Alterations at brain A_1 adenosine receptors, adenosine metabolising enzymes and adenylate cyclase in hypothyroidism

THE EFFECTS OF HYPOTHYROIDISM ON CERTAIN ASPECTS OF ADENOSINE METABOLISM IN THE RAT BRAIN

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ABSTRACT

Rat brain tissue was used to study alterations at adenosine A_1 receptors, in adenosine metabolising enzymes and adenylate cyclase in hypothyroidism.

Rats were made hypothyroid by treatment with propylthiouracil and a low iodine diet for four weeks. Brain synaptic membranes were isolated from synaptosomes after fractionation on Ficoll gradients.

Saturation binding analysis of the synaptic A_1 adenosine receptor was performed using the agonist [^{3}H] phenylisopropyladenosine (PIA), whilst competition binding analysis was performed using [^{3}H] diethylphenylxanthine ([^{3}H] DPX) as the radiogland and PIA as the competing ligand. Hypothroidism resulted in a significant decrease in [^{3}H] PIA maximum binding, and of [^{3}H] DPX maximum binding in the presence of GTP. No significant differences in the dissociation contants were found in either case.

Measurements of adenylate cyclase activity revealed an enhanced inhibition of forskolin stimulated adenylate cyclase by PIA. Forskolin activation was comparable in euthyroid and hypothyroid states. Inhibition of adenylate cyclase by GTP was also comparable in euthyroid and hypothyroid states, although hypothyroidism impaired the inhibitory action of sodium on forskolin stimulated adenylate cyclase.

The adenosine metabolising enzymes were assayed in extracts from six regions of rat brain namely the cortex,

cerebellum, medula oblongata, striatum, hippocampus and hypothalamus. Adenosine deaminase and adenosine kinase were assayed in a supernatant fraction, whilst 5'-nucleotidase was assayed in myelin and synaptic membrane fractions. The three fractions were prepared by a combination of density centrifugation and Ficoll gradients. Hypothyroidism resulted in an increase in 5'-nucleotidase activity in the cerebullum, cortex, hippocampus and striatum; only the medulla oblongata showed an increase in myelin 5'-nucleotidase activity. Conversely a decrease in adenosine kinase activity was seen in the cerebellum, hippocampus, hypothalamus and striatum, whilst adenosine deaminase displayed no change in any of the six rat brain supernatant fractions studied.

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ABBREVIATIONS

ADA	adenosine deaminase
AK	adenosine kinase
AD	adenosine
BBOT	2,5-di[5'-tert-butylbehz-oxazoyl(2')thiopen
Bmax	maximum binding capacity
2-CADO	2-chloroadenosine
CHA	N^6-cyclohexyladenosine
CPA	N^6-cyclopentyladenosine
DTNB	5,5' dithiobis-2-nitro-benzoic acid
EC_{50}	the concentration of agonist that causes 50% of its maximum effect
DPX	1,3-diethylphenylxanthine
EDTA	ethylenediamimetetraacetic acid
Gpp(NH)p	guanyl-5'-(β,-imino) triposphate
GTPγS	guanosine 5'-(3-0-thio) triposphate
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride)
K_D	dissociation constant
K_H	high affinity dissociation constant
K_L	low affinity dissociation constant
NECA	5'-N-ethylcarboxamide-adenosine
NCPCA	5'-N-cyclopropylcarboxamide-adenosine
PTU	6-n-propyl-2-thiouracil
Rf	the ratio of the distance travelled by a substance to the distance travelled by the solvent front
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine

To my Mother

Mamo, serdecznie Ci dziękuje za wszelką pomoc przez te moje szęśliwe i miłe lata studiowania.

CHAPTER 1

I N T R O D U C T I O N

1.1 GENERAL INTRODUCTION

Chemically adenosine consists of the purine base adenine linked to ribose, hence it is designated a nucleoside. It is one of the most ubiquitous metabolic intermediates in the body. Adenosine functions in nucleic acid biosynthesis and is also prominent in the formation of ATP and thence cAMP.

Although adenosine was reported to cause vasodilation and to have a negative inotropic effect in the heart as long ago as 1929 (Drury & Szent-Gyorgyi, 1929) and ATP was shown to cause sedation in cats (Feldberg & Sherwood, 1954) and proposed as neutotransmitter candidate at primary afferent fibres in the 1950's (Holton & Holton, 1954) it was not until 1970, following from the seminal study of Sattin & Rall (1970) on adenosine-evoked increases in cAMP production in mammalian brain slices, that the role of adenosine and adenine nucleotides in CNS function has been studied to any great extent.

The extensive studies of Burnstock and his colleagues (Burnstock, 1972,1978,1979) on "non-cholinergic, non-adrenergic" neurotransmission and from studies performed by McIlwain (1972), Phillis et al., (1979), Phillis & Wu (1981), Daly, (1977) and Snyder et al., (1981) on the central actions of these compounds have supported the concept that adenosine can modulate cellular function in a physiologically relevant manner via extracellular receptors sensitive to blockade by the alkylxanthines. The ubiquitous distribution of adenosine coincides with a

diversity of physiological modulatory actions including vascular tone, hormone action, platelet function and lymphocyte differentiation, (Newby, 1984). From a behavioural standpoint it appears likely that adenosine functions as a potent central depressant, (Williams, 1984).

Coincidentally, there has been a great deal of interest in the incidence and co-existence of thyroid dysfunction and depressive illness because of the known effects of decreased thyroid function on mood and the similarity of symptoms found in both hypothyroidism and depression (Gold et al., 1981). The effects of decreased thryoid hormone levels on brain function are different at different stages in life. Deficiency of thyroid hormone at critical stages of brain development results in an aberrant maturation of the cerebral and cerebellar cortex and the chemical syndrome of cretinism. Experimental animals made hypothyroid in early infancy tend to be retarded in their behavioural development, are more placid and easily handled but learn new skills with difficulty, (Gold et al., 1981; Reus, 1986).

The behavioural changes associated with long-standing hypothyroidism are classic and seldom misdiagnosed. Signs of fatigue, decreased libido, memory impairment and disruption of sleep pattern are most characteristic. In severe cases, patients may present with a true organic psychosis characterised frequently by paranoid delusions or cortical dementia. Unlike thyroid effects in early life, thryoid hormone replacement

generally improves emotional state and mental functioning (Reus, 1986). The mechanism/s by which thyroid hormone modulates mood and cognitive function is speculative, but mediation of β-adrenergic receptor response is thought to be a prime candidate (Whybrow et al., 1981). In view of the fact that adenosine is a potent sedative, eliciting a "hypnotic state" in various species including mammals and has been shown to decrease spontaneous motor activity as well as eliciting a central depressive effect (Williams, 1987), it is possible that altered sensitivity and/or biovailability to adenosine in the CNS may contribute to the clinical expression of some of the symptoms seen in hypothyroidism.

1.2 ADENOSINE RECEPTORS

In 1970, Sattin and Rall proposed a biochemical basis for the mechanism of action of adenosine. They observed that exogenous adenosine and some adenine nucleotides were potent stimulators of cAMP accumulation in brain slices. They subsequently proposed the existence of an external adenosine receptor which stimulates cAMP production in a hormone-like manner.

It was subsequently proposed (Burnstock, 1978) that two types of purinergic receptor could be distinguished. Their subclassification into P_1 and P_2 purinoceptors were based on four criteria: the relative potency of the adenine compounds, selective antagonism,

activation of adenylate cyclase by adenosine and induction of prostaglandin synthesis by ATP and ADP (see table 1.1).

Table 1.1

	Antagonists	Agonist Potencies	Changes in cAMP
P_1	Methylxanthines	ADO≥AMP>ADP≥ATP	Yes
P_2	Quinidine		
	2 substituted imidazolines	ATP≥ADP>AMP≥ADO	No
	2'2 - Pyridilisatogen		

The P_1 purinoceptors were further subclassified by looking at structure affinity relationships with respect to adenosine interactions with cAMP systems. Londos et al, (1980) examined the actions of L-PIA, NECA and adenosine on three cell types: rat liver cells; 1-10 Leydig cells and rat adipocytes. The agonists stimulated adenylate cyclase in the liver and Leydig cells and inhibited the enzyme in ADO adipocyte membranes. The potency series NECA>ADO>L-PIA was found in the stimulatory cell systems whereas the converse ADO was found for the inhibiting system, L-PIA>ADO>NECA. The terms Ra and Ri were proposed to describe the receptors mediating the stimulatory and inhibitory actions on adenylate cyclase respectively. A parallel study on adenosine receptors was conducted on

cultured glial cells from mouse brain (Van Calker et al., 1979). They also observed that adenosine acted at two cell surface receptors: one high affinity and inhibitory to adenylate cyclase and the other low affinity and stimulatory to adenylate cyclase. The nomenclature assigned to these receptors was A_1 and A_2 respectively. Agonist potency profiles at the A_2 receptor were ADO, 2-CADO > PIA, N^6-methyladenosine and at the A_1 receptor PIA > ADO, 2-CADO > N^6-methyladenosine. For the purposes of clarity the nomenclature proposed by Van Calker et al., (1979) shall be used throughout. Affinity constants for adenosine and analogues at the A_1 receptor are in the nanomolar range whilst those for A_2 are in the micromolar range (Bruns, 1980; Daly et al., 1981).

There is also a third "P" site which mediates the inhibition of adenylate cyclase and requires that the ligand maintain the integrity of the purine ring for activity. This is not a receptor site but rather an intracellular site, perhaps directly associated with the catalytic subunit of the enzyme (Wolff et al., 1978). The affinity as assessed in terms of IC50 values for adenosine and its analogues are in the low to high micromolar range. The physiological significance of this site remains unknown since it seems unlikely that free adenosine levels ever reach high micromolar concentrations.

A wide range of adenosine analogues have been used on many different tissue preparations to generate structure activity profiles with respect to cAMP,

physiological responses and also in terms of inhibition constants. Extensive classification of A_1 and A_2 receptors has been formed in terms of structure activity relationships (SAR's). This method has been used since it cannot be supposed that any one adenosine receptor subtype is linked exclusively to a single effector/transduction system. Burnstock & Buckley, (1985) showed agonist potency orders to be extremely variable. Even though the N^6-substituted adenosine analogues are generally the most active at A_1 sites there appears to be little consenus on the order of potency within this group. As a consequence two main criteria have been used to distinguish between the two receptors. At A_1 receptors L-PIA is more potent than NECA, whilst at A_2 receptors NECA is more potent than PIA. The other criterion for classification is the stereoselective nature of the A_1 site. The initial discovery by Smellie et al., (1979) that the A_1 adenosine receptor is highly stereoselective for the L-PIA isomer whereas the A_2 receptor shows little stereoselectivity has since been demonstrated in several laboratories. L-PIA is approximately 40 times more potent than its diastereoisomer D-PIA at A_1 receptors, but only 5 times more potent at A_2 receptors. A summary of the characteristics of adenosine receptor subtypes is presented in table 1.2.

Table 1.2

Receptor type	Location	Potency order	Stereo-Selectivity	K_D range (M)
A_1	Cell Surface	L-PIA>CHA> NECA, NCPCA	L-PIA>D-PIA	10^{-9}
A_2	Cell Surface	NECA,NCPCA> L-PIA, CHA	LPIA>D-PIA	10^{-6}
P	Intracellular	2'-Dideoxy-adenosine>ADO	-	10^{-5}

The antagonists used to study adenosine receptors have for the most part been xanthine derivatives. Structure activity correlations for xanthines have been studied very thoroughly by Bruns, (1980). The following is the rank order of potencies at both A_1 and A_2 receptor-mediated effects on adenylate cyclase:

8 - phenyltheophylline > 1,3 dibutylxanthine > 3 - isobutyl -1-methylxanthine > theophylline > theobromine.

1.2.1 CHARACTERISATION OF ADENOSINE RECEPTORS BY RADIOLIGAND BINDING

Initial attempts to identify adenosine receptors by radioligand binding utilised [^{3}H] adenosine as the radioligand. Binding sites for adenosine in membranes from fat cells showed that binding was rapid and reversible, although it appeared nonsaturable and did not possess the characteristics that one would expect of binding to

ectoreceptors (Malbon et al., 1978). Subsequent studies in the brain (Newman et al., 1981) and heart (Dutta & Mustafa, 1980) again showed unexpected binding characteristics as evidence by low affinities and unusually large numbers of binding sites. These difficulties stemmed from the fact that adenosine itself is liable to uptake by high affinity transport systems and also to extensive metabolism to other nucleosides and other degradation products.

A satisfactory binding protocol therefore would seem to involve radioligands that are not subject to metabolism by adenosine deaminase or to high affinity transport systems and have an affinity for adenosine receptors greater than adenosine itself. In 1980 four laboratories reported the development of protocols for the binding of radioactive adenosine agonists and antagonists to brain membranes. These ligands were:- [^{3}H]2-chloroadenosine (Wu et al., 1980; Wu & Phillis, 1982; Williams & Risely, 1980) [^{3}H] N^6-cyclohexyladenosine (Bruns et al., 1980) [^{3}H] L-N^6 - phenylisopropyladenosime (Schwabe & Trost, 1980) and [^{3}H] 1,3 - diethylphenylxanthine (Bruns et al., 1980).

Pretreatment of membranes with adenosine deaminase or the copresence of adenosine deaminase during binding appears to be necessary therefore to remove endogenous adenosine and thereby prevent its competitive binding to receptors. In all cases mentioned, ligands bound to A_1 receptors as defined by the criteria for classification, previously outlined. Overall the data

obtained from radioligand binding studies to A_1 adenosine receptors are in good agreement. Estimates of K_D and Bmax values are summarised in table 1.3.

Table 1.3

Radioligand	Tissue	K_D (nM)	Bmax fmol/mg Protein	
[^{3}H] 2-CADO	Rat brain	16	426	Williams &
		1.3	207	Risely (1980)
		23.5	476	Wu et al., (1980)
N^6 - CHA	Guinea Pig brain	6	370*	Bruns et. al., (1980)
	Bovine brain	0.3)	340*	
		1.8)	200*	
	Rat brain	0.7)	230	Patel et al.,
		2.4)	120	(1982)
[^{3}H](-)N^6-PIA	Rat brain	5	810	Schwabe & Trost, (1980)
[^{125}I](-)N^6-PIA	Rat brain	0.48	230	Schwabe et al., (1982)
[^{3}H] CPA	Rat brain	0.48	416	Williams et al., (1986)
[^{3}H] DPX	Guinea Pig brain	70	416	
	Bovine brain	5	500*	Bruns et al.,
			1000*	(1980)

* B max values recalculated on the basis 1g brain tissue ≡ 100 mg protein

Two groups developed 2-chloradenosine as a radioligand for binding studies. It is a potent agonist at the A_1 receptor but is also relatively potent at A_2 sites. The protocols used by the two laboratories differed somewhat as did the results. Unlike Williams & Risely (1980), Wu et al.,

(1980) did not treat their membranes with adenosine deaminase. It would seem that binding of 2-CADO to membranes in the presence of high concentrations of divalent cations (25 mM of $CaCl_2$ and $MgCl_2$) at 0-4°C for 30 min, masks a second lower affinity site (Wu et al., 1980). This discrepancy has not been fully clarified. The presence of more than one affinity site is probably due to high and low affinity states of one receptor type rather than an indication of receptor subtypes, (Williams & Risely 1980).

Dual affinities were also seen with the binding of [^{3}H] CHA to brain membranes. The presence of more than one site being dependent on the species studied, (Bruns et al., 1980). [^{3}H] CHA binding to guinea pig membranes revealed that the radioligand bound to two sites; an A_1 receptor with a high affinity for CHA and a second site with a much lower affinity for CHA. Although the second site was initially thought to be an A_2 receptor, structure activity analysis indicated that most of this binding was not to adenosine receptors. CHA binding to bovine brain revealed only one A_1 site, (Bruns et al., 1980).

The first laboratory to successfully label A_2 receptors was that of Yeung & Green (1981). Subsequent work (Yeung & Green, 1984) demonstrated the binding of [^{3}H] NECA to both A_1 and A_2 type receptors in guinea pig striatal membranes. Structure activity relationships and saturation binding of [^{3}H] NECA were performed on control and NEM treated membranes. The purpose of using NEM was to

selectively eliminate the A_1 component of binding (NEM is a sulphydryl alkylating agent which is thought to act by covalently modifying the Gi-GTP binding protein in brain membranes). Agonist inhibition profiles of [^{3}H] NECA binding to striatal membranes were shallow (Hill coefficient <0.8) and in some cases appeared biphasic. Repetition of these inhibition profiles on NEM-pretreated membranes resulted in the curves being steepened and shifted to the right. These results being in accord with what one would expect if NECA bound to both A_1 and A_2 sites. Agonist potency profiles in NEM-pretreated striatal and hippocampal membranes (the latter, according to Yeung & Green, 1984 containing only A_1 receptors, a theory disputed by, Fredholm et al., 1983) gave the following potency inhibition profiles: hippocampus, L-PIA ≥ CHA > NECA ≥ D-PIA striatum, NECA > L-PIA > CHA >> D-PIA. These pharmacological profiles support the notion that in NEM-pretreated striatal membranes, binding of [^{3}H] NECA was to A_2 type receptors whilst in hippocampal membranes it was to A_1 type receptors. Saturation binding and stereoselectivity data also supported the notion that A_2 receptors as well as A_1 receptors were labelled by [^{3}H] NECA in striatal membranes.

1.2.2 SUBCLASSIFICATION OF A_1 AND A_2 RECEPTORS

Whilst the evidence for the subclassification of A_2 receptors is reasonably convincing, it is more difficult from information presently available to propose the

existence of multiple A1 type receptors.

In brain membranes there is considerable evidence for inter species heterogencity of A_1 binding sites. Murphy & Snyder (1982) found that although adenosine agonists displayed little species variation in affinity for A_1 receptors, binding of DPX to adenosine receptors displayed significant differences. In all species examined binding of analogues was consonant with binding at A_1 receptors. DPX competing for A_1 sites labelled with [^{3}H] CHA showed a 250 fold lesser affinity in guinea pig and human than in calf brain. Similar species variation in binding properties occurred for [^{3}H] DPX binding to brain membranes.

A more extensive study examining species differences in structure/activity relationships of adenosine agonists and antagonists was carried out by Ukena et al., (1986). Bulky N^6 substitutions of agonists greatly increased affinity at the calf brain A_1 receptor compared to guinea pig. The presence of an 8-substituent at antagonists also enhanced affinity at calf compared to guinea pig brain. It should be noted that the presence of multiple binding sites with different affinities for radioligands in brain membranes from a single species and brain region, as found by:- Bruns et al., (1980); Marangos et al., (1983; Patel et al., (1982) and Williams & Risely, (1980), may reflect only different states of a single receptor rather than different receptors.

Some suggestion of A_1 receptor heterogenicity within a single species has come from behavioural studies.

Dunwiddie & Worth, (1982) examined differences in anticonvulsant properties of CHA, L-PIA and 2-CADO. Their relative potencies as anticonvulsants varied depending on how seizures were induced. Such differences indicated that two distinct receptor sites which differ in affinities for these drugs probably exist in brain.

Daly et al., (1983) proposed that A_2 adenosine receptors could be further divided into two classes based upon observations made by Prémont et al., (1979) that some A_2 receptors have EC_{50} values for adenosine in the high nanomolar range (0.1 - 1μM) rather than in the micromolar range. The high affinity A_2 receptors exist in the striatum where they appear to be localised on intrinsic neurons (Wojcik & Neff, 1983) and can be observed in the brain in broken cell adenylate cyclase assays (Prémont et al., 1979) but not in brain slices Daly et al., 1983). The low affinity A_2 receptor activates cAMP generation from almost all brain regions but cannot be detected in broken cell adenylate cyclase preparations (Daly et al., 1983).

Daly's initial proposal for subclassification was later confirmed by a study examining the binding of [^{3}H] NECA to rat striatal membranes (Bruns et al., 1986). Unlike Yeung & Green, (1984) who used NEM to eliminate the A_1 component of binding Bruns et al., used 50nM CPA. They calculated that this amount when added to [^{3}H] NECA incubation would displace about 98% of the A_1 binding of [^{3}H] NECA but only 7% of A_2 binding. The site labelled by [^{3}H] NECA in the presence of 50nM CPA was identified as an

A_2 receptor by its distinctive SAR. NECA was about 12 times as potent as L-PIA at the [^{3}H] NECA binding site in agreement with adenylate cyclase results (Londos et al., 1980). From saturation binding analysis they identified that the A_2 receptor labelled by [^{3}H] NECA belongs to the high affinity subclass. Comparing SAR's of this high affinity form with the low affinity form of human fibroblasts (Bruns, 1980) they noted significant differences. Whereas most agonists were 200-400 times more potent in [^{3}H] NECA binding to high affinity receptors than in fibroblasts, 1,N^6-etheno-2-oxoadenosine had an affinity ratio of only 11, while 2-(4-methoxyphenyl) adenosine, had a ratio of close to 10,000. It is difficult to explain these huge differences in selectivity in terms of different affinity states of a single receptor. Also the radical difference in distribution of the two A_2 receptor subtypes in brain would be difficult to explain in terms of a simple coupling difference. These results indicate that the A_{2a} (high affinity) and A_{2b} (low affinity) adenosine receptors are different proteins and therefore distinct subtypes.

1.2.3 LOCALISATION OF A_1 AND A_2 RECEPTORS IN THE BRAIN

Minor differences in distribution of A_1 receptors were seen when [^{3}H] 2-CADO was used as the radioligand (Williams & Risely, 1980). There was only about a 2-fold difference between the lower values of the cortex, hypothalamus, spinal cord, medulla and pons and the higher values detected in the thalamur, cerebellum and hippocampus.

In contrast marked differences were seen using [^{3}H] CHA to radiolabel A_1 adenosine receptors by an in vitro autoradiographic technique, (Goodman and Snyder, 1982). The highest densities of A_1 receptors were found in the molecular layer of the cerebellum, the molecular and polymorphic layers of the hippocampus and dentate gyrus, the medial geniculate body, certain thalamic nuclei and the lateral septum. High densities were also found in certain layers of the cerebral cortex, the piriform cortex and caudate-putamen. Most white matter areas, as well as certain grey matter areas such as the hypothalamus were found to have negligible receptor concentrations. Similar results were obtained by Weber et al., (1988) with [^{125}I]PIA as radioligand using an in vitro autoradiographic technique. In contrast human cerebellum displayed low levels of density and binding was concentrated to a narrow band corresponding to the Purkinje cell layer (Fastbom et al., 1986). The molecular and polymorphic layers of the hippocampus and dentate gyrus were also seen to possess high densities.

A study performed by Bruns et al., (1986) labelling rat brain A_2 receptors with [^{3}H] NECA showed that binding was highest in striatum but detectable at much lower levels in each of seven other brain regions.

1.2.4 RECEPTORS AS TWO STATE SYSTEMS

Receptors may exist in at least two states: active and inactive. To activate either the stimulatory or inhibitory

guanine nucleotide binding proteins (Gs or Gi, see section 1.8) receptors must be occupied by an agonistic ligand. Agonists under appropriate conditions act to stabilise i.e. favour the formation of the active form while antagonists favour the formation of the inactive form.

The simplest model for hormone receptor interactions which can explain and reproduce the experimental data currently available is the ternary complex model (DeLean et al., 1980; Lefkowitz et al., 1982). This model was derived from computer analysis of agonist and antagonist binding to frog erythrocyte β-adrenergic receptors.

Interaction of hormone/ligand with the free receptor results in the formation of the "low affinity" form of receptor HR. This HR complex may combine with G protein in a Mg^{2+} dependent fashion to form a ternary complex HRG - the "high affinity" form of the receptor. It should be noted that it is a unique property of agonists (as opposed to antagonists) to be able to induce, stabilise or recognise the high affinity form of a receptor. Interaction of the HRG complex with guanine nucleotide results in a reduction promoting formation of the low affinity form of the receptor.

A_1 receptors of guinea pig and bovine brain membranes labelled with [^{3}H] CHA are modulated by guanine nucleotides and divalent cations (Goodman et al., 1982) as are A_1 receptors of rat and bovine brain membranes labelled with [^{3}H] PIA (Lohse et al., 1984). The former group found

that GTP decreased the affinity of [^{3}H] CHA with no profound influence on Bmax values. In agreement Lohse et al., (1984) found that GTP reduced the affinity of agonist by a factor of 90-150 in rat brain and 10 in bovine brain with no effect on Bmax. Both groups found no change in Bmax and K_D values of antagonist binding by guanine nucleotides. Lohse et al., (1984) like Goodman & Snyder, (1982) found that GTP did not influence the binding of antagonists. In contrast Yeung and Green, (1983) found a marked increase in Bmax of [^{3}H] DPX binding in the presence of GTP and 4mM $MgCl_2$. An increase in [^{3}H] DPX binding of approximately 8% has also been reported by Bruns et al., (1980).

In addition to the two agonist affinity states a state of medium affinity for agonists has been seen in saturation binding experiments with [^{3}H] CHA in the presence of Gpp(NH)p, (Yeung & Green, 1984). In the same experiment this group also observed a marked reduction in Bmax. The most likely explanation for this discrepancy is the limited concentration range of the ligand used by these authors (10 - 100 nM) which is beyond the K_H (1.8 nM) and below the K_L (430 nM) reported.

Removal of endogenous divalent cations with EDTA inhibits [^{3}H] CHA but not [^{3}H] DPX binding, (Goodman et al., 1982), thus suggesting that endogenous divalent cations regulate agonist affinity at adenosine receptors. [^{3}H] CHA binding is selectively augmented by manganese, magnesium and calcium. The primary effect of divalent cations seems to be to decrease the affinity (i.e. increase the

proportion of receptors in the high affinity state) and also in some systems to increase Bmax values (Goodman et al., 1982; Lohse et al., 1984). As has been demonstrated in numerous other receptor systems sodium also selectively decreases binding to adenosine receptors with lithium and potassium being inactive, (Goodman et. al., 1982).

1.2.5 THE GLYCOPROTEIN NATURE AND SIZE OF THE A_1 ADENOSINE RECEPTOR

Adenosine A_1 receptors from different tissues were photoaffinity labelled and the carbohydrate content examined by both enzymatic and chemical treatment. Experiments of this type (Klotz & Lohse, 1986; Stiles 1986) demonstrated the glycoprotein nature of the receptor. SDS-PAGE electrophoresis showed the protein comprising the A_1 adenosine binding subunit of rat brain to migrate with Mr 38,000, (Stiles, 1986). Deglycosylation of the receptor binding subunits resulted in a single polypeptide of Mr 32,000 (Klotz & Lohse 1986; Stiles 1986) in both fat and brain, showing that the carbohydrate and protein components of the receptor-binding subunit are similar.

That adenosine A_1 receptors are glycoproteins has also been demonstrated by photoaffinity labelling of the receptor from different tissues and examining the carbohydrate content by enzymatic and chemical treatment (Klotz & Lohse, 1986; Stiles, 1986).

A more direct approach to identifying the size of the functional ligand binding moiety is made possible

with the high energy-irradiation/inactivation method (target size analysis). This allows for an assessment of molecular size according to sensitivity to loss of biological activity after irradiation, without the necessity for prior purification.

Using this method Frame et al., (1986) reported that the target size of the high affinity form of the adenosine A_1 receptor was 63,000 Da. A value comparable to that of β-adrenergic receptors, but smaller than dopaminergic D_2 and muscarinic cholinergic receptors, (Frame et al., 1986).

A receptor size of approximately 60,000 Da may represent a minimal size that conveys an adequate surface recognition area, membrane insertion domains and GTP-regulatory protein interacting domain. The size reported in this study is approximately 2-fold that as described by photoaffinity labelling. This may be due to the fact that adenosine receptors are dimeric or composed of subunits other than those that can be photolabelled by adenosine analogues.

1.3 PATHWAYS AND ADENOSINE METABOLISM

Most tissues are capable of producing, releasing, taking up and metabolising adenosine. This nucleoside can be synthetised via a number of pathways, some of which make a negligible contribution (for review see Arch & Newsholme, 1978b). The enzymes of importance are 5'-nucleotidase, adenosine deaminase, adenosine kinase and to a lesser

extent adenylate deaminase. Production of adenosine from S-adenosylhomocysteine (SAH) is another important synthetic pathway. SAH is formed from S-adenosylmethionine (SAM) in the course of methylation reactions (see fig. 1.2).

Even though 5'-nucleotidase is not the sole enzyme involved in dephosphorylating AMP, intracellular alkaline and acid phosphatases also being present, it is quantitatively the most important, as the non-specific phosphatases at physiological pH's have very low activities. 5'-Nucleotidase conversely is specific for 5'-nucleotides, (Bodansky & Schwartz, 1968) also its pH optimum is close to the physiological pH (Arch & Newsholme, 1978). At least three major forms of 5'-nucleotidase exist, one form is an ectoenzyme localised on the outer surface of cells (De Pierre & Karnovsky 1974) the others are intracellular forms with completely different kinetic properties to the ectoenzyme (Itoh 1981a,b; Newby, 1988). Adenosine kinase, adenosine deaminase and SAH hydrolase are all located mainly in the soluble fraction (Phillips & Newsholme, 1978a,b; Schrader et al., 1981; Pull and McIlwain, 1974). The important pathways of adenosine metabolism are summarised in figs. 1.1 and 1.2.

The intracellular production of adenosine has been shown to be effected by a reduction in the energy change of the cell i.e. (ATP + ½ADP/ATP + ADP + AMP), (Atkinson, 1968; Itoh, 1981b; 1986; Newby, 1983). An initial hypothesis to explain the actions of adenosine was put forward by Berne, (1964) who stated that adenosine was

Fig. 1.1 Possible pathways of adenosine metabolism

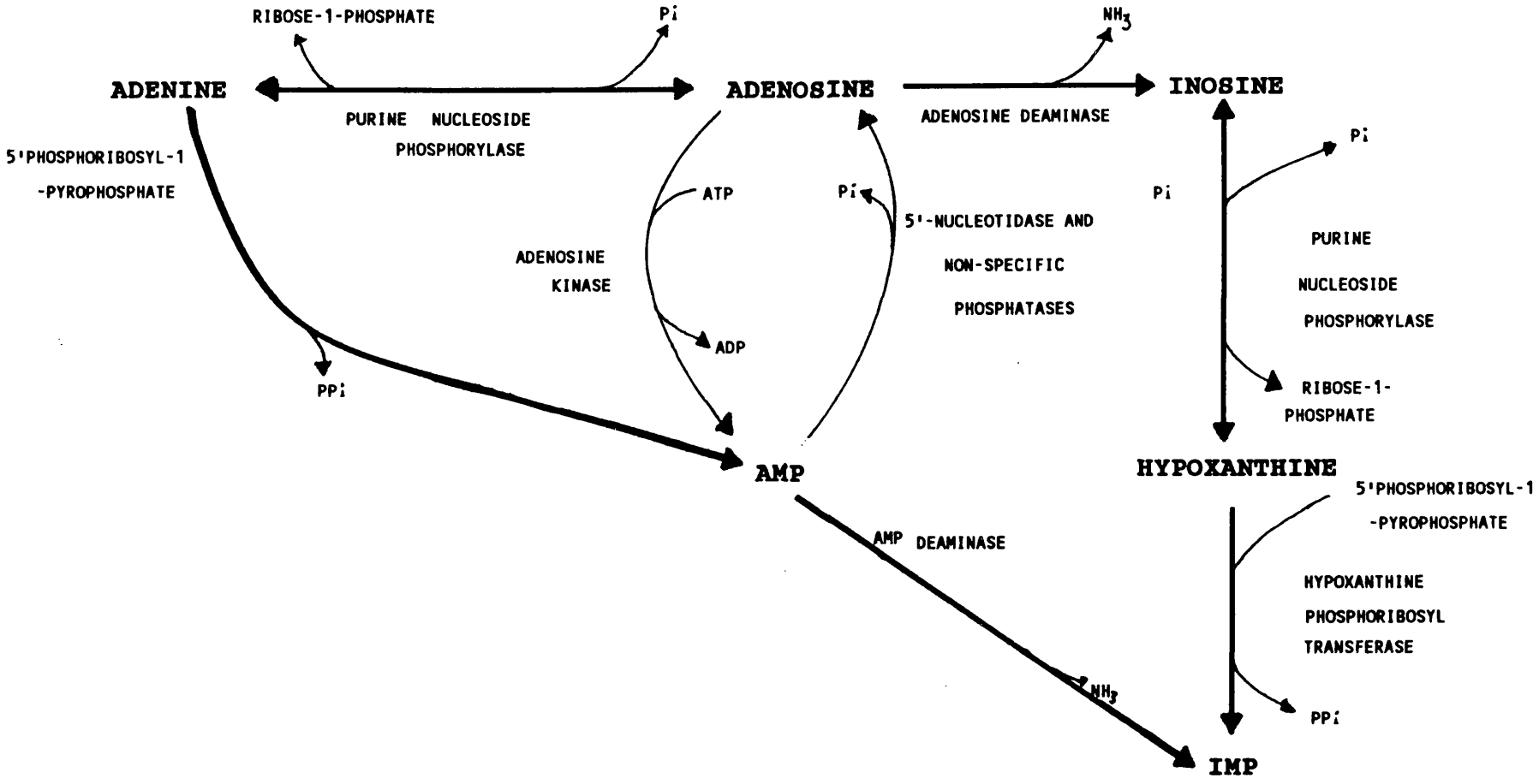


Fig. 1.2 Pathway of adenosine and homocysteine production from the transmethylation pathway.

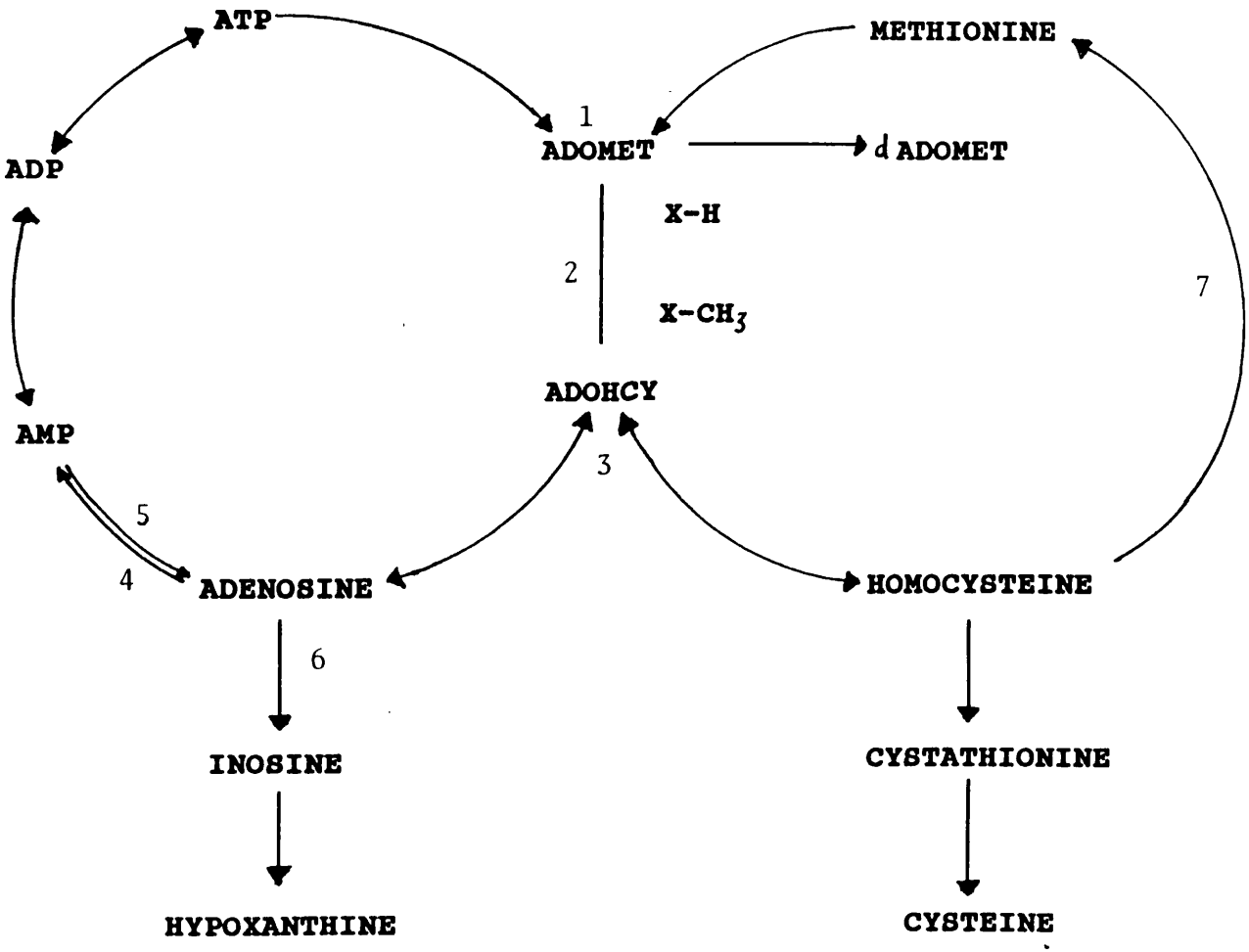
KEY

dADOMET = deoxy ADOMET

X = methyl acceptor.

Enzymes:

1. ATP; L-methionine adenosyltransferase (EC 2.5.1.6)
2. S-adenosyl-L-methionine: X methyl-transferase (EC 2.1.1)
3. S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1)
4. adenosine kinase (EC 2.7.1.20)
5. 5'-nucleotidase (EC 3.1.3.5)
6. adenosine deaminase (EC 3.5.4.4)
7. 5'-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13)


ATP
METHIONINE
1
ADOMET
d ADOMET
ADP
X-H
2
X-CH3
7
ADOHCY
AMP
3
5
4
ADENOSINE
HOMOCYSTEINE
6
INOSINE
CYSTATHIONINE
HYPOXANTHINE
CYSTEINE

formed as a result of net ATP breakdown in myocardial cells under conditions where oxygen supply did not meet oxygen demand. Overall therefore any perturbation that decreases the energy charge of the cell may result in increased adenosine production. The nucleoside then acts via one of its receptors to effect a response such that the metabolic homeostasis of a tissue can be maintained. Adenosine can therefore be seen as a 'local hormone' or retaliatory metabolite (Arch & Newsholme, 1978a, b; Newby, 1984). Since the purine is rapidly metabolised by mammalian tissues it can not therefore have a systemic effect. The many and varied effects of adenosine such as: modulation of platelet aggregation, its antilipolytic effect, neuromodulation, hypnotic effects and promotion of astrocyte glycogenolysis can all be rationalised in terms of the retaliatory metabolite concept.

Within the central nervous system two routes of extracellular provision of adenosine appear likely. First adenosine may be produced as the end product of an extracellular purine nucleotide phosphohydrolase pathway. Second, in response to metabolic insults, adenosine may be produced intracellularly after a decrease in the energy change of the cell and a resulting rise in AMP content. (Richardson et al., 1987b; Jonzon & Fredholm, 1985; McIlwain and Poll, 1986).

1.4 THE EXTRACELLULAR FORMATION OF ADENOSINE

The "purinergic neuron" hypothesis whereby ATP is the sole

neurotransmitter as proposed by Burnstock (1972) is still relatively speculative. There is however both direct and indirect evidence for the co-release of ATP and acetylcholine (Ach) from cholinergic nerve terminals, (Zimmerman & Whittaker, 1974; Giompres et al., 1981). These two groups using synaptosomes isolated from Torpedo electric organ were able to demonstrate the stimulation-induced release of both ATP and Ach. The molar ratio of the released substances was due to that of storage inside synaptic vesicles (Morel & Meunier, 1981). However it has still not been possible to identify ATP as the sole transmitter at any central or peripheral synapses. To show that ATP can act as a transmitter/cotransmitter it is necessary to demonstrate that it is released presynaptically in a calcium dependent fashion. Despite initial contradictory results (see Phillis & Wu, 1981; Stone, 1981) evidence points to the fact that ATP is released in a transmitter-like fashion (Silinsky, 1975; Richardson and Brown, 1987a,b). However data obtained from the synaptic junction of the electric organ of Torpedo, suggests that ATP released by nerve stimulation can also have a post-synaptic origin, (Israel et al., 1976).

A study of ATP release from immunoaffinity purified rat cholinergic nerve terminals demonstrated unequivocally the co-release of ATP and Ach, (Richardson & Brown, 1987a,b). However it was not possible from their data to conclude that the release of ATP was due to vesicular exocytosis. Observations concerning the

different kinetics of ATP and Ach release (Stone, 1981) and variations in the ratio of released Ach/ATP depending on the method of stimulation (Morel & Meunier, 1981) suggest that ATP and Ach can be released by different mechanisms.

The released ATP is rapidly degraded by a series of ectoenzymes including 5'-nucleotidase. The concentration of ATP in the synaptic cleft can reach a concentration of 10^{-4}-3 x 10^{-3}M depending on the assumptions made as to the dimensions of the cleft (Richardson & Brown, 1987a,b). The ectonucleotidases located on the postsynaptic membrane, 5'-nucleotidase being located on glial cell structures (Kreutzberg & Barron, 1978; Kreutzberg et al., 1978, 1986; Heymann et al., 1984) have high affinities for their substrates Km≈ 10^{-5}M (Nagy, 1986). Since 5' AMP was seen to accumulate (30% of the extracellular label accumulated as AMP; Richardson & Brown 1987b) it would indicate that the 5'-nucleotidase may be the rate limiting step in the extracellular production of adenosine from ATP. Adenosine has been shown to inhibit the release of Ach, noradrenaline, dopamine and 5-hydroxytryptamine in rat brain preparations (Harms et al., 1979; Fredholm & Dunwiddie, 1988). It is possible therefore that synaptic ectophosphohydrolases have a general function in the autoinhibition of neuro-transmission. That it is adenosine that evokes this inhibitory response and not ATP was shown in the study by Richardson & Brown (1987b). A perfusion of cholinergic nerve terminals with 1mM ATP resulted in 90% hydrolysis of

the ATP and 36% inhibition of Ach release, whereas the non-hydrolysable analogue $\alpha\beta$ methylene ATP had no effect. Both addition of theophylline and inhibition of 5'-nucleotidase by a polyclonal antiserum (by 90%) prevented any effect of ATP on Ach release.

Richardson (1983) showed that 5'-nucleotidase is not present on presynaptic membranes of cholinergic nerve terminals. Indeed cytochemical evidence suggests that much of the enzyme is present on a wide variety of glial cells (Kreutzberg et al., 1978; 1986). Certain studies have suggested a synaptic localisation for 5'-nucleotidase, it seems likely that the enzyme present in their synaptic fractions was probably due to glial contamination (Heymann et al., 1984). A significant proportion of the 5'-nucleotidase is also present on myelin fibres; in fact as much as 30% of total brain 5'-nucleotidase is to be found on this locus (Cammer et al., 1980). Consequently, the presence or absence of 5'-nucleotidase on glial membranes close to the synaptic cleft (and perhaps post-synaptic membranes; Richardson & Brown, 1987a, b) may control the adenosine concentration near cholinergic nerve terminals. This would result in glial cells being able to modulate synaptic activity.

Whilst the inhibitory effect of adenosine is seen in cholinergic nerve terminals of striatal origin it is not seen in those of cortical origin, the latter lacking the complete ectophosphohydrolase pathway (Richardson & Brown, 1987a). Therefore when ATP is the major source of

adenosine, modulation of neurotransmission in various brain regions may be controlled by the presence and activity of synaptic ectophosphohydrolases.

1.4.1 UPTAKE AND RELEASE OF ADENOSINE

Adenosine is taken up across plasma membranes by a carrier mediated process. In one study using synaptosomes, an apparent Km value of 2μM for adenosine uptake was equal to that of the high affinity uptake system for choline. The maximal uptake capacity Vmax was 30 pmol min^{-1} mg^{-1} protein. This rate has been found to be 1000-fold lower than the maximum velocity of ATP hydrolosis by ectoenzymes (Zimmerman et al., 1986). A rapid uptake process into rat cerebral cortical synaptosomes has a Km of 0.9μM and a Vmax of 10.52 pmol min^{-1} mg^{-1} protein. Uptake was inhibited at low temperatures and by various agents known to inhibit nucleoside transport in other tissues (e.g. dipyridamole, papaverine). Cytidine, uridine and hypoxanthine were rather less effective at inhibiting uptake (Bender et al., 1980; 1981). These observations are generally consistent with the view that adenosine uptake is mediated by a nucleoside carrier in the membrane. Simple diffusion may also account for a proportion of nucleoside movement across plasma membranes, but it is differentiated experimentally as its rate is proportional to nucleoside concentration and is unaffected by other substrates or nucleoside transport inhibitors (Clanachan et al., 1987).

Overall therefore the functional significance of

ecto-5'-nucleotidase activity in the brain can be considered from two points of view. First, nucleotides appearing in the extracellular space may be utilised to form nucleosides like adenosine, which can act on extracellular receptors producing physiological effects, such as neuromodulation or changes in local cerebral blood flow. These humoral actions are then terminated by removing adenosine from the extracellular compartment via a high affinity uptake system. Secondly, 5' - nucleotidase might form part of a retrieval mechanism for purine or pyrimidine nucleotides. These are converted into a membrane-permeable form and can therefore be removed from the extracellular space. Evidently in terms of both of these functions, the relationship between 5'-nucleotidase activity and the nucleoside uptake system is a principle factor determining the extracellular concentration of nucleosides, (Kreutzberg et al., 1986).

1.4.2 RELEASE OF ADENOSINE FROM INTRACELLULAR STORES

The question arises as to whether adenosine is formed solely from intact ATP liberated from cells or whether it is also released from intracellular stores formed as a consequence of changes in the energy status of the cell. A working hypothesis states that electrical stimulation increases the activity of e.g. a brain slice, partly by the action of established transmitters and partly as a result of direct depolarisation. This increased activity leads to

an increase in intracellular AMP. Some of this AMP may be dephosphorylated intracellularly to adenosine and released from the cell, (Fredholm et al., 1984). Several lines of evidence suggest that this is in fact the case. Pons et al., (1980) showed that a veratridine evoked release of adenosine from brain slices resulted in a stimulation of adenylate cyclase, (veratridine acts by blocking the inactivation of Na^+ conductance). This increase in cAMP was prevented by incubation with adenosine deaminase, but not by inhibiting the 5'-nucleotidase ectoenzyme with a combination of inhibitors. Thus adenosine released by veratridine in brain slices cannot have been formed extracellularly from ATP or another nucleotide and must have been released as intact adenosine. Furthermore, in superfused synaptosomes nucleotides accounted for only some 6% of the total radioactive purines released by depolarisation but about 75% of the radioactivity released by hypo-osmotic shock, (Fredholm & Vernet, 1979). Pull & McIlwain, (1977) found that theophylline (0.5mM) increased the amount of adenosine in the superfusate from electrically stimulated cortical slices, but decreased the nucleotide content. Theophylline at these concentrations is a potent inhibitor of 5'-nucleotidase (Tsuzuki & Newburgh, 1975). Potentiation of adenosine release by theophylline is therefore not compatible with the opinion that all the adenosine (and inosine as well as hypoxanthine) derives from extracellularly released ATP.

1.4.3 RELEASE OF PURINES FROM NEURONAL TISSUE

In a study comparing the release of noradrenaline (NA) and GABA to that of purines from rat hippocampal slices, Jonzon & Fredholm, (1985) found that NA release, evoked by field stimulation was Ca^{2+} and frequency dependent and was maximal at 10V. GABA on the other hand may be released from different pools: nerve terminals, cell bodies and dendrites as well as glial cells. The evoked release of GABA is partially calcium sensitive. Certainly all of the NA and much of GABA release show characteristics of transmitter release (Orrego, 1979).

Electrical pulses were found to evoke a voltage and frequency dependent release of purines, consisting of hypoxanthine, inosine and adenosine. Less than 5% of the [^{3}H] purine outflow could be accounted for by nucleotides. By contrast, the bulk of the purines remaining in the slices after stimulation were nucleotides. The energy charge of the slice was low, which is probably why most of the non-nucleotide material was present as hypoxanthine. However when the effluent from the slices was rapidly cooled by ice the proportion of nucleosides present as hypoxanthine decreased. Fredholm & Jonzon, (1985) therefore concluded that the purines are most probably released as adenosine.

Potassium-evoked purine release from hippocampal slices has been found to be Ca^{2+} dependent, (Jonzon & Fredholm, 1985) likewise from cortical synaptosomes (Kuroda & McIlwain, 1973). On the other hand veratridine evokes a

Ca^{2+} independent release of purines (Jonzon & Fredholm, 1985). It seems likely therefore that purines are not exclusively released by exocytosis and that a major part of the release occurs by adenosine being transported out of the cell by a carrier mediated process.

1.5 ENZYMES INVOLVED IN ADENOSINE METABOLISM

1.5.1 ADENOSINE DEAMINASE

Adenosine deaminase (ADA) is primarily a cytoplasmic enzyme, there is no evidence to suggest that it is present as an ectoenzyme in the brain (Pull & McIlwain, 1974; Phillips & Newsholme, 1979). ADA has been shown to exist in at least three forms, amphibian ADA for instance revealed three molecular forms of Mr 30,000, 100,000 and 180,000 Da (Ma & Fisher, 1968). Most mammalian tissues studied seem potentially to possess two forms of the enzyme (Andy & Kornfeld, 1982). The small C form has a molecular weight of 30 - 40,000 (Ma & Fisher, 1968). This C form has the ability to bind to an ADA binding protein (BP) forming a complex of Mr 200,000, (Andy & Kornfeld, 1982). It has been suggested that in human fibroblasts the BP may function to anchor ADA to the membrane and thus it may play some role in the extracellular metabolism of adenosine. Since it was found that 16-30% of total fibroblast ADA was inhibited by an antiserum to the enzyme, it was suggested that this proportion may be in the form of an ectoenzyme (Andy & Kornfeld, 1982). However little is known about the

expression and cellular localisation of the BP in the brain.

The activity of the enzyme in a sucrose dispersion of rat cerebral cortex is 115 nmol min^{-1} g^{-1} tissue with a Km of 54-57μM, (Pull & McIlwain, 1974). The activity of ADA in rat brain homogenate gave a Km of 17μM and a Vmax of 54 nmol g^{-1} wet weight (Phillips and Newsholme, 1979). Activity in these regions of the brain was investigated namely: Cortex, cerebellum and hindbrain; no regional differences were observed (Phillips and Newsholme, 1979). Several groups, (Pull & McIlwain, 1974; Phillips & Newsholme, 1979; Centelles et al., 1988) have shown this enzyme to be enriched in synaptosomal and synaptic vesicle fractions. Rat glial cells have also been found to contain ADA activity (Hertz, 1978).

Species differences in maximum levels of ADA activity vary significantly, (values given in parentheses are activities in n moles/30 min/mg protein), rabbit (1252; superior colliculus) > rat (279; olfactory bulb) > guinea pig (273; cerebellum) > mouse (106; olfactory bulb), (Yamamoto et al., 1987). Like Phillips & Newsholme, (1979) this group also found no regional differences in activity between: Cortex, cerebellum and hind brain in any of the species studied. However high levels of ADA activity were found in the posterior and anterior hypothalamus as compared to other regions. Maximal activities of ADA from nine regions of human brain showed some degree of variation, the highest activity being in the hypothalamus

(350 nmol min^{-1} g wet $weight^{-1}$) the lowest in the hind brain (38 nmol min^{-1} g wet $weight^{-1}$), (Phillips and Newsholme, 1979).

1.5.2 ADENOSINE KINASE

The properties of adenosine kinase (AK) in different tissues of several species have been studied by Arch & Newsholme, (1978b) and Phillips & Newsholme, (1979). Rat brain AK (as measured in homogenates) has a Km of 2μM and Vmax of 25.02 nmol min^{-1} g wet $weight^{-1}$. Subcellular localisation studies have shown AK to be located mainly in the soluble fraction. A comparison of activity in the soluble fraction from rat: cortex, cerebellum and hindbrain revealed a 3.6 fold difference between the highest value in cerebellum and the lowest value in hindbrain (2.31 compared to 0.64 nmon min^{-1} mg $protein^{-1}$). Cortex gave a value of 1.16 nmol min^{-1} mg $protein^{-1}$, (Phillips & Newsholme, 1979).

Partially purified AK from rat brain was shown to have a Km for adenosine of 20μM (Shimzu et al., 1972), whilst that of the more fully purified rat brain enzyme a Km of 0.2μM was determined, (Yamada et al., 1980). The enzyme itself has been found to be a monomeric structure with Mr 40,000. No differences in properties were observed between the enzyme from brain and that of liver (Yamada et al., 1980).

1.5.3 S-ADENOSYLHOMOCYSTEINE HYDROLASE (SAH HYDROLASE)

SAH hydrolase is a soluble enzyme (Broch & Ueland, 1980). Studies examining regional variations in activity are rather discrepant. In one study in adult rat brain the highest activity was found in the hypothalamus and bulbus olfactorius and the least in pons and medulla, (Broch & Ueland, 1980). Schatz et al., (1977) however found the hypothalamus to be the region of lowest activity. Generally very little difference in magnitude of activity between various regions of rat brain was seen by either group. In the heart the Km for hydrolysis of SAH is approximately 12μM (Achterberg et al., 1985), whilst that of guinea pig heart is 0.39μM (Schrader et al., 1981).

1.5.4 5' - NUCLEOTIDASE

The four main forms of 5'-nucleotidase have been widely studied, two of which are cytoplasmic, one is localised on the plasma membrane and one is lysosomal. The plasma membrane form of the enzyme has been categorised as an ectoenzyme in several cell types e.g. polymorphonuclear leucocytes (De Pierre & Karnovsky, 1974), fat cells (Newby et al., 1975) and rat brain myelin (Casadó et al., 1988). The molecular weight of the ectoenzyme from rat heart is 74,000 Da whilst that of the liver is 70,000. Cross-linking studies have indicated that the native form is a homodimer (Naito & Lowenstein, 1981. AMP is the normal physiological substrate for the enzyme (Km 20-40μM), (Newby

et al., 1987). The 5'-nucleotidase enzymes studied from different tissue sources display similar kinetics in that they are inhibited by ADP, ATP and their analogues (Burger & Lowenstein, 1975). Both ADP and the ATP analogue AOPCP are competitive inhibitors of the rat heart enzyme with Ki values of 83 and 6nM respectively. ATP is a competitive inhibitor with a Ki of 4.4μM while the $\alpha\beta$ - diphosphate analogue inhibits with a ki value of 120nM (Naito & Lowenstein, 1985).

A cytoplasmic 5'-nucleotidase which favours IMP as a substrate over AMP has been studied in rat and chicken heart and rat liver (Itoh, 1981a, 1981b, 1982; Itoh and Oka, 1985; Itoh et al., 1986) human placenta (Berry et al., 1986; Madrid-Marina & Fox, 1986) and brain (Montero & Fes, 1982). The rat liver and heart enzyme characterised by Itoh (1981a) and Itoh et al., (1986) gave a hyperbolic substrate velocity plot when IMP was used as a substrate and a sigmoidal plot with AMP as substrate. In the first instance ATP, NaCl and Tris maleate activated the enzyme by increasing the V value and decreasing the Km value. In the second instance these three activators decreased the sigmoidicity of the plot but did not alter the V value. ADP is also an activator of the enzyme (Van den Berghe et al., 1977; Itoh et al., 1986), whilst Pi is an inhibitor, at least in the heart. When IMP is substrate Pi acts by changing the plot from a hyperbolic to a sigmoidal form and increasing the Km (Itoh et al., 1986).

By contrast the IMP preferring rat heart enzyme purified by Truong et al., (1988) displayed sigmoidal saturation behaviour with IMP but not AMP at low substrate concentrations. The IMP preferring 5'-nucleotidase has four subunits and a Km for AMP in the millimolar range (2.6 - 10mM) (Worku & Newby, 1983; Itoh & Oka 1985; Itoh et al., 1986).

The cytoplasmic 5'-nucleotidase purified from rat brain by Montero and Fes (1982) is somewhat atypical in that it has a μmolar Km value (10.4μM) and did not express an absolute requirement for Mg^{2+} for activity, characteristics more typical of the ecto-enzyme than the cytosolic form.

Recently a cytosolic 5'-nucleotidase that prefers AMP to IMP as substrate has been characterised from rat heart (Truong et al., 1988) from pigeon heart (Newby, 1988) and from rabbit heart (Collinson et al., 1987). The Km values for rabbit, rat and pigeon heart in the presence of ATP are 2.6, 1.2 and 4.6 - 5.2 mM respectively. ATP was found to be stimulatory in each case. The effects of ADP on the rabbit enzyme are somewhat complex in that it inhibits at high and activates at low concentrations of AMP. It has been suggested that ATP acts only at an activator site, while ADP acts as an activator at the activator site and as an inhibitor at the catalytic site. An inhibitor of the IMP-5'-nucleotidase, 5'-deoxy-5'-isobutyl-thioadenosine did not inhibit the pigeon heart 5'-nucleotidase (Newby, 1988), suggesting that the two enzymes

are separate entities and are not some modification of one form as a consequence of a purification procedure.

1.6 PRESENCE AND DISTRIBUTION OF ADENOSINE AND ADENINE NUCLEOTIDES IN BRAIN, EFFECT OF HYPOXIA

The accurate determination of ATP and consequently adenosine is very difficult since ATP is extremely labile. It is of paramount importance that the tissue should be frozen or fixed without interruption of the circulation or traumatisation of the cerebral tissue. If adequate precautions are taken by freezing the brain in situ with an intact circulation or by freeze blowing, adenosine concentrations in rat brain do not exceed 1μmol/kg and ATP concentrations are of the order 3 mmol/kg, see table 1.4.

Table 1.4 Brain Levels of Adenosine and its Metabolites

Purine	Rat Cerebral Cortex (1)		Rat Brain (2)	
ATP	3.00 ± 0.03	mmol/kg	2.67 ± 0.12	mmol/kg
ADP	2.43 ± 0.009	mmol/kg	0.416 ± 0.034	mmol/kg
AMP	0.039 ± 0.001	mmol/kg	0.046 ± 0.005	mmol/kg
cAMP	1.66 ± 0.03	μmol/kg	-	
Adenosine	0.6 ± 0.00	μmol/kg	0.52 ± 0.11	μmol/kg
Inosine	-		3.76 ± 0.43	μmol/kg
Hypoxanthine	-		0.09 ± 1.32	μmol/kg

1 = Rehncrona et al., (1978)

2 = Winn et al., (1980)

During complete brain ischaemia, AMP, IMP, adenosine, inosine, hypoxanthine and xanthine accumulate in the tissue as a result of ATP catabolism (Berne et al., 1974). Hagberg et al., (1987) monitored the in vivo concentrations of these breakdown products in rat striatum during normoxia, 15 min of complete brain ischaemia and during four hour reflow. Extracellular purines were sampled by microdialysis and tissue adenine nucleