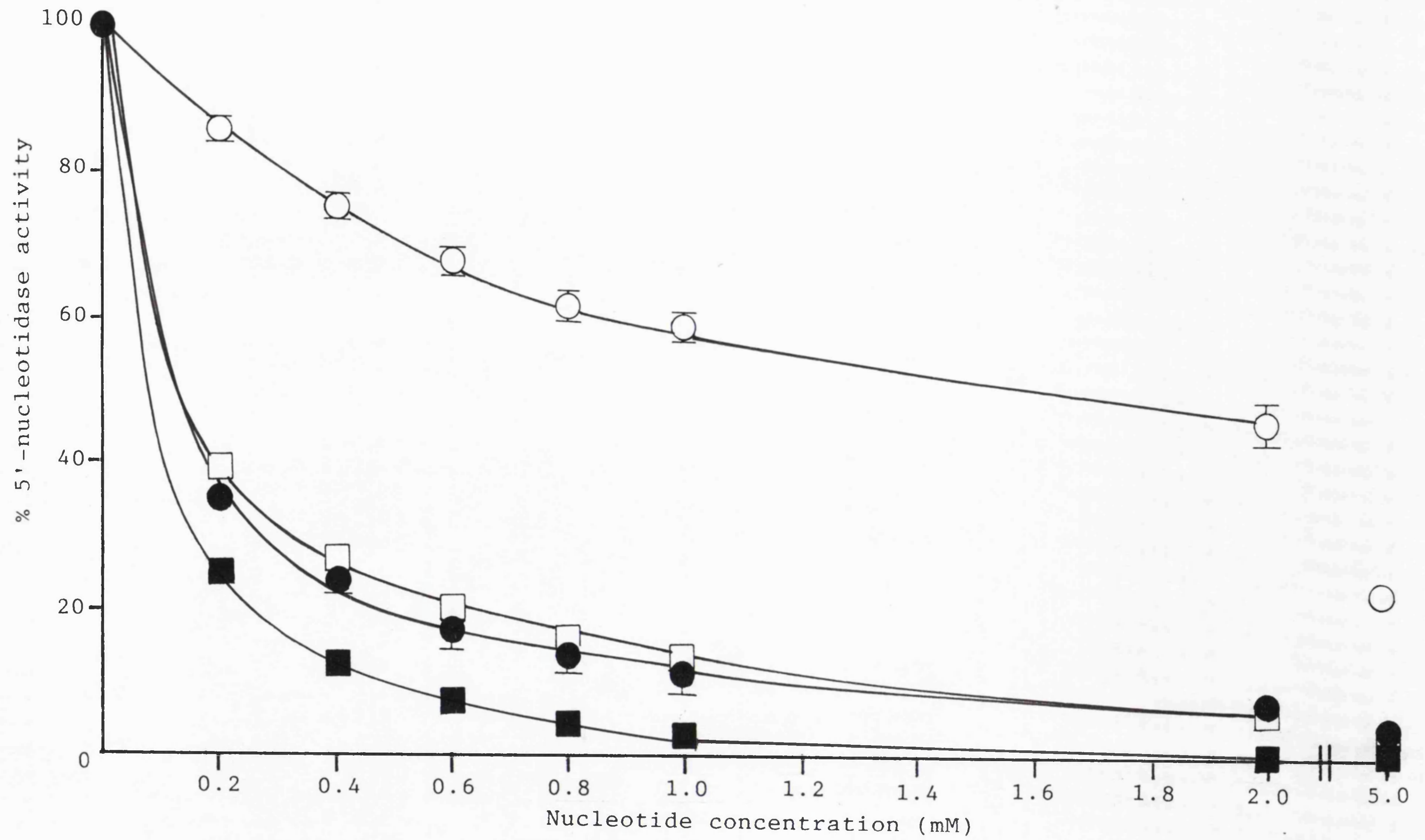
○, 'bound' enzyme with ATP

●, 'unbound' enzyme with ATP

□, 'bound' enzyme with ADP

■, 'unbound' enzyme with ADP

Differences in sensitivity of 'bound' and 'unbound' forms of 5'-nucleotidase to ATP and ADP



in the two forms were still apparent but not as marked as for ATP (figure 4.7). Later studies have revealed that the bound and unbound forms are likely to be the same enzyme. It appears moreover that they can be interconverted by binding to an uncharacterized soluble factor. 5'-nucleotidase in the absence of the factor is capable of binding to the ion exchanger, whereas enzyme which is associated with the factor is excluded from the column. Moreover the factor enhances the sensitivity of the enzyme to inhibition by ATP thereby explaining the different sensitivities of the bound and unbound populations. This offers an explanation as to the differences in sensitivity of the 'bound' and 'unbound' populations to ATP inhibition. The evidence for these findings will be further discussed in section 4.6.

4.3 PURIFICATION OF SOLUBLE 5'-NUCLEOTIDASE

The soluble 5'-nucleotidase was purified to apparent homogeneity by a combination of affinity and ion exchange chromatography. As described above, ammonium sulphate fractionation was omitted from the purification scheme due to low recovery of the enzyme following dialysis. In a preliminary study it was observed that the lectin Concanavalin A (Con A) inhibited both the bound and unbound populations of 5'-nucleotidase generated by mono Q ion exchange chromatography (figure 4.8). This surprising finding indicated that the soluble enzyme was glycosylated and possibly contained its glycans near to the active site. The presence of glycosylation is a common feature of the

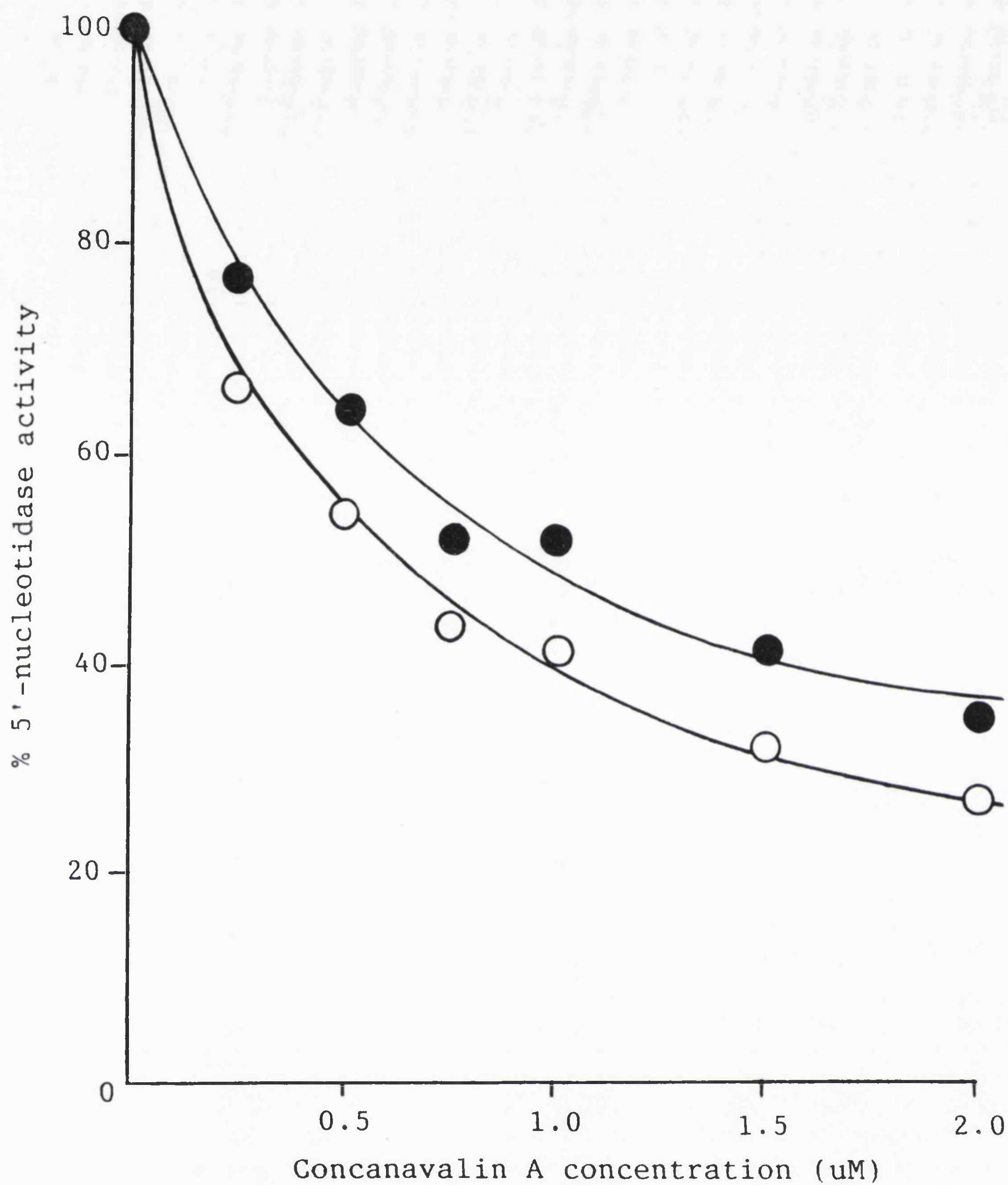


Fig 4.8 Inhibition of bound and unbound 5'-nucleotidase by Concanavalin A

The bound and unbound populations of 5'-nucleotidase produced by mono Q ion exchange were assayed in the presence of various amounts of Concanavalin A at pH 8.0. The results are from a single representative experiment assayed in duplicate.

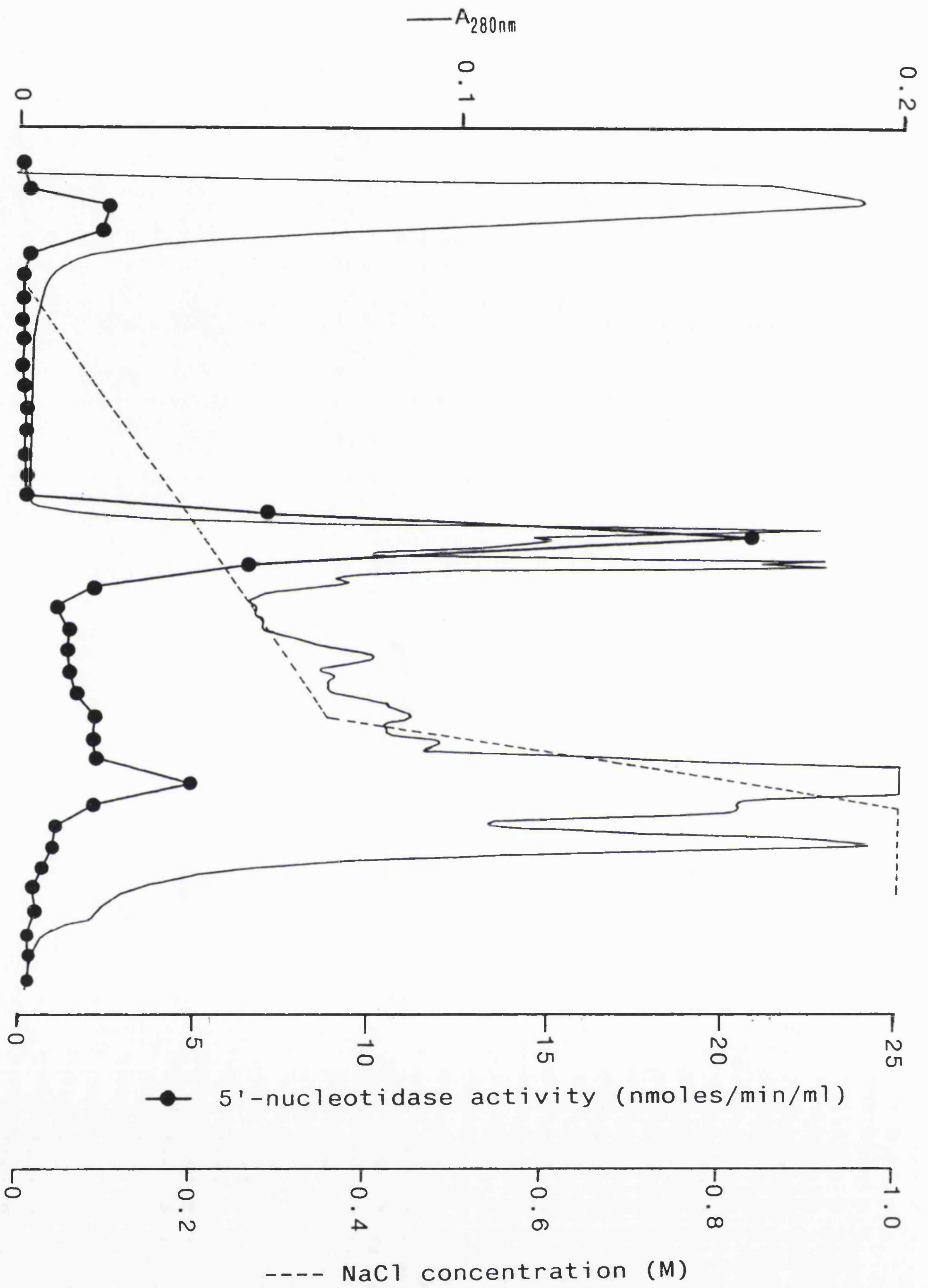
●, bound 5'-nucleotidase activity

○, unbound 5'-nucleotidase activity

ectoenzyme which has been shown to contain 4-5 N-glycosylation concensus sequences. It is not however certain whether the soluble variety reported here is more or less sensitive to Con A inhibition than the ectoenzyme. Assuming the soluble enzyme is derived from the ectoenzyme by PIPLC cleavage, it would be expected to be more sensitive towards inhibition by this lectin as Stochaj *et al.* (1989) reported that cleavage of the PIG anchor resulted in an enhanced sensitivity towards Con A inhibition. Although the activity was recovered in the soluble subcellular fraction of the cell, the presence of glycosylation raises the question as to whether it is a truely cytosolic enzyme or originated from another subcellular compartment or the interstitial fluid. However, it was reasoned that Con A immobilized on a solid sepharose support would provide a useful tool for purification purposes. In fact affinity chromatography of this type has been previously used for the isolation of the ectoenzyme as well as for the low Km soluble enzyme from bovine liver (Zekri *et al.*, 1988) and human placenta (Madrid-Marina & Fox, 1986). Since most cytosolic proteins are expected to lack covalently bound carbohydrate, Con A sepharose was used as the first step in the purification and consequently resulted in a 40 fold purification of the enzyme. Following elution from Con A sepharose, 5'-nucleotidase was applied to a mono Q column at pH 8.5 as previously described. Figure 4.9 shows a typical mono Q run at this stage in the purification. It should be noted that following Con A affinity chromatography, very little activity

Figure 4.9 Mono Q HR 5/5 ion exchange chromatography of partially purified 5'-nucleotidase

Following elution of 5'-nucleotidase from concanavalin A sepharose, the preparation was dialysed against 20mM Tris-HCl, pH 8.5, 0.1% (v/v) Tween 20 and applied to a 1mlMono Q HR5/5 column which had been pre-equilibrated in the same buffer (see section 2.15.3). The concanavalin affinity step removed a soluble factor which appeared to be associated with a population of the enzyme and hence prevented its binding to the ion exchanger. Consequently, the unbound fraction of activity has been eliminated and is now capable of binding (compare to figure 4.6).

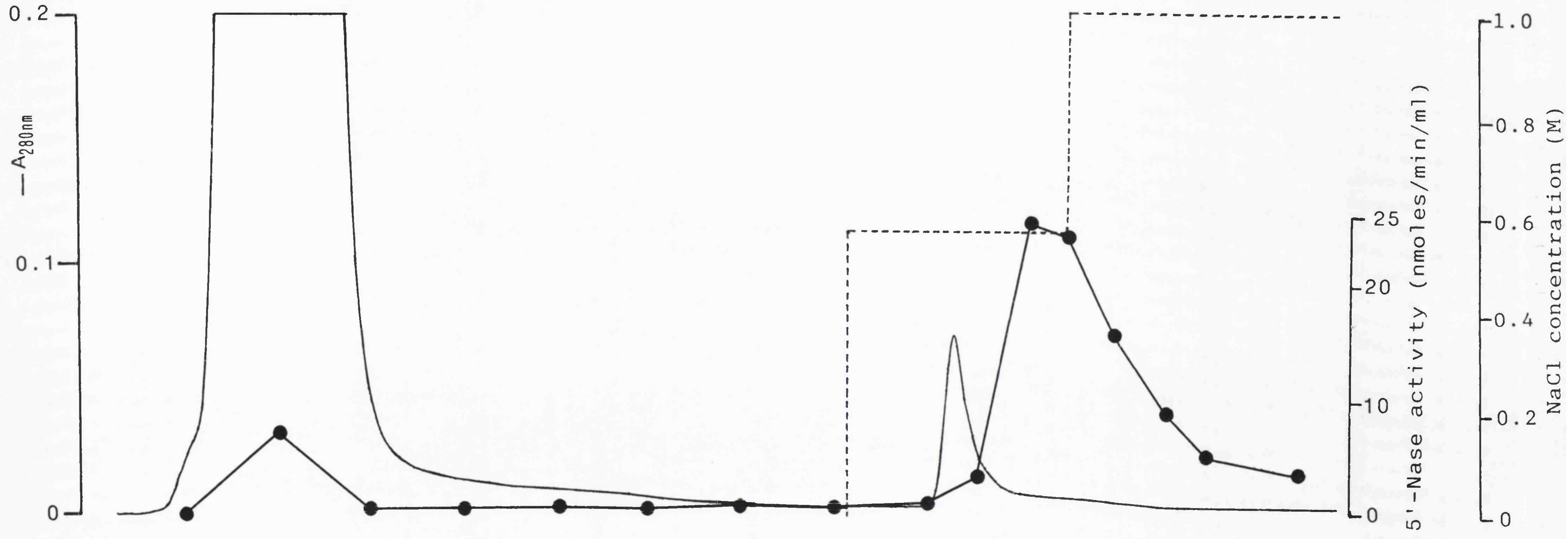


A280nm
0
0.1
0.2
0
5
10
15
20
25
5'-nucleotidase activity (nmoles/min/ml)
0
0.2
0.4
0.6
0.8
1.0
---- NaCl concentration (M)

was excluded from the ion exchanger. This should be directly compared to figure 4.6 where the column was run prior to the Con A step, and approximately 50% was observed in this unbound fraction. Con A sepharose did not however serve to remove the unbound activity as all 5'-nucleotidase activity bound to this matrix and was consequently eluted. However, later experiments revealed that Con A sepharose removes a soluble factor which binds to a population of the enzyme and prevents its binding to mono Q. Therefore by removing the factor all the enzyme was free to bind to the ion exchange resin (see section 4.6). The final purification step employed the use of AMP-sepharose as a specific adsorbant for 5'-nucleotidase. Figure 4.10 shows a typical column run using this final affinity matrix. It can be seen that a great deal of purification can be achieved using a two step elution process. A large proportion of material showed no affinity for AMP and was consequently excluded from the column. Bound proteins showing a lower affinity for the matrix could be eluted with 0.6M NaCl, which also initiated the desorption of 5'-nucleotidase. By raising the NaCl concentration to 1M, 5'-nucleotidase was rapidly eluted from the matrix. In several purification strategies reported in the literature where AMP-sepharose has been used, desorption was generally achieved using either AMP or ATP. It was decided to preferentially use a salt elution however as nucleotides absorb at 280nm and would therefore interfere with the detection of protein at that wavelength. In addition nucleotides used to elute the enzyme would have to

Figure 4.10 AMP-Sepharose affinity chromatography during the course of purification

The peak of 5'-nucleotidase activity from the Mono Q column was dialysed against 50mM Tris-HCl, pH 7.4, 0.1%(v/v) Tween 20 and applied to a 2ml column of AMP-Sepharose which had been pre-equilibrated in the same buffer (see section 2.15.4). Proteins showing a lower affinity for the matrix were eluted with a 0.6M NaCl wash, which also initiated the desorption of 5'nucleotidase. The enzyme was then rapidly eluted with 1M NaCl and assayed for 5'-nucleotidase activity.



0.2
0.1
0
— A_{280nm}
25
20
10
0
5'-Nase activity (nmoles/min/ml)
1.0
0.8
0.6
0.4
0.2
0
NaCl concentration (M)

be removed by dialysis prior to assay due to their interference in the assay. The resulting final preparation was free from non-specific phosphatase activity and appeared to be homogeneous on the basis of SDS-PAGE following silver staining (figure 4.11). It should be noted that the AMP-sepharose column appeared to have a limited lifetime presumably due to degradation of the covalently bound substrate by the action of 5'-nucleotidase. Therefore a fresh column was used for each successive purification to ensure maximum efficiency and reproducible results.

Table 4.2 shows the results of a typical purification experiment. The increase in yield following the initial Con A-sepharose step is probably attributed to the removal of adenine nucleotides from the cytosolic fraction which are known to inhibit the enzyme. For this reason, the final purification fold of 1770 and 68% yield are likely to be overestimates since the pure preparation was compared to the cytosol where the enzyme is in an inhibited state. By comparing the relative mobility of the pure enzyme to proteins of standard molecular weight, the subunit size was estimated to be 54,300 ± 800 Da from 4 separate experiments (figure 4.13). Interestingly, Lai & Wong (1991b) purified a very similar enzyme from rat brain cytosol. These authors reported a subunit molecular weight of 131kDa attributed to the major band of that weight visible on their gels. However, a minor band was additionally visible with a molecular weight of 53kDa which they reported to be a contaminant. Furthermore, the specific activity of their

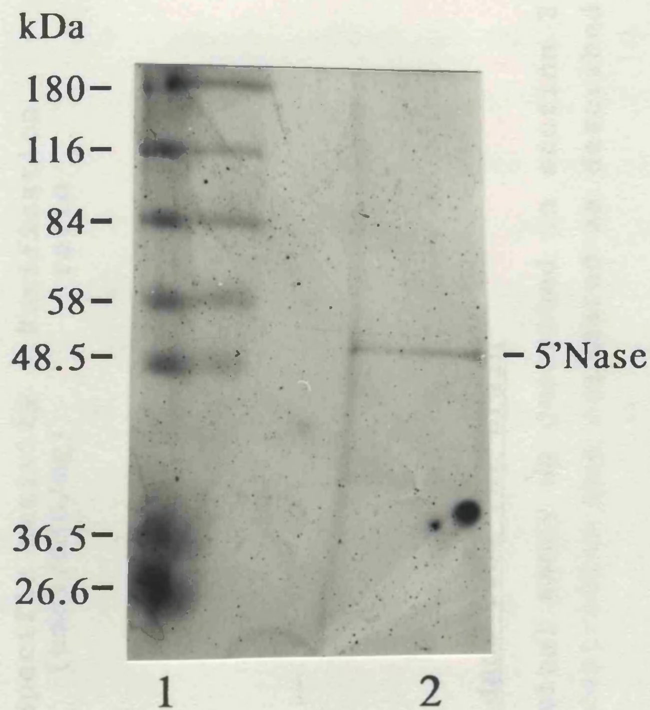


Figure 4.11 SDS-PAGE of purified soluble 5'-nucleotidase

Approximately 10ng of purified soluble 5'-nucleotidase was resolved in 10% SDS-polyacrylamide gels. Proteins were visualized with neutral silver staining as described in section 2.10.2 and shows a single band of molecular weight 54kDa.

Lane 1, Prestained molecular weight markers

Lane 2, Purified soluble 5'-nucleotidase

Fraction	Enzyme activity (μmol/min)	Protein (mg)	Specific activity (μmol/min/mg)	Purification ratio	Yield (%)
Cytosol	1.300	147.25	0.009	1.0	100.0
ConA-Sepharose	4.938	14.00	0.353	40.0	380.3
Mono Q	3.066	0.94	3.254	369.0	236.1
AMP-Sepharose	0.884	0.06	15.635	1772.7	68.1

Table 4.2 <u>Purification of soluble 5'-nucleotidase from rat brain</u>

Specific activity was measured by the radiochemical assay as described in section 2.12.1 at pH 7.4 using AMP as substrate. Protein concentration was estimated as described in section 2.4.2.

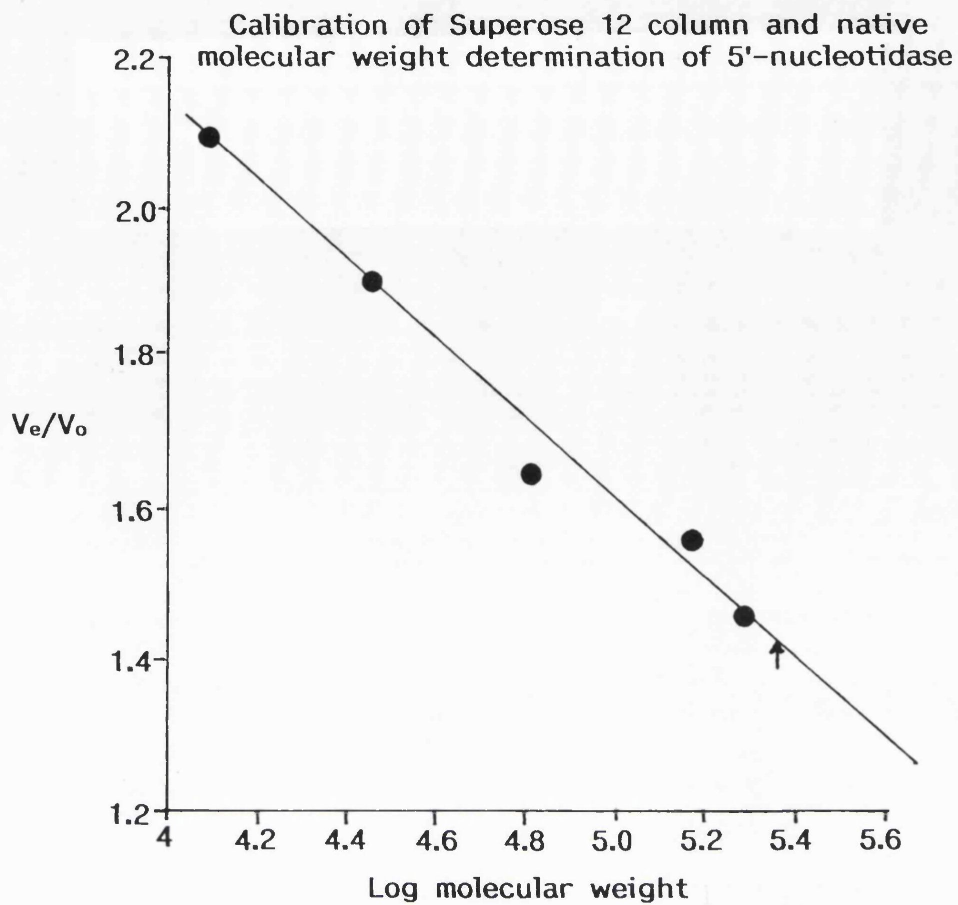
purified enzyme was some 2 fold lower than that reported here. No high molecular weight band was seen in this preparation and its removal would account for the higher specific activity observed. It seems likely therefore that the 131kDa band in their preparation was the contaminating species and the presence of a 53kDa band confirms the observations reported here. The subunit size of the purified brain enzyme is smaller than reported values for the ectoenzyme or low Km soluble 5'-nucleotidase. These enzymes are generally the same size and fall in the range 70-74kDa (Thompson *et al.*, 1987; Klemens *et al.*, 1990; Piec & Le Hir, 1991; Vogel *et al.*, 1992). This finding would be expected if the soluble form originated as a result of PIPLC cleavage of the ectoenzyme. However, the smaller subunit size of the purified brain enzyme is inconsistent with these findings and is unlikely to represent either of these forms reported. Furthermore, it is also unlikely that the brain enzyme represents a deglycosylated form of the ectoenzyme which could account for a lower subunit weight. In a totally deglycosylated form, the ectoenzyme shows a subunit size of 56-59kDa (Wada *et al.*, 1986; Van den Bosch *et al.*, 1986; Baron & Luzio, 1987; Klemens *et al.*, 1990), a size still larger than that reported in this study. Moreover, if the enzyme was completely deglycosylated, it would fail to interact with Con A. However, the purified enzyme might represent a proteolytically truncated form of the ectoenzyme or a novel form resulting from an alternative splicing mechanism. It should be noted that the same results were

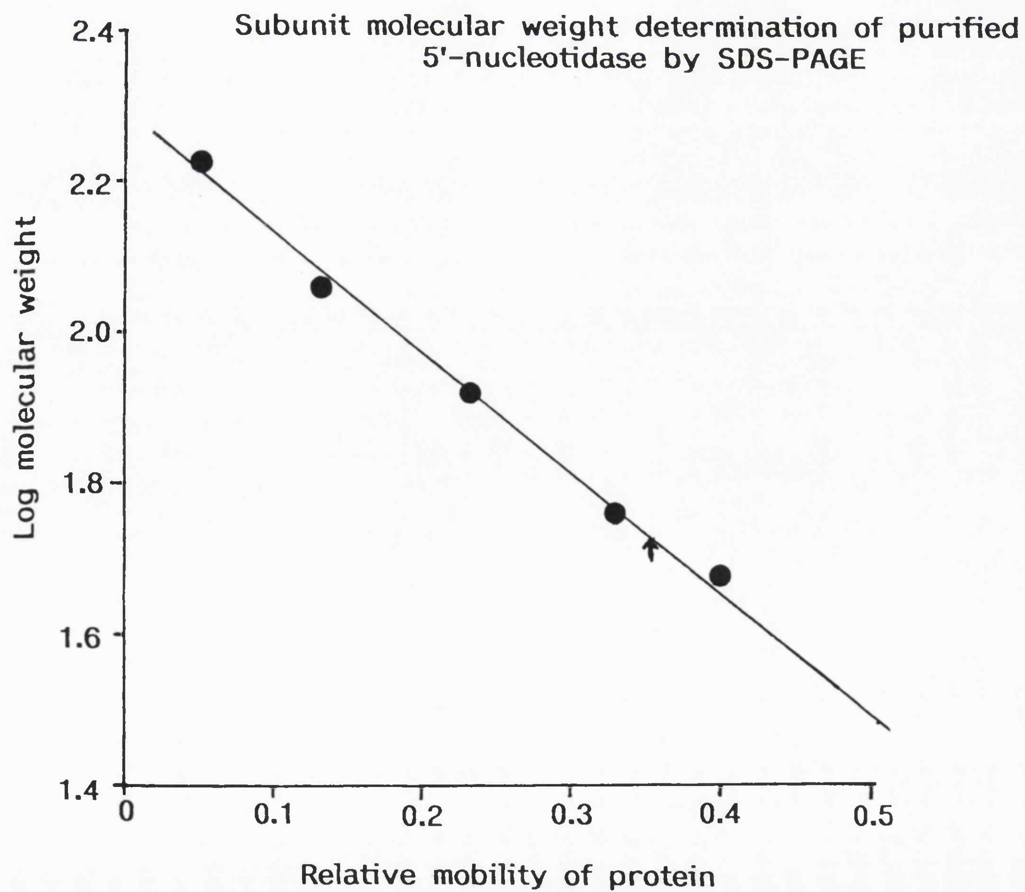
Figure 4.12 Calibration of Superose 12 gel filtration column and native molecular weight determination of purified 5'-nucleotidase

Gel filtration chromatography was carried out in 50mM Tris-HCl, pH 7.4, 0.1%(v/v) Tween 20, 1M NaCl as described in section 2.15.5. Standard proteins used consisted of; cytochrome C (12.4kDa), carbonic anhydrase (29kDa), albumin (66kDa), alcohol dehydrogenase (150kDa) and β-amylase (200kDa). The void volume was estimated using blue dextran (molecular weight 2000kDa). 5'-nucleotidase eluted with a retention time consistent with a molecular weight of 230kDa (n=1) as shown (↑).

Figure 4.13 Subunit molecular weight determination of purified 5'-nucleotidase by SDS-PAGE

SDS-PAGE was carried out in 10% gels as described in section 2.7. Prestained molecular weight markers consisted of subunits of; α_2-macroglobulin (180kDa), β-galactosidase (116kDa), fructose-6-phosphate kinase (84kDa), pyruvate kinase (58kDa) and fumarase (48.5kDa). 5'-nucleotidase migrated as a single band of 54,300 ± 800 (n=4).


Calibration of Superose 12 column and native molecular weight determination of 5'-nucleotidase
2.2
2.0
1.8
Ve/Vo
1.6
1.4
1.2
4
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6
Log molecular weight


Subunit molecular weight determination of purified 5'-nucleotidase by SDS-PAGE
2.4
2.2
2.0
1.8
1.6
1.4
Log molecular weight
0
0.1
0.2
0.3
0.4
0.5
Relative mobility of protein

* Pepstatin A (100μM)
  Leupeptin (100μM)
  Antipain (100μM)
  Phenylmethanesulphonyl fluoride (0.1mM)

obtained when the purification was repeated in the presence of a cocktail of proteolytic inhibitors.* Therefore if proteolytic truncation is the reason for the smaller size, it is likely to be an event mediated *in vivo* rather than due to degradation during the purification procedure. From the experiments however, it could not be concluded that the enzyme initiated from the cytosol as the high speed supernatant from whole brain homogenate would additionally contain extracellular soluble proteins. It may be possible that the enzyme is a secreted form of the ectoenzyme which functions to dephosphorylate AMP in the extracellular compartment, possibly in areas devoid of the ectoenzyme. Further studies however are need to conclusively confirm the true location of the enzyme within the tissue.

The native molecular weight of the purified enzyme was estimated by gel filtration chromatography on a calibrated superose 12 column. These experiments were problematic because of the tendancy of the enzyme in its highly purified state to form high molecular weight aggregates. Consequently it appeared in the void volume of the column in each experiment, indicating a molecular weight of greater than 2 million. However, by repeating the gel filtration in buffers containing high salt (1M NaCl) in addition to 0.1%(v/v) Tween 20 this aggregation could be eliminated. In a single representative experiment the enzyme eluted with a retention time consistent with a molecular weight of 230kDa (figure 4.12). Therefore the enzyme may exist as a tetramer, although this result should be treated with caution due to

the relatively harsh conditions used to determine the native size. This finding is in partial agreement with the finding that the low Km soluble enzyme exists as both dimers and tetramers linked by interchain disulphide linkages. Moreover, it has been reported that intact disulphide bridges are essential for enzyme activity (Worku *et al.*, 1984; Fini *et al.*, 1985). It was therfore necessary to test whether this was the case for the purified brain enzyme. As figure 4.14 demonstrates, reduction by dithiothreitol (DTT) results in loss of enzyme activity. In fact pre-treatment with 2mM DTT inhibited the enzyme by 50% thereby showing that intact disulphide bridges may be required for activity in common with the findings reported previously.

4.4 IMMUNOLOGICAL STUDIES

With the availability of a pure preparation of the enzyme, it was decided to raise a polyclonal antiserum for further studies. With such an antiserum it would therefore be possible to expand the studies concerning the activity of the enzyme in the various brain regions. Such studies were to be directed towards quantitation of the amounts of 5'-nucleotidase protein in the control and hypothyroid states, and to correlate these results with changes seen in the activity to ascertain whether the activity changes observed were attributed to altered abundance of 5'-nucleotidase protein. Therefore an attempt was made to raise a polyclonal antiserum directed towards the purified brain enzyme. Following four innoculations of 50µg of the enzyme into a

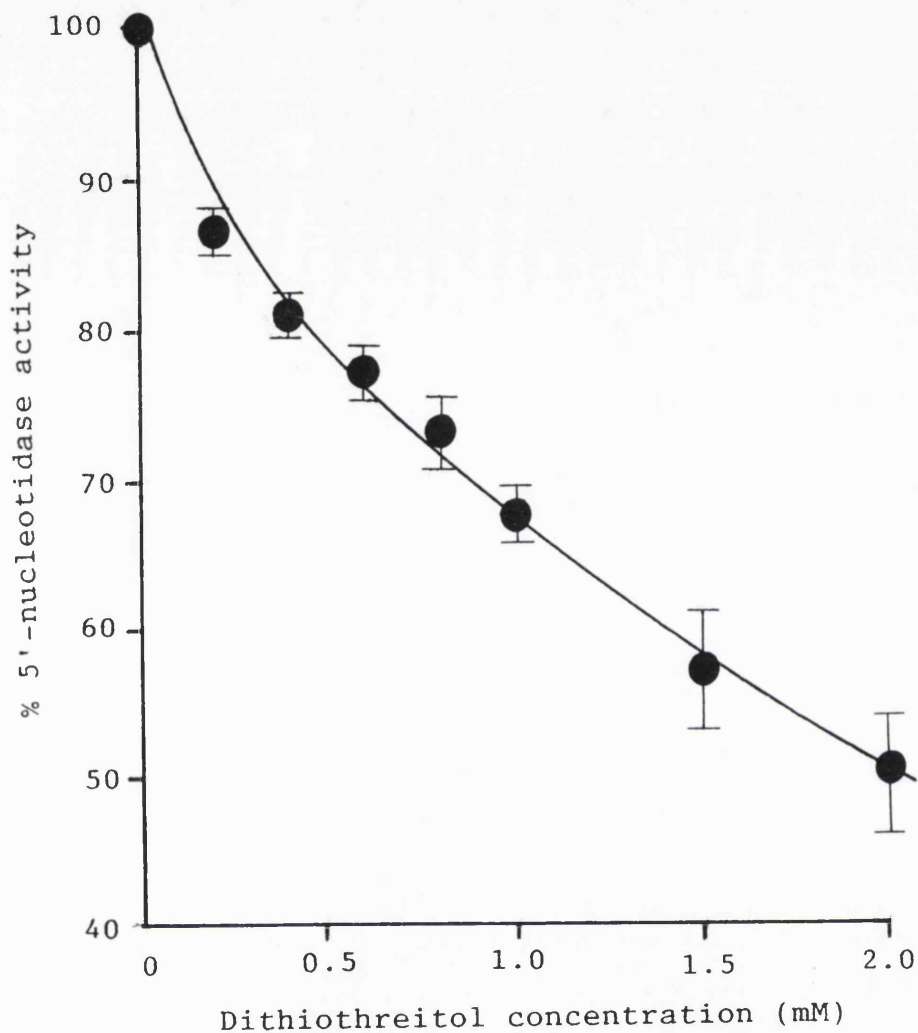


Fig 4.14 EFFECT OF DITHIOTHREITOL ON PURIFIED SOLUBLE 5'-NUCLEOTIDASE ACTIVITY

The purified soluble enzyme was incubated with varying amounts of DTT for 5 minutes at 37°C prior to assay for 5'-nucleotidase activity. The results are means ± SEM for three separate experiments each assayed in duplicate.

Dutch-Cross rabbit, such an antiserum was produced which cross-reacted with the purified enzyme on a western blot and consequently confirmed the subunit weight of the purified enzyme (figure 4.15). However, on western blots the antiserum appeared to cross react with many proteins present in the cytosol (results not shown) and its use was therefore limited. It was possible that the antiserum recognised epitopes common to many proteins, for example the Rossman fold motif in the nucleotide binding region of many enzymes. For this reason it would not be a useful reagent for quantitation of the enzyme in cytosolic preparations and its use was consequently abandoned. A strong similarity between the soluble brain enzyme and the ectoenzyme was suggested by the finding that the purified enzyme cross-reacted with a polyclonal antiserum raised against the rat liver ectoenzyme (figure 4.16). In a series of experiments 0.3µg of the purified enzyme was pre-incubated with varying amounts of the antiserum prior to enzyme assay. This revealed that 40µg of antiserum protein inhibited greater than 90% of the enzyme activity. Furthermore, a parallel experiment was performed where the antigen:antibody complex was precipitated with protein A (immobilized on acrylic beads). Under these conditions similar results were obtained although slightly smaller amounts of antiserum were required to inhibit the enzyme (30µg of antiserum protein inhibited 95% of enzyme activity). This antiserum was a generous gift from Dr AC Newby, and at the time of the study no pre-immune serum was available for the control experiment. However these results

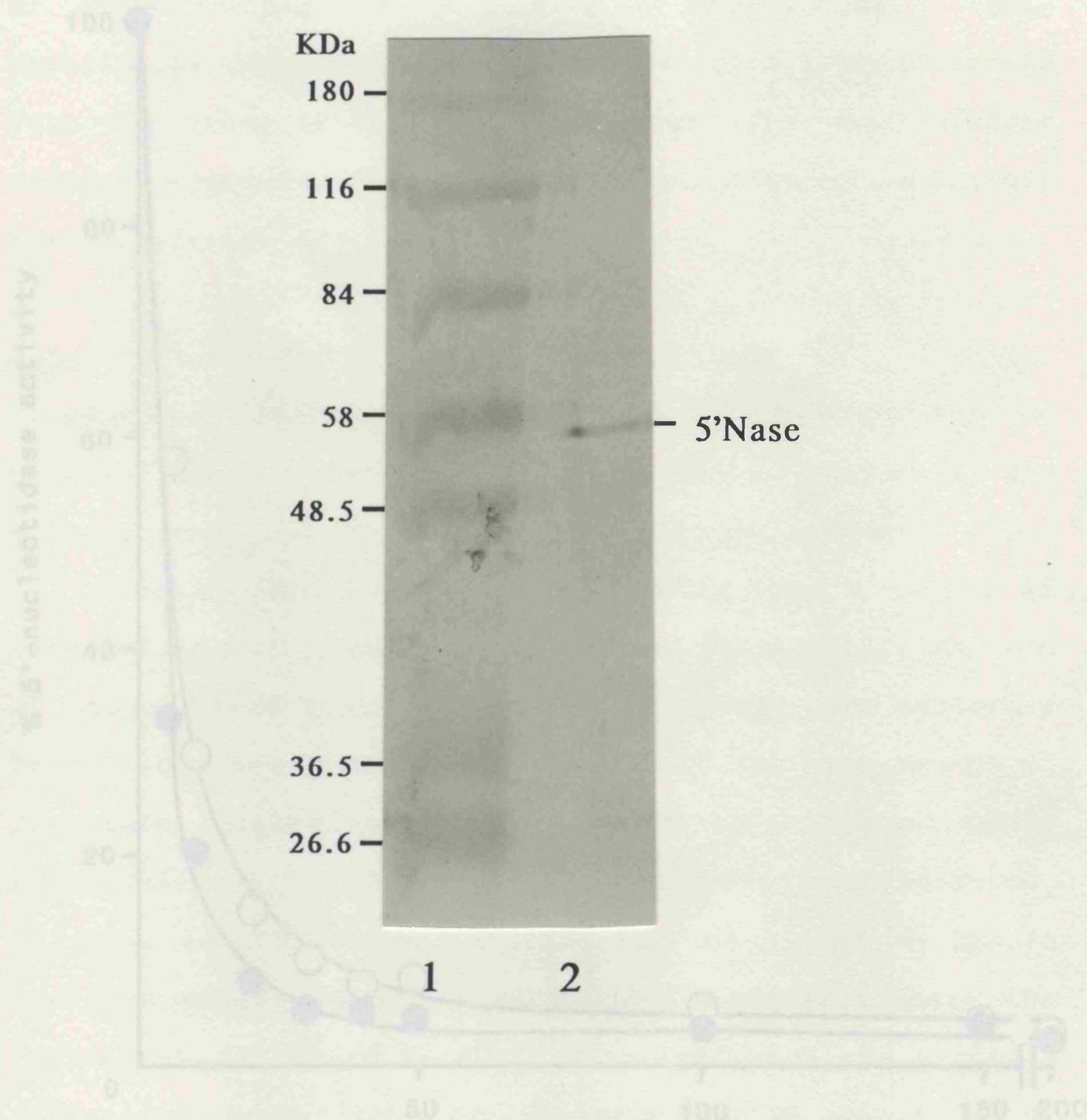


Figure 4.15 Immunoblotting of purified soluble 5'-nucleotidase

Approximately 10ng of purified soluble 5'-nucleotidase was resolved in a 10% SDS-polyacrylamide gels and transfered to nitrocellulose (see sections 2.7 and 2.8). A polyclonal antibody raised against the same enzyme (section 2.16) was used to probe the blot at 1:25 dilution by the protocol described in section 2.10.1.

Lane 1, Prestained molecular weight markers

Lane 2, Purified soluble 5'-nucleotidase

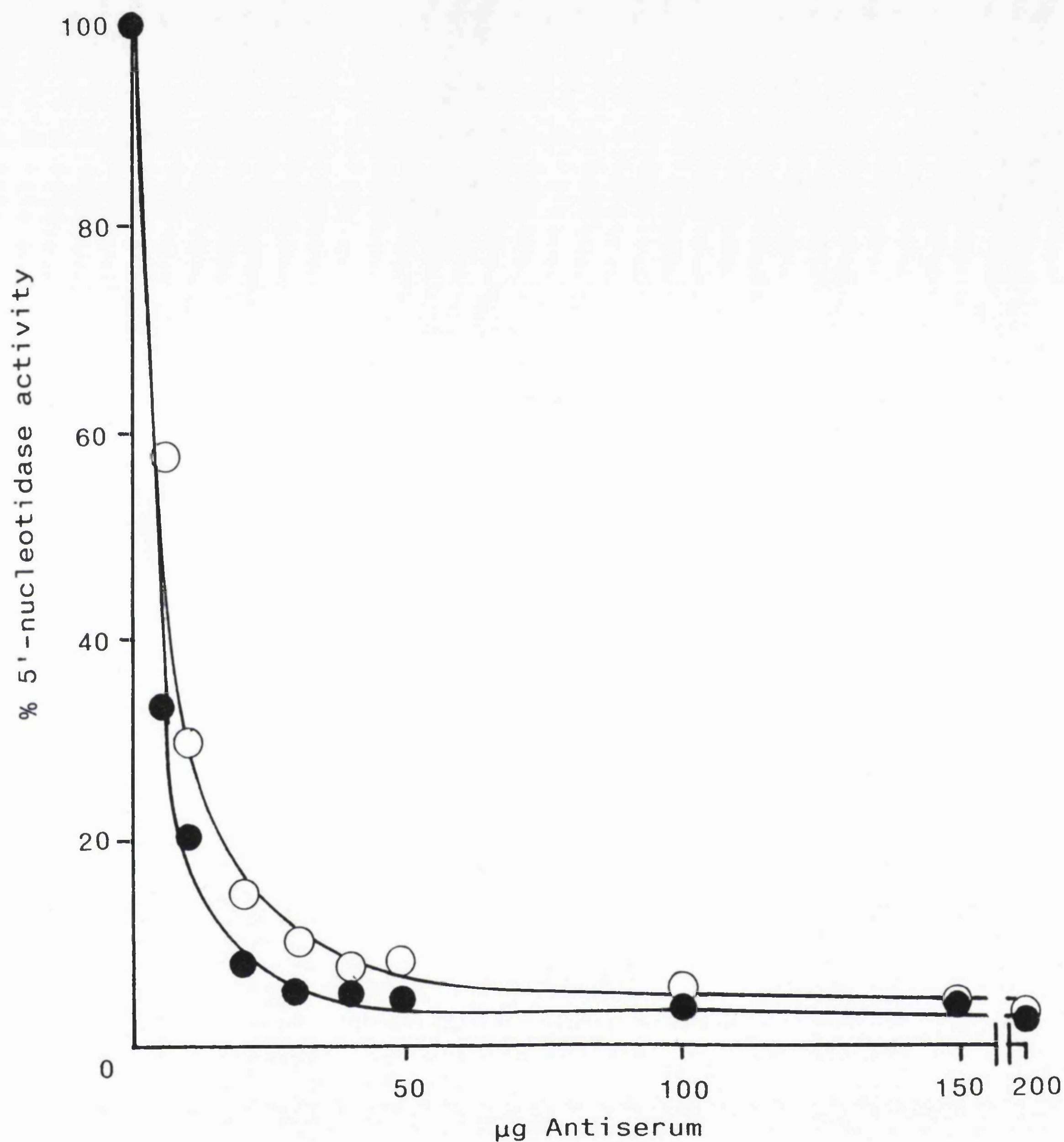


Fig 4.16 <u>Inhibition and immunoprecipitation of purified soluble 5'-nucleotidase by anti rat liver ecto 5'-nucleotidase antiserum</u>

0.3µg of purified enzyme was incubated with the indicated amounts of antiserum at 37°C for 2 hours. It was either assayed directly for 5'-nucleotidase activity (○), or further incubated for 30 minutes at room temperature with protein A immobilized on acrylic beads. Following centrifugation the supernatant was assyed for 5'-nucleotidase activity (●).

do tend to suggest that the antiserum recognises epitopes at or close to the catalytic site of the enzyme. These experiments discriminate the purified soluble brain enzyme from the other truly cytosolic forms of 5'-nucleotidase which show no cross-reactivity with antibodies raised against the ectoenzyme.

4.5 KINETICS OF THE PURIFIED ENZYME

In general, a majority of the kinetic data observed for the purified brain enzyme were similar to those reported for the ectoenzyme or low Km soluble form of 5'-nucleotidase.

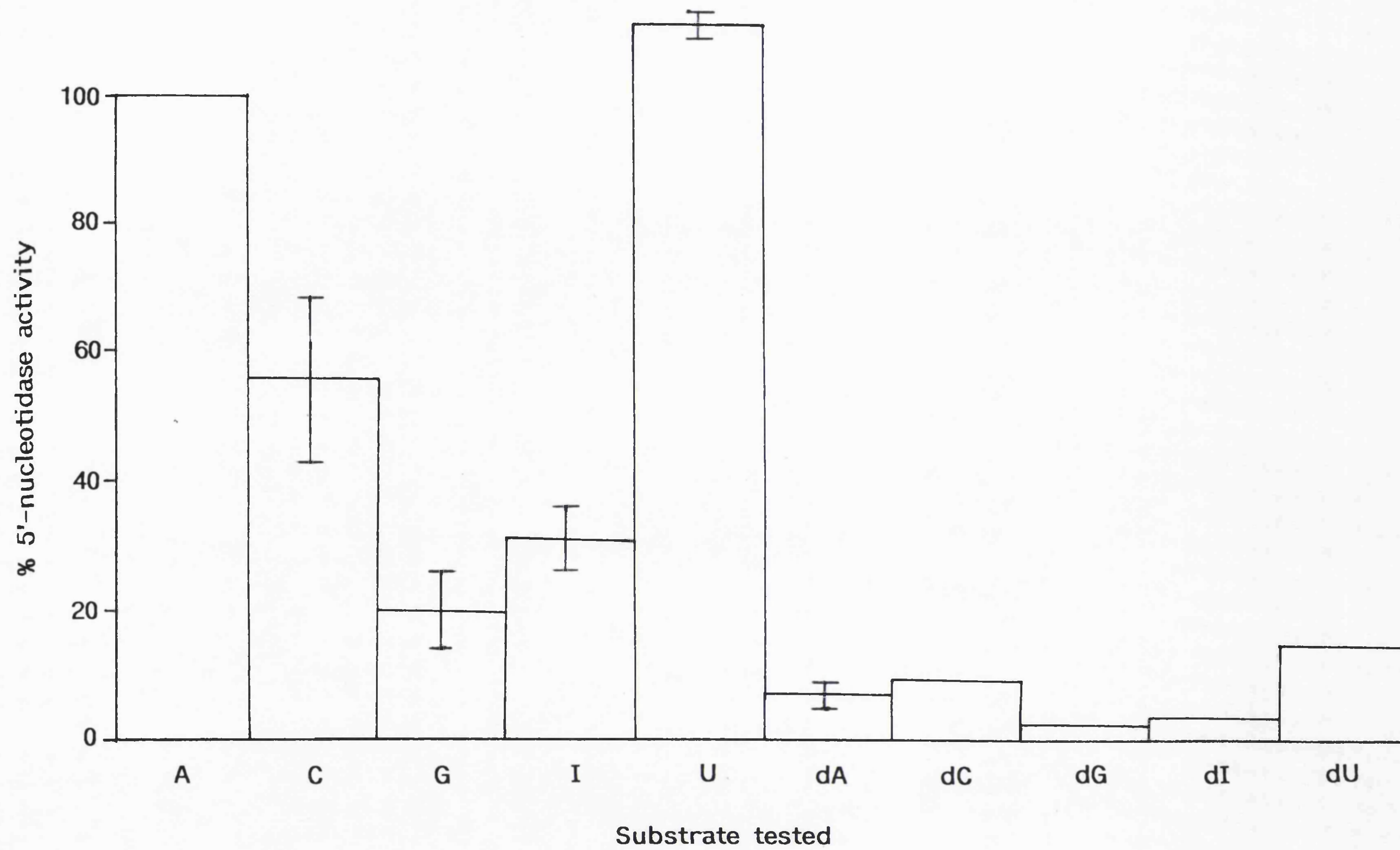
Figure 4.17 summarises the results from a series of experiments performed to establish the substrate preference of the purified brain enzyme. These assays were performed by a colourimetric assay which involved the measurement of phosphate released as opposed to the radiochemical assay which was used for all the other experiments. In addition, a higher substrate concentration was used (2mM) to ensure that the assays were conducted under conditions where the enzyme was likely to be fully saturated with the proposed substrate. Under these conditions it can be seen that the purified enzyme displays activity towards most nucleoside 5'-monophosphates, but shows a clear preference for AMP and UMP. No activity was detected with TMP, XMP or cyclic AMP and the enzyme clearly showed a strong preference for ribo- than deoxyribo nucleoside 5'-monophosphates as substrate. The results presented here for the purified brain enzyme are in agreement with those reported for the rat liver enzyme

Figure 4.17 Substrate specificity of purified 5'-nucleotidase

The purified enzyme was assayed for 5'-nucleotidase activity at pH 7.4 using various nucleoside 5'-monophosphates at a concentration of 2mM. Phosphate liberated in terminated assay supernatants was consequently measured as described in section 2.5, and activity expressed as a percentage of the activity measured with AMP as substrate. Values are means of 1-3 separate experiments each assayed in duplicate.

A: Adenosine 5'-monophosphate (AMP)
C: Cytidine 5'-monophosphate (CMP)
G: Guanosine 5'-monophosphate (GMP)
I: Inosine 5'-monophosphate (IMP)
U: Uridine 5'-monophosphate (UMP)
dA: 2'-deoxyadenosine 5'-monophosphate (dAMP)
dC: 2'-deoxycytidine 5'-monophosphate (dCMP)
dG: 2'-deoxyguanosine 5'-monophosphate (dGMP)
dI: 2'-deoxyinosine 5'-monophosphate (dIMP)
dU: 2'-deoxyuridine 5'-monophosphate (dUMP)

Substrate specificity of purified 5'-nucleotidase



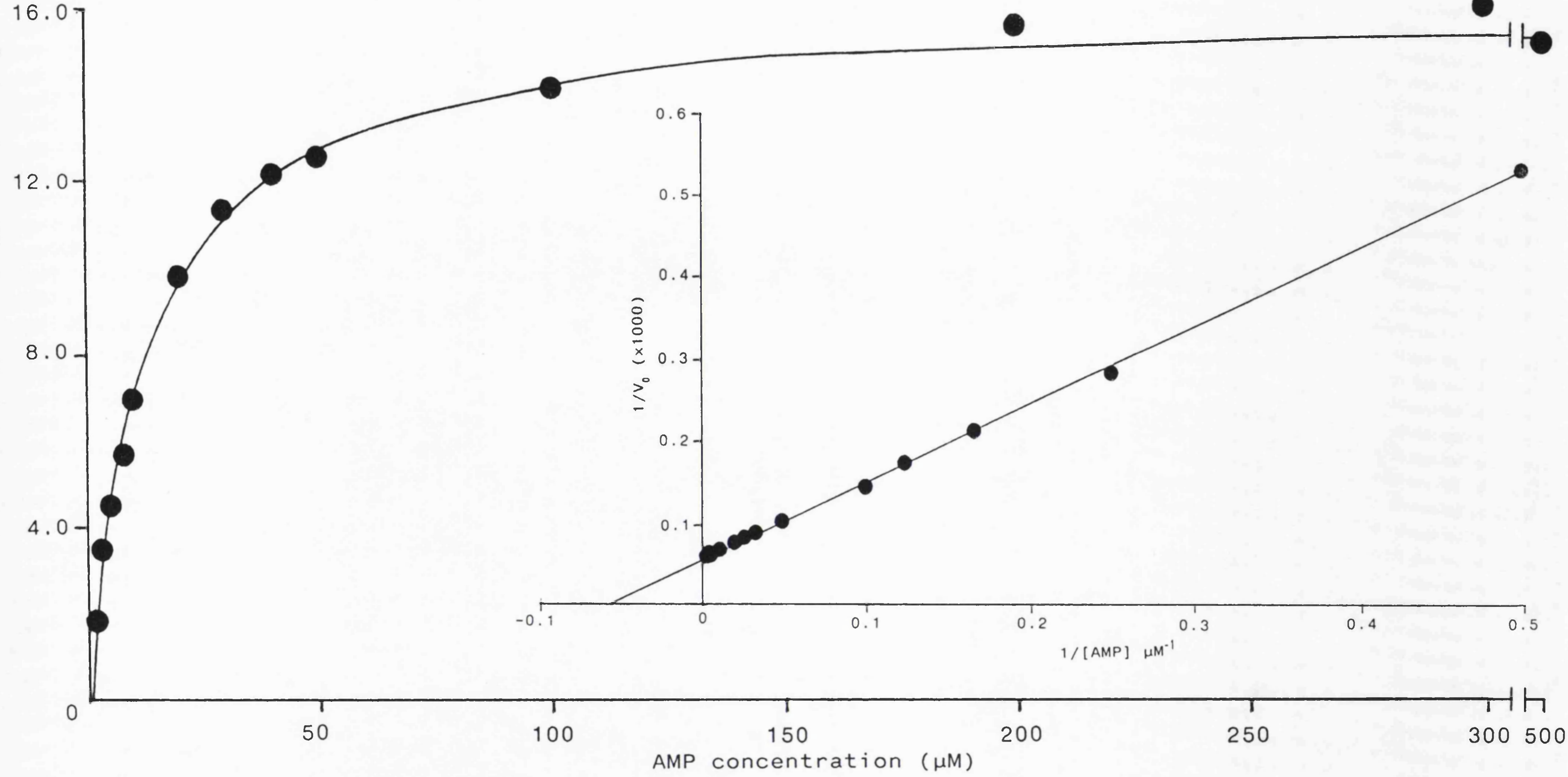
(Fritzson *et al.*, 1986) and the enzyme purified from rat brain (Lai & Wong, 1991b), but contrast somewhat to those observed with the enzyme from human placenta (Madrid-Marina & Fox, 1986) or bovine brain (Montero & Fes, 1982). These discrepancies may however reflect differences in assay conditions used by different authors.

The kinetics of the purified enzyme were studied in the absence of ATP at pH 7.4 using AMP as substrate. Hyperbolic V/S plots were observed with respect to increasing concentrations of AMP and Michaelis-Menton kinetics were obeyed following linear transformation of the data (figure 4.18). From the intercept on Lineweaver-Burke plots the Km for AMP was estimated to be 15μM, a value closely resembling that of the low Km soluble enzyme measured in a variety of tissues (Montero & Fes, 1982; Vogel *et al.*, 1992; Fritzson *et al.*, 1986; Spychala *et al.*, 1989; Madrid-Marina & Fox, 1986). With the assumption of one active site per subunit, the Kcat was calculated to be 14 S^{-1}.

On the basis of pH optima, the two cytosolic forms differ greatly from the ectoenzyme or low Km-soluble variety. Whereas the former cytosolic types display sharp pH optima in the range 6.5-7.5 the latter types show a broad activity in the neutral to alkaline end of the pH scale. From figure 4.19, it can be seen that the purified soluble brain enzyme falls into the latter category with the highest activity observed between pH 7-9. Incidently, the same observation was apparent for both the 'bound' and 'unbound' populations of 5'-nucleotidase generated on mono Q, a finding that

Figure 4.18 Effect of AMP concentration on purified soluble 5'-nucleotidase

0.6μg of the purified enzyme was assayed for 5'-nucleotidase activity in 50mM Tris-HCl, pH 7.4 as described in section 2.12.1. The values are the means of two separate experiments each assayed in duplicate. The Km (14.75μM) and Vmax (17.3μmoles/min/mg) were determined from the inset Lineweaver-Burk plot (Γ=0.996).



5'-nucleotidase activity (µmoles/min/mg)
16.0
12.0
8.0
4.0
0
50
100
150
200
250
300
500
AMP concentration (µM)
1/V0 (x1000)
0.6
0.5
0.4
0.3
0.2
0.1
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[AMP] µM-1

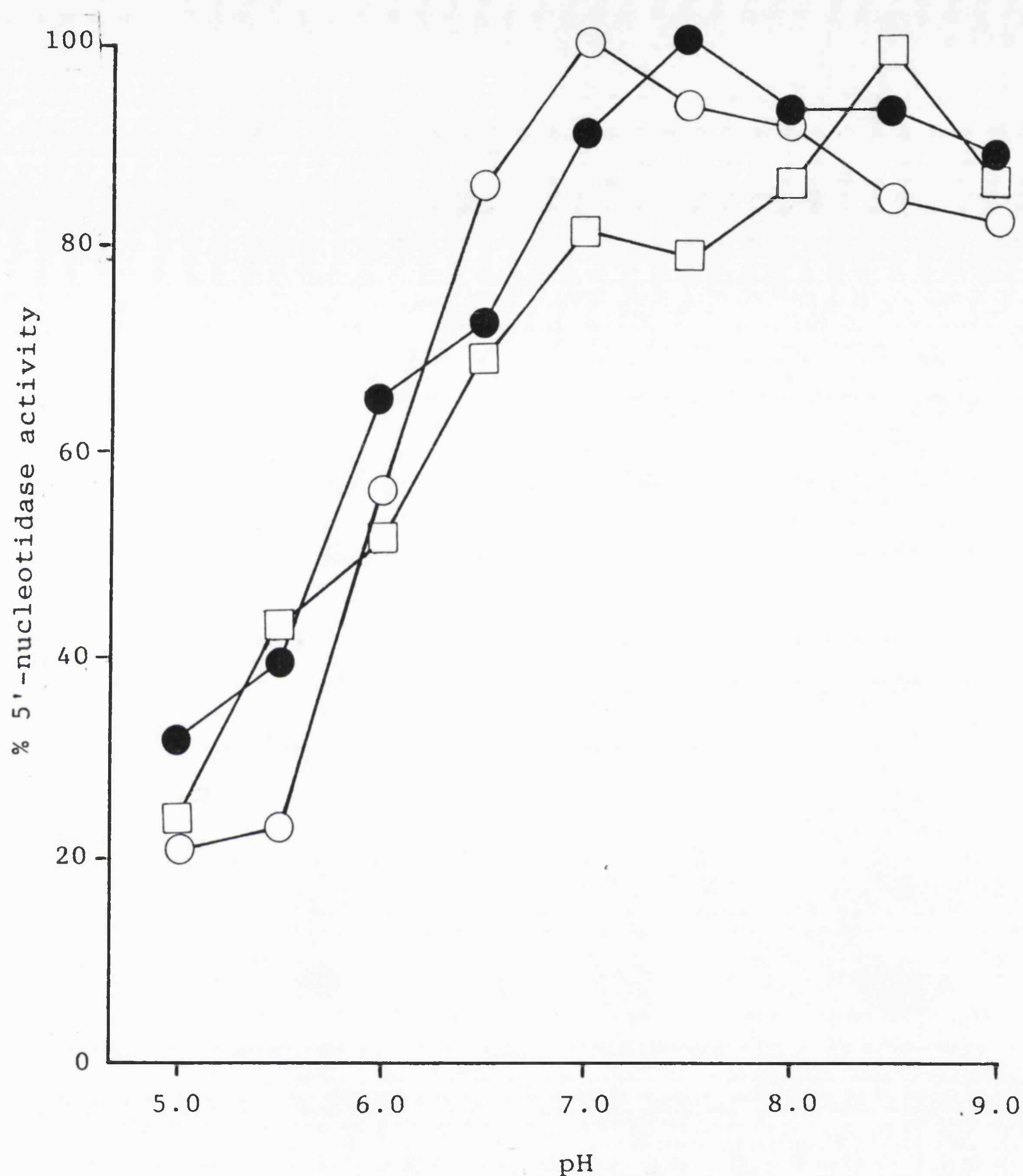


Fig 4.19 <u>Effect of pH on soluble 5'-nucleotidase</u>

Fractions of bound and unbound 5'-nucleotidase from mono Q were assayed alongside the purified enzyme at various pH values. The assay buffers contained 50mM Tris-HCl, 50mM Glycine, 50mM MES, 20mM β-glycerophosphate adjusted to the respective pH value. 5'-nucleotidase activity was assayed in duplicate as described in section 2.12.1. The results are from a single representitive experiment.

○ Bound activity

● Unbound activity

□ Purified enzyme

further supports the conjecture that that these two are the same enzyme. Furthermore, these results indicate that the presence of the soluble factor is ineffective in altering the enzyme activity with respect to pH.

Assays performed in the presence of phosphate (1-10mM) do not appear to affect the enzyme activity (results not shown). This finding is in general agreement with previous reports for the ectoenzyme, and the low Km-soluble form, but contrasts with the cytosolic forms where phosphate inhibits by increasing the sigmoidicity of the V/S plot, resulting in decreased activity at low substrate concentrations.

In contrast to the membrane-bound ectoenzyme, cytosolic 5'-nucleotidase activity shows an absolute requirement for divalent cations. The physiological metal ion responsible is likely to be magnesium as cytosolic levels occur in the millimolar region, a concentration more than sufficient to ensure enzyme activity. With regards to the low Km-soluble form, magnesium does not appear to be essential for catalysis, but is likely to modulate enzyme activity indirectly by reversing ATP and ADP inhibition. One such study performed on the enzyme isolated from bovine brain clearly demonstrated this effect (Mallol & Bozal, 1983). The authors measured 5'-nucleotidase activity in the presence of 2μM ATP and varying amounts of magnesium chloride. It was observed that ATP inhibition was lowered by the addition of increasing amounts of magnesium ions. As figure 4.20 shows, this effect is also apparent for the enzyme purified from rat brain. Assays were performed with varying amounts of ATP at

different fixed concentrations of magnesium chloride. A family of curves were generated which indicated that magnesium ions lessened the inhibitory action of ATP. A more striking observation however was noted when the concentration of free ATP in each of the assays was calculated. It was noticed that on replotting the data from figure 4.20 against the calculated free ATP concentration, all the curves were shifted onto a single curve indicating that the magnesium effect had been lost (figure 4.21). These results confirm the findings of Mallol & Bozal (1983) and suggest that free ATP was the inhibiting species. Physiologically it is likely that magnesium functions to lower ATP inhibition presumably by binding to it thereby lowering the free ATP concentration rather than affecting the enzyme directly. The inhibitory effect of ATP was further studied to determine the exact nature of the inhibition. Assays were therefore performed with varying amounts of free ATP at distinct concentrations of AMP. Analysis of the data in the form of a Dixon plot suggested that inhibition was competitive in nature with a Ki of 67μM (figure 4.22). This value is almost identical to that of 61μM measured by Lai & Wong (1991b) for the soluble rat brain enzyme and 100μM for the human placental enzyme (Madrid-Marina & Fox, 1986), but appreciably lower than the value of 410μM measured for the brain ectoenzyme. These authors reported that the inhibition of the soluble enzyme was of a competitive type. However, analysis of the data by Dixon plots will not discriminate between competitive and mixed type inhibition. Therefore the data obtained for the

Figure 4.20 <u>Inhibition of purified 5'-nucleotidase by ATP at different concentrations of Mg^{2+}</u>

Purified 5'-nucleotidase was assayed at pH 7.4 with 200μM AMP as substrate and the indicated concentrations of ATP and $MgCl_2$. The values are means of two separate experiments each assayed in duplicate.

●, 0mM $MgCl_2$

▼, 1mM $MgCl_2$

+, 2.5mM $MgCl_2$

■, 4mM $MgCl_2$

✱, 6mM $MgCl_2$

Figure 4.21 <u>Inhibition of purified 5'-nucleotidase by free ATP</u>

These results are the same data as above, but calculated for free ATP concentration in each assay. The same symbols have been used to show the origin of each data point from the above data set.

✱, 0mM $MgCl_2$

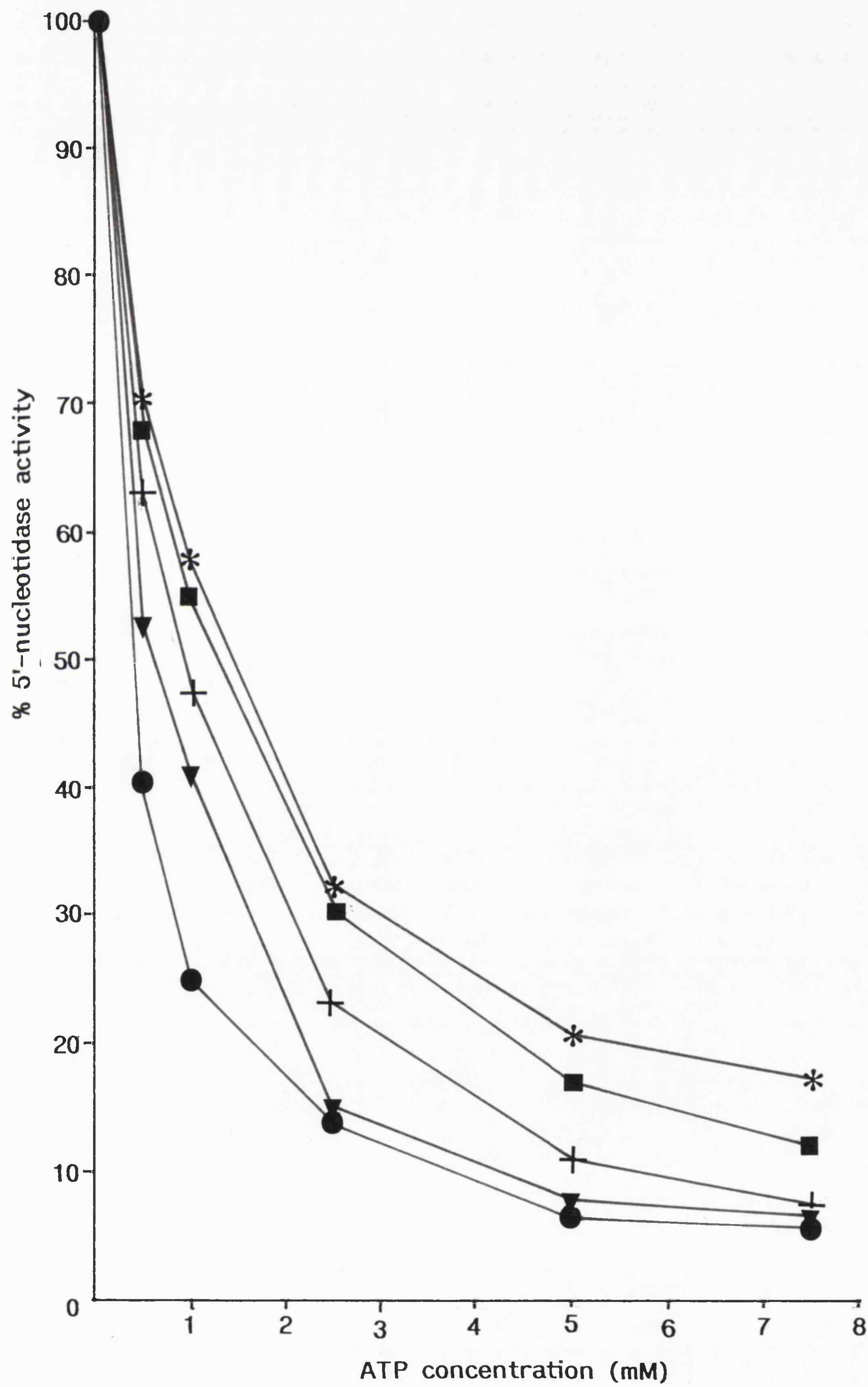
■, 1mM $MgCl_2$

+, 2.5mM $MgCl_2$

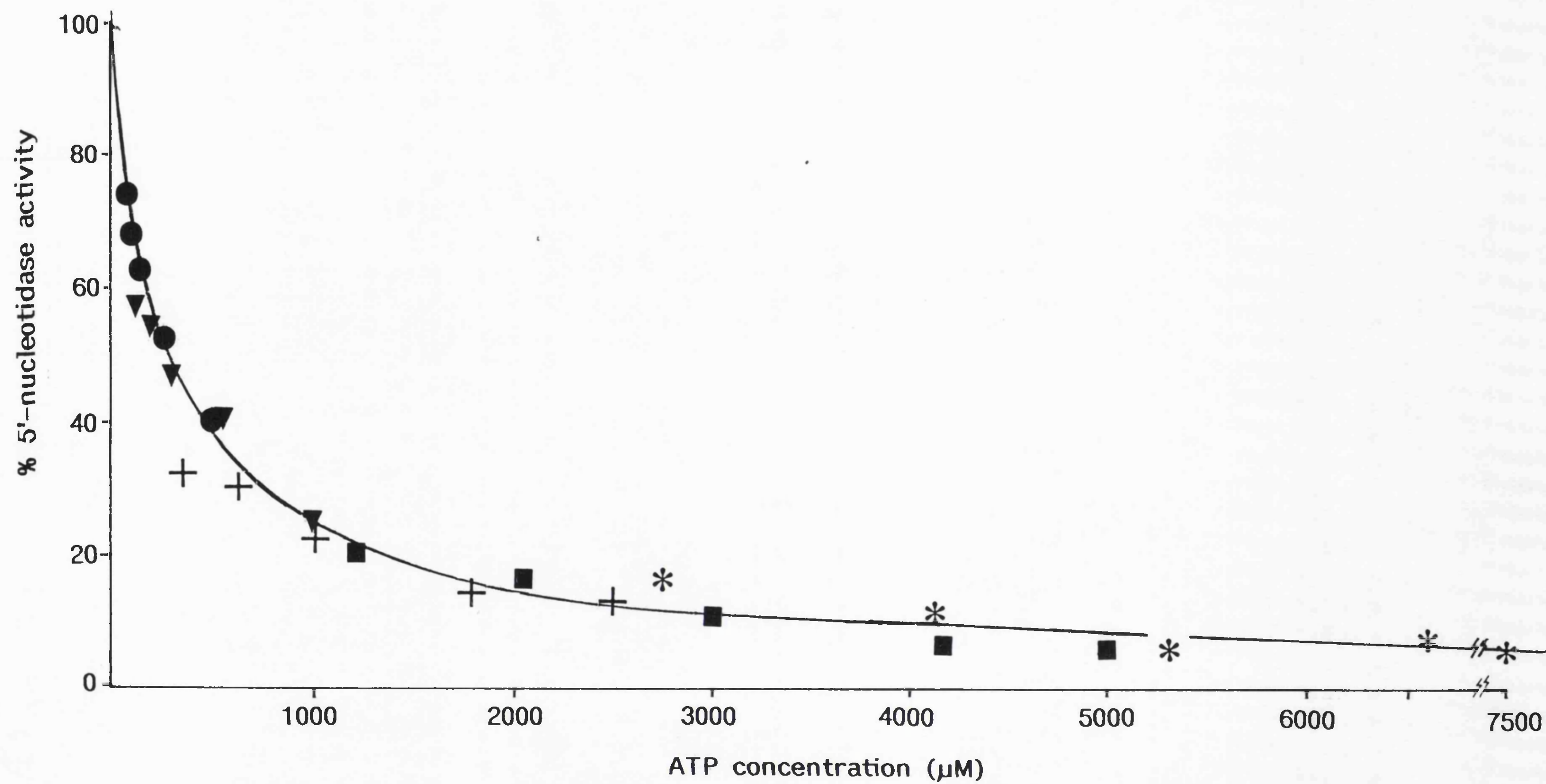
▼, 4mM $MgCl_2$

●, 6mM $MgCl_2$

Effect of magnesium ions on the inhibition of purified 5'-nucleotidase by ATP



Inhibition of purified 5'-nucleotidase by free ATP



inhibition of the purified enzyme was re-examined by Lineweaver-Burke plots and plots of S/V versus ATP concentration. This indicated that inhibition by ATP was of the mixed type (figs 4.23 & 4.24). In addition, the Ki' (inhibition constant for the formation of EIS complex) of 450µM was deduced from plots of this kind. This result contrasts the finding of Lai & Wong (1991b) who reported competitive inhibition, but is in agreement with observations seen with the renal enzyme (Le Hir & Dubach, 1988). Mixed type inhibition is expected to result from the formation of the dead end EI and EIS complexes. However Le Hir & Dubach (1988) suggested a two site model with competition at the catalytic site at low ATP concentration, and mixed at higher ATP concentration possibly due to an additional interaction of ATP at a second distinct site. At present however the precise mechanism is unknown. A further feature common to the ectoenzyme and the low Km soluble enzyme was observed in the form of potent inhibition by α,β-methylene ADP (figure 4.25). As little as 0.5µM of this compound inhibited 50% of the purified activity, a value comparable to that measured for the human placental enzyme (Madrid-Marina & Fox, 1986). Generally, this compound is a more potent inhibitor of the ectoenzyme than ADP and Ki values usually lie in the nanomolar range. As can be seen for the purified brain enzyme, α,β-methylene is more potent than ADP or ATP although no such Ki estimates were measured in this study.

Figure 4.22 Dixon plot for the inhibition of purified 5'-nucleotidase by free ATP

0.3µg of purified 5'-nucleotidase was assayed at pH 7.4 in the presence of the indicated concentrations of ATP. The Ki for ATP was estimated from the point of intersection of lines above the ATP axis. The values are from a representative experiment assayed in duplicate.

●, 25µM AMP

○, 50µM AMP

■, 100µM AMP

□, 200µM AMP

Dixon plot for the inhibition of purified soluble 5'-nucleotidase by ATP

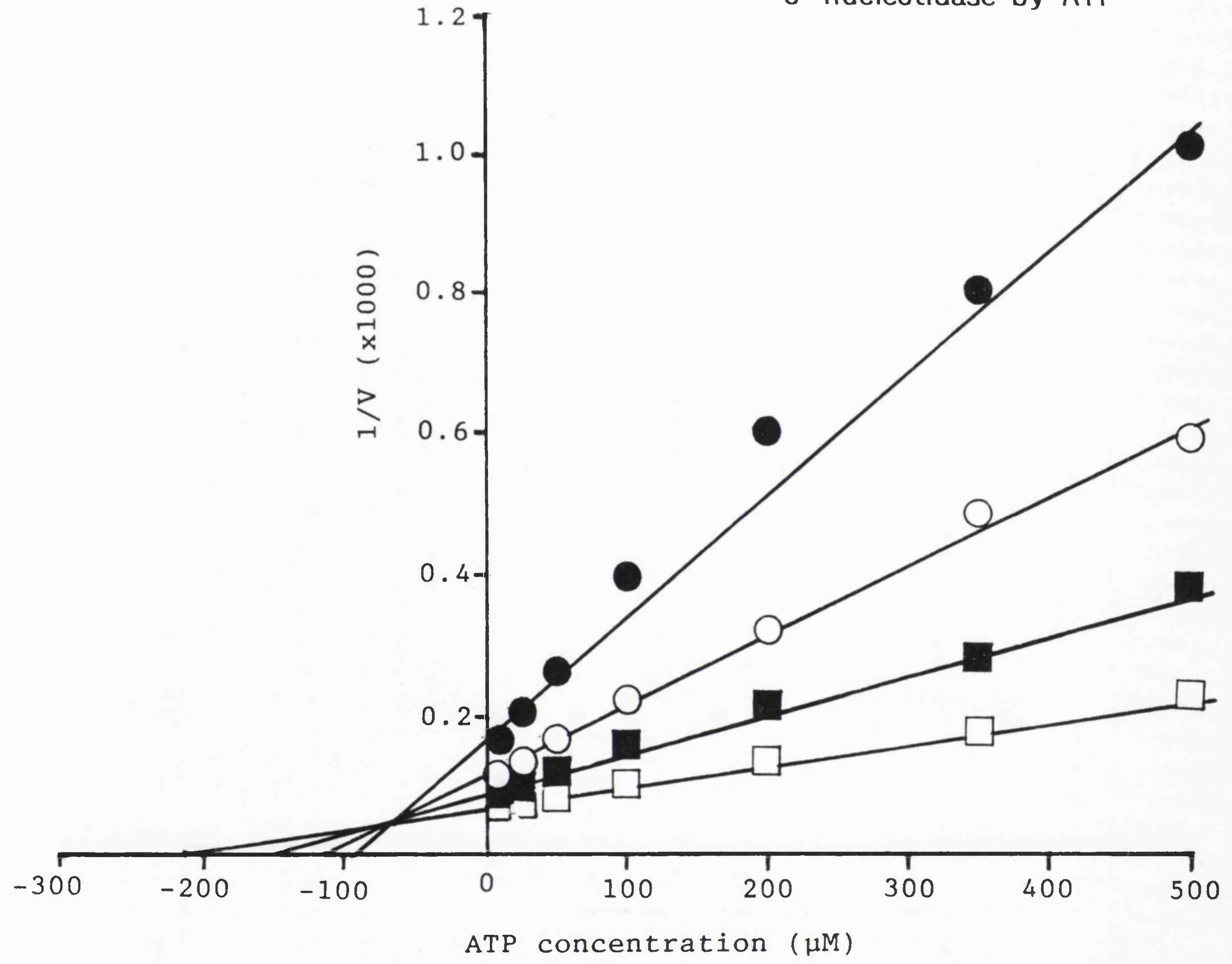


Figure 4.23 <u>Lineweaver-Burke plot demonstrating mixed type inhibition of purified 5'-nucleotidase by ATP</u>

The data from figure 4.22 was further analysed in the form of Lineweaver-Burke plots to demonstrate that inhibition of soluble 5'-nucleotidase by free ATP is of the mixed type.

+, 0μM ATP

▽, 10μM ATP

▼, 25μM ATP

□, 50μM ATP

■, 100μM ATP

○, 200μM ATP

●, 350μM ATP

Figure 4.24 <u>Demonstration of mixed type inhibition of purified 5'-nucleotidase by ATP</u>

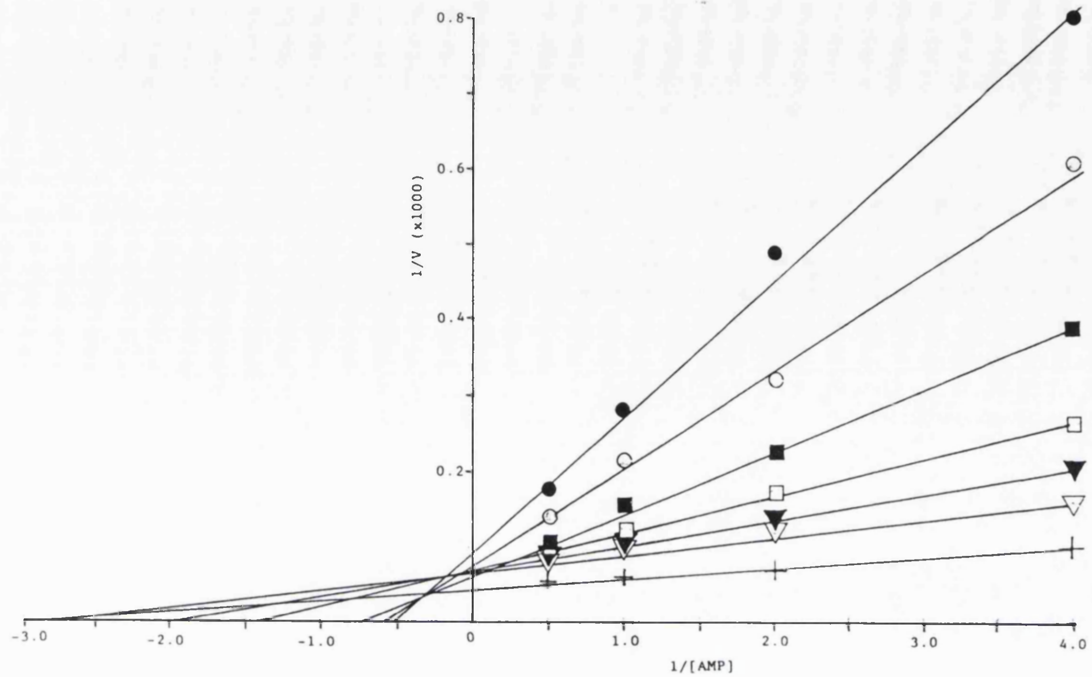
The data from figure 4.22 was further analysed in the form of a plot of S/V against ATP concentration. The lines converge in the lower left hand quadrant of the plot indicating mixed type inhibition. Moreover, the point of intersection indicates a Ki'value (dissociation of the EIS complex) for ATP of 450μM.

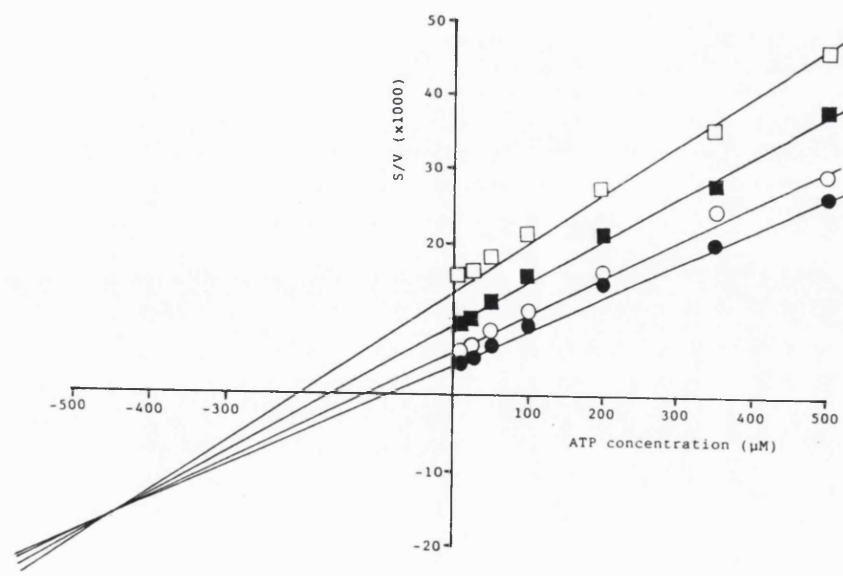
●, 25μM AMP

○, 50μM AMP

■, 100μM AMP

□, 200μM AMP


1/V (x1000)
0.8
0.6
0.4
0.2
-3.0
-2.0
-1.0
0
1.0
2.0
3.0
4.0
1/[AMP]


S/V (x1000)
50
40
30
20
-10
-20
-500
-400
-300
100
200
300
400
500
ATP concentration (μM)

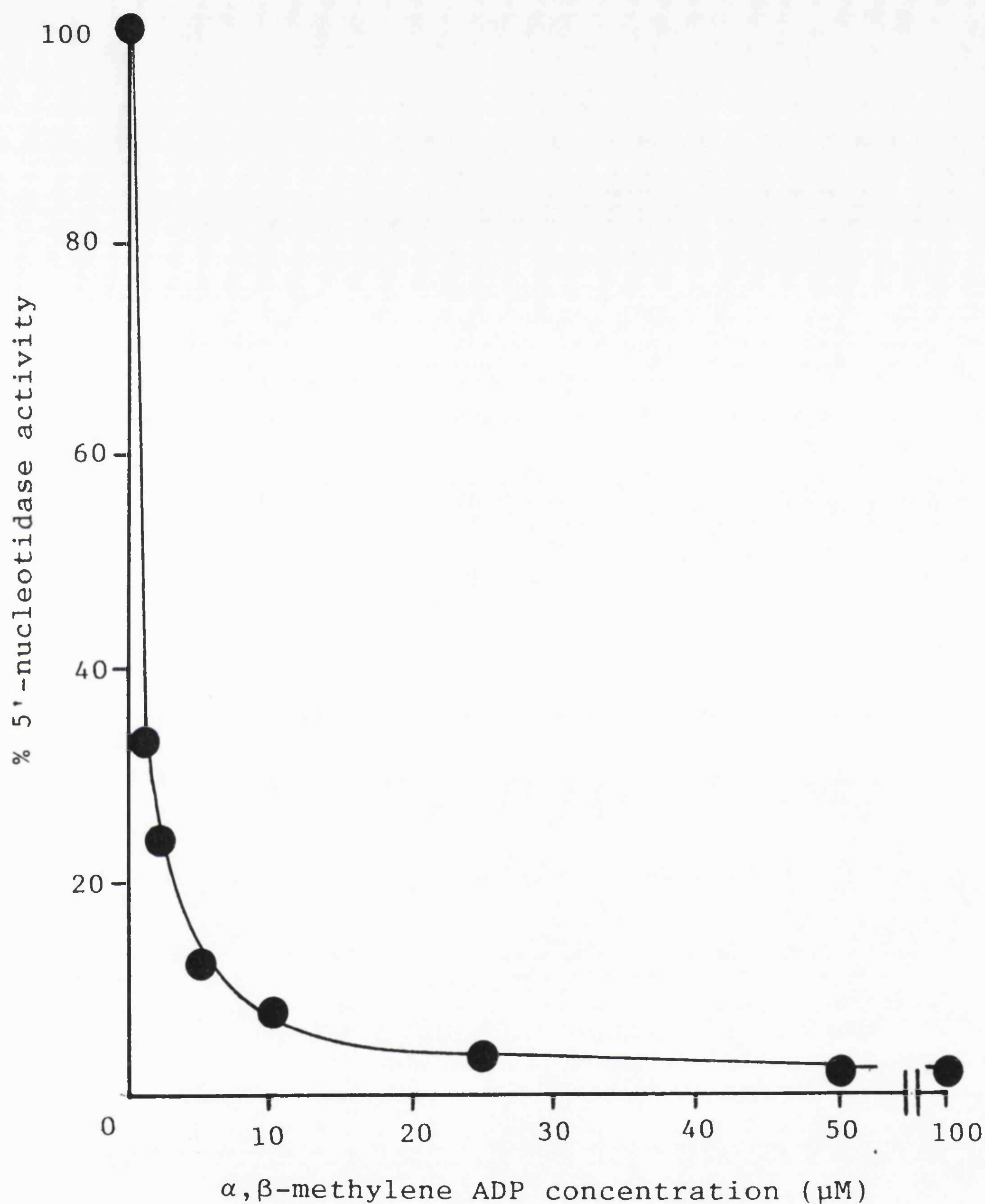


Fig 4.25 INHIBITION OF PURIFIED SOLUBLE 5'-NUCLEOTIDASE BY α,β-METHYLENE ADP

The purified enzyme was assayed for 5 minutes with various amounts of α,β-methylene ADP. Results shown are the means of two separate experiments each assayed in duplicate as described in section 2.12.1.

Following linear transformation of the data, an IC_{50} value of 560nM was obtained for this inhibitor (Γ=0.996).

4.6 REMOVAL OF A SOLUBLE FACTOR FROM THE 'UNBOUND' ENZYME WHICH ENHANCES ATP-SENSITIVITY

When soluble brain proteins were subjected to ion exchange chromatography at pH 8.5, two distinct populations of 5'-nucleotidase were generated, one which bound and the other which was excluded from mono Q. These two pools of the enzyme furthermore displayed different sensitivities to inhibition by ATP and ADP (section 4.2). It was noted however, that when the ion exchange was performed on 5'-nucleotidase which had been previously chromatographed over Con A-sepharose, this unbound population had been eliminated (figure 4.9). This observation could not be attributed to the Con A column removing the unbound activity, as all 5'-nucleotidase activity bound to the lectin column and could be subsequently eluted. It was reasoned that the lectin column was removing a factor which bound to a population of the enzyme and prevented it from binding to mono Q. Moreover, previous studies (see section 4.2) suggested that the factor could enhance the sensitivity of the enzyme to inhibition by ATP and ADP. It was therefore decided to test this idea by further studying the mono Q-unbound fraction. This pool of the enzyme was chromatographed over Con A-sepharose in an attempt to remove the factor. The results showed that the enzyme bound to the lectin column in the same manner as did the mono Q-bound enzyme. When the inhibition characteristics of the enzyme were compared before and after the lectin column, it was noticed that the ATP sensitivity had changed. The unbound enzyme following Con A-sepharose chromatography behaved in a similar manner to the mono Q-

bound or purified enzyme on the basis of its inhibition by ATP. The proteins which were excluded from the lectin column were therefore collected, concentrated and tested for their ability to enhance ATP-sensitivity of the 5'-nucleotidase which bound to the column. By pre-incubating the enzyme for 10 minutes with 50µg of the crude factor preparation prior to assay, it was noted that the enhanced ATP sensitivity had been restored to that of the original unbound enzyme (figure 4.26). Assays with varying amounts of free ATP showed that 60µM ATP inhibited 50% of the unbound activity once it had been chromatographed over Con A-sepharose. This figure paralleled with that of the purified enzyme which required 40µM ATP to inhibit 50% of its activity. In contrast, as little as 5µM was required to elicit the same response prior to the lectin column. In the presence of 50µg of the factor, the original sensitivity of the unbound enzyme was restored (10µM ATP inhibited 50% of the activity). Furthermore, adding the factor back to the enzyme alone did not inhibit the enzyme when assayed in the absence of ATP (figure 4.27 inset). Assays conducted in the presence of 50µM ATP with varying amounts of the factor demonstrated that it could be reconstituted back in a saturable manner (figure 4.27). A concentration of 50µM ATP was chosen because it was a submaximal concentration required to inhibit completely the unbound enzyme but not the bound or purified form. It would therefore show the greatest differences between enzyme assayed in the presence and absence of the factor. As expected, the crude factor could be reconstituted with the purified enzyme

Figure 4.26 Removal of a soluble factor that increases the sensitivity of 5'-nucleotidase to ATP inhibition

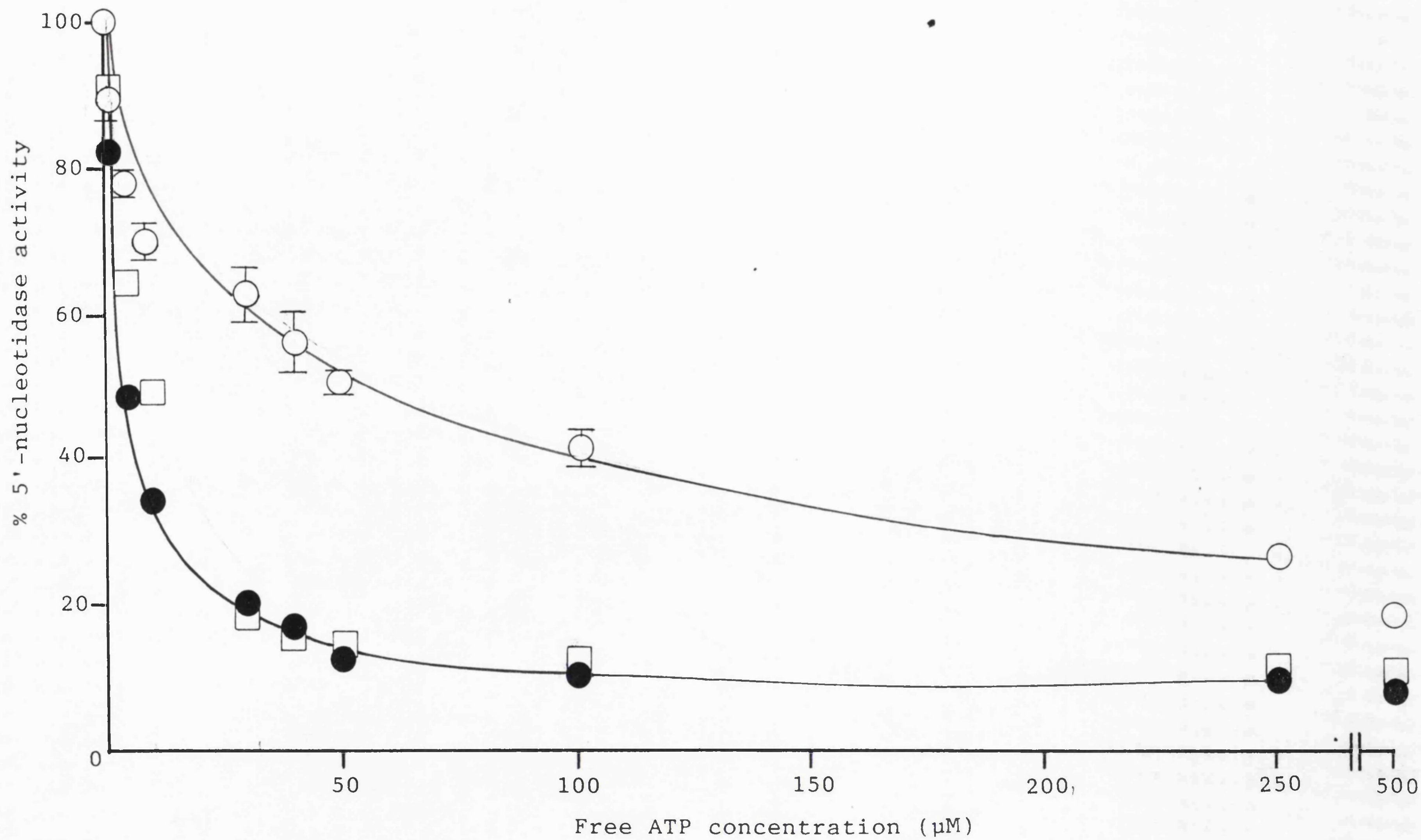
The ATP sensitivity confering factor was stripped from the 'unbound' form of 5'-nucleotidase as described in section 2.15.6. The resulting preparations were dialysed for 12 hours against 50mM Tris-HCl buffer, pH 7.4. Assays were performed at pH 7.4 with 200μM AMP as substrate. In addition, 20mM β-glycerophosphate and the indicated concentrations of ATP were also present in the assay buffer.

●, 5'-nucleotidase prior to Con A chromatography (n=1)

○, 5'-nucleotidase eluted from the Con A column (n=2)

□, 5'-nucleotidase eluted from the Con A column, but assayed after 10 minutes preincubation at 37°C with approximately 50μg of the soluble factor. This factor was contained in the fraction of proteins which were excluded from this lectin column (n=1)

Removal of soluble factor that increases the sensitivity of 5'-nucleotidase to ATP inhibition



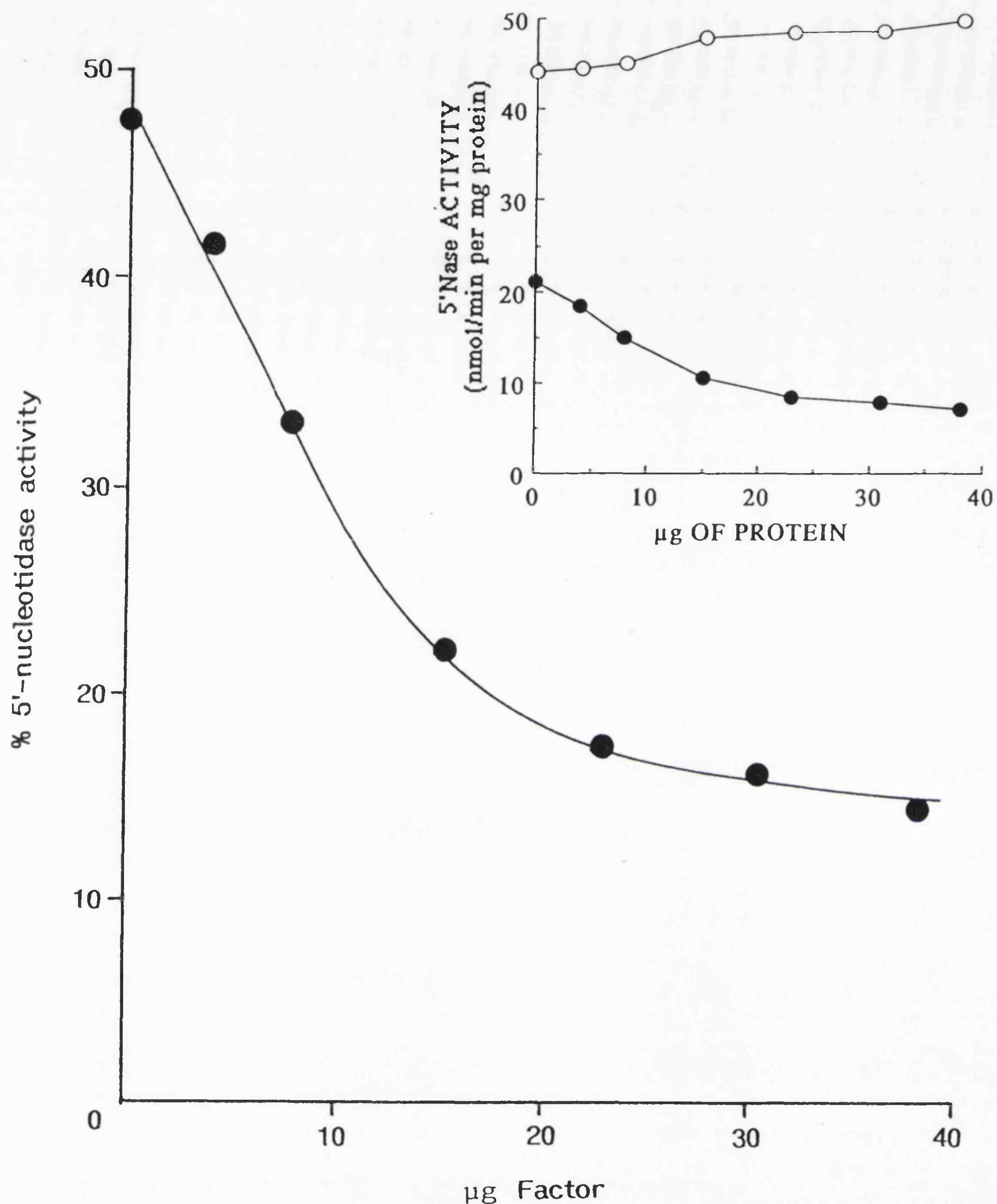


Fig 4.27 Concentration dependence of the ATP-sensitivity conferring factor

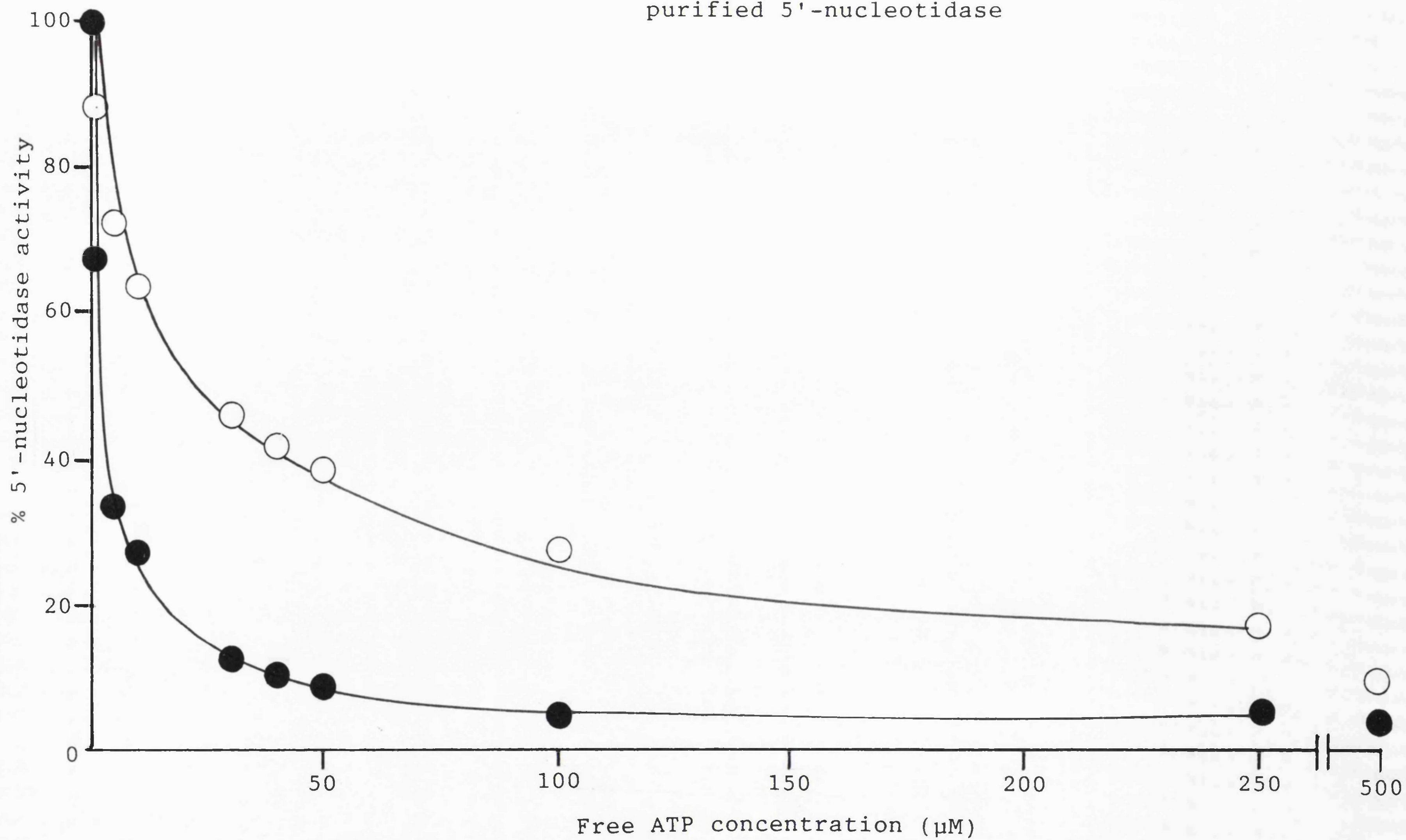
'Unbound' fraction of 5'-nucleotidase activity from Mono Q was chromatographed on Concanavalin A sepharose to remove the soluble factor. Following dialysis, the enzyme was preincubated at 37°C for 10 minutes with various amounts of the soluble factor. 5'-nucleotidase assays were then performed as described in section 2.12.1 in the presence of 50μM free ATP (n=1).

Inset: In the absence of ATP (○), the factor is ineffective at modulating 5'-nucleotidase activity, although its presence (●) greatly enhances the ATP inhibition.

to enhance the ATP inhibitory effect. In the absence of the factor, the purified enzyme was 50% inhibited by 40µM ATP. However, in the presence of 50µg of the crude factor, this was reduced to 2µM to elicit the same response (figure 4.28). This supported the idea that the bound, unbound and purified enzymes were identical, but the presence of the factor modifies the ATP sensitivity and its behaviour on the ion exchange column. Very little is known about the properties of the ATP sensitivity-conferring factor. Since Con A was required to separate it from 5'-nucleotidase it may suggest that the factor is itself an endogenous lectin. It certainly seems to be positively charged at pH 8.5 since its interaction with 5'-nucleotidase prevents the enzyme binding to mono Q at that pH. In the course of concentating the factor prior to reconconstitution experiments, it was noted that the factor failed to pass through an ultrafiltration membrane with a cut-off of 30kDa. It therefore seems likely that the minium size of the factor is 30kDa. When attempting to precipitate the factor by lowering the pH to 5.0 the ability of the factor to enhance the ATP sensitivity was lost. The factor retained its properties for periods up to 6 months when stored at -70°C in the presence of 5'-nucleotidase (ie as the mono Q-unbound fraction). However, following its removal from the enzyme by Con A-sepharose affinity chromatography, its ability to enhance ATP inhibition of 5'-nucleotidase was abolished with 2-3 cycles of freezing and thawing. Owing to its labile nature and minimum size, it may be a protein, although this idea is

Figure 4.28 Effect of the ATP sensitivity confering factor on purified cytosolic 5'-nucleotidase

0.6μg of purified enzyme was preincubated at 37°C for 10 minutes with (●, n=1) or without (○, n=4) 50μg of protein from the fraction containing the ATP sensitvity confering factor. 5'-nucleotidase assays were performed at pH 7.4 with 200μM AMP as substrate. ATP was varied as indicated.


Reconstitution of ATP sensitivity confering factor on
purified 5'-nucleotidase
% 5'-nucleotidase activity
100
80
60
40
20
0
50
100
150
200
250
500
Free ATP concentration (μM)

purely speculative. It does however retain its properties following incubation with trypsin. Future studies should therefore be directed towards purifying and characterizing this component. Although no reports of such a factor have appeared in the literature to date, it has been reported that the ectoenzyme can be activated or inhibited by laminin or fibronectin respectively (Dieckhoff *et al.*, 1986). As yet it is not known whether these compounds might confer extra ATP sensivity to the enzyme. However, it is unlikely that the factor represents either of these compounds since alone it does not affect the enzyme. Its effect is only apparent in the presence of adenine nucleotides.

Although ATP is rapidly degraded by the extracellular phosphohydrolase pathway to adenosine, it can also exert its own effects via P_2 purinergic receptors. This latter effect is harder to demonstrate due to its rapid metabolism, and often the inhibitory response observed could be attributed to the action of adenosine so formed at P_1 purinergic receptors. However, evidence also exists that it is capable of acting per se to inhibit transmitter release in the peripheral nervous system (see Westfall *et al.*, 1991) or conversely provoking excitatory effects on cortical neurones when applied by iontophoresis (Phillis *et al.*, 1979). Both adenosine and ATP are capable of inducing vasodilation which thus permits an increase in local blood flow (hyperaemia) at P_1 and P_2 receptors respectively. Whereas adenosine exerts a direct vasodilatory effect, ATP activates P_{2Y} receptors on vascular endothelial cells resulting in the release of

endothelium-derived relaxing factor (EDRF). Considering the greater potency of ATP over adenosine to mediate vasodilation of cerebral arteries (Toda *et al.*, 1982), it appears that ATP may be an important mediator of hypoxic or ischaemic hyperaemia. As demonstrated for the ectonucleotidase pathway on striatal cholinergic nerve terminals (James & Richardson (1993), inhibition of 5'-nucleotidase by ATP and ADP increases the temporal separation between the appearance of extracellular ATP and its subsequent degradation to adenosine. This would consequently enable ATP to transiently act at P_2 sites prior to its degradation to adenosine where it would then either be taken up or provoke responses at adenosine receptors. The ATP-sensitivity conferring factor would therefore be expected to increase this temporal separation by delaying the action of 5'-nucleotidase by maintaining it in an inhibited state for a longer period. This action could therefore prolong the effect of ATP at P_2 purinoceptors and delay those mediated by adenosine at its own receptors. However, it is not certain at present whether the ATP-sensitivity conferring factor has an extracellular location. It is therefore of paramount importance to ascertain the true location of the factor within the cell or tissue.

CHAPTER FIVE

GENERAL DISCUSSION

5. GENERAL DISCUSSION

The developing brain shows an absolute requirement for thyroid hormone for normal maturation processes. In contrast, it is frequently reported that the adult brain appears unresponsive, despite the presence of a significant intracellular thyroid hormone content (Heninger & Albright, 1975; Obregon *et al.*, 1978) and high affinity receptors for the same (Oppenheimer *et al.*, 1974; Valcana & Timiras, 1978; Kolodny *et al.*, 1985). It can be reasoned that many of the events associated with perturbed thyroid status may arise due to altered cell signalling processes. In a preliminary study Mazurkiewicz & Saggerson (1989a) provided good evidence for such an altered pathway with the observation that synaptic membranes isolated from hypothyroid rats displayed an enhanced responsiveness to inhibition of adenylyl cyclase by the adenosine A1-receptor agonist L-PIA. With the use of radioligand binding studies, it was further concluded that changes were likely to occur at the post receptor level, the results of which could be explained by an up-regulation of G_i. In the present study direct immunological evidence for the up-regulation of $G_i1\alpha$, $G_i2\alpha$ and $G_0\alpha$ in the hypothyroid brain has been provided, thereby confirming this hypothesis. Not all the regions of the brain were equally affected however. The greatest increases were observed in the cerebral cortex and the striatum where an increase in all the α-subunits was apparent. In contrast, the hypothalamus and cerebellum displayed the least changes in abundance showing only significant increases in $G_i2\alpha$ and $G_0\alpha$ respectively. In

a further study, it was additionally noted that significant decreases of $G_i1\alpha$ and $G_i2\alpha$ in the cerebral cortex were apparent after three days administration of T3 to rats. In contrast to the hypothyroid brain (Mazurkiewicz & Saggerson, 1989a), altered G-protein abundance in hyperthyroidism could not be correlated with functional changes in the adenosine A1-receptor mediated signalling pathway. From these experiments, it therefore seems likely that changes in G-protein abundance may serve to strengthen or weaken inhibitory inputs into cells in hypo- and hyperthyroidism respectively and provide means whereby the functioning of neurones may alter in these perturbed metabolic states. The precise receptor-mediated signalling pathways affected however remain to be elucidated. The nucleoside adenosine provides one such inhibitory input to neurones and its action at adenosine A1 receptors results in a decrease of neurotransmitter release. It is interesting to note that alterations in this adenosine-mediated pathway are paralleled by increases in the activity of ecto 5'-nucleotidase in the hypothyroid rat brain (Mazurkiewicz & Saggerson, 1989a,b). It may therefore be possible that an increased extracellular adenosine concentration, in conjunction with a more sensitive signalling pathway may strengthen this particular inhibitory input, and thereby contribute to the decreased brain excitability associated with myxoedema. Despite a decrease in the abundance of G_i in the hyperthyroid brain, no alterations in the adenosine mediated pathway were observed in this study. However many signalling pathways are likely

to be altered in both hypo and hyperthyroid states and many further studies are needed to elucidate precisely which pathways are affected. Significant changes in the hyperthyroid model were apparent after a period of 3 days treatment of rats with T3. It would however be of interest to repeat these studies over a longer time scale, for example with the use of subcutaneous 'mini-pumps' to slowly release T3 over a longer time scale. Such an experiment may detect greater changes in G-protein down-regulation and provide a necessary model of long term thyrotoxicosis. With a significant decrease in G_i in hyperthyroidism potentially reducing inhibitory inputs into neurones, it would be of particular importance to quantitate G_s in the hyperthyroid brain in an attempt to look for changes opposite to that observed for G_i. In addition, quantitation of G_q in the hypo- and hyperthyroid brain may produce rewarding results. Lastly, more detailed studies concerning the location of these changes would be of interest whereby the brain regions are further divided into individual neurone types, for example cholinergic or muscarinic neurones.

The nucleoside adenosine exerts a wide but varied range of effects in the central nervous system via extracellular receptors. It is produced by the phosphohydrolytic cleavage of AMP catalysed by the enzyme 5'-nucleotidase, an enzyme which appears to exist in at least four forms. As previously stated, the ectoenzyme responds in the hypothyroid state as observed by increased activity in various regions of the adult brain (Mazurkiewicz & Saggerson, 1989b). It is not

certain however whether this increased activity is attributed to enzyme activation or due to a raised concentration of 5'-nucleotidase protein. To date no measurements of the cytosolic activity appear to have been made in the hypothyroid brain. Examination of the post microsomal supernatant of rat brain indicated the presence of two soluble activities which vary in their distribution between the brain regions and seem to be independently regulated in hypothyroidism. One activity which appears to be ATP stimulated is likely to represent the high Km AMP-specific cytosolic enzyme. This enzyme although only studied in cardiac tissue to date is believed to be the enzyme responsible for intracellular adenosine production during periods of depleted cellular energy such as ischaemia (Newby, 1988; Yamazaki *et al.*, 1991). Examination of the other enzyme in a purified form showed that it closely resembled the ectoenzyme or low Km soluble enzyme on the basis of its kinetics, inhibitability by adenine nucleotides and immunological cross reactivity with anti-ectoenzyme antibodies. However the purified enzyme showed a significantly smaller subunit size than the previously reported forms and may represent a proteolytically truncated form of the ectoenzyme or even a novel isoform resulting from an alternative splicing of the 5'-nucleotidase gene product. It is interesting to note that the regional distribution of this enzyme correlates very well with that of the ectoenzyme as measured by Mazurkiewicz & Saggerson (1989b). In the hypothyroid state this correlation broke down, further

supporting the idea that the two forms are distinct in nature. It is unlikely that the purified soluble enzyme represents a truly cytosolic enzyme for two reasons. Firstly it appears to be glycosylated, an uncommon feature of enzymes found in this subcellular compartment. Secondly, due to kinetic parameters including potent inhibition by ATP and ADP it seems unlikely that the enzyme would be active under physiological conditions if located within the cytosol. It may however be an extracellular enzyme functioning to dephosphorylate extracellular AMP possibly in areas of the brain devoid of the ectoenzyme. The precise location of the enzyme within the cell or tissue however is uncertain at present and further studies are essential to determine its true tissue location possibly employing the use of a monoclonal antibody. With a subunit size considerably smaller than the ectoenzyme or low Km soluble enzyme it is also crucial to obtain a complete amino acid sequence of the purified enzyme and to check for identity with the other forms.

The discovery of a soluble factor that enhances extra sensitivity of 5'-nucleotidase to ATP and ADP inhibition is particularly exciting. At present its location within the brain is unknown. Since the factor physically associates with a portion of the soluble enzyme, it is likely that the two species are located in the same cellular compartment of the brain. Further studies however are required to test this conjecture. Evidence indicates that the soluble 5'-nucleotidase purified from brain is possibly located in the

extracellular compartment. It would therefore seem reasonable to expect the factor to be extracellular as well. Moreover, if located within the extracellular compartment a potential physiological role can be hypothesised. It is reported that cerebral arteries are more sensitive to ATP than adenosine in mediating vasodilation (Toda *et al.*, 1982). The presence of the factor would delay the formation of adenosine when ATP or ADP is additionally present. This increase in the temporal separation between the appearance of ATP and the formation of adenosine may therefore serve to prolong the action of ATP at P_2-purinoceptors. Consequently, the ATP-sensitivity conferring factor may be of particular importance in enhancing ischaemic or hypoxic hyperaemia.

At present, little is known about the factor, although it may be proteinaceous on the basis of its minimum size and lability. Further work should therefore be concentrated on its purification and characterization. In a purified form, antibodies could be raised against the factor to aid in the elucidation of its precise tissue location. In addition it would be of interest to study its abundance in perturbed metabolic or disease states. With the soluble 5'-nucleotidase closely resembling the ectoenzyme, it would be of great interest to see whether the factor can associate with the membrane associated form to modulate its ATP and ADP inhibitability in a similar fashion. This is likely as indicated by a preliminary study where the soluble factor was capable of interacting with, and enhancing ATP inhibitability of the ectoenzyme isolated from white adipose tissue (Gang

& Saggerson, personal communication). It would therefore be of great interest to perform both *in vivo* and *in vitro* studies with the factor to modulate neuronal activity. Finally, it would be of interest to ascertain whether the soluble factor is capable of interacting with either of the 'true' cytosolic enzymes to alter the modulation exerted by adenine nucleotides.

CHAPTER SIX

REFERENCES

6. **REFERENCES**

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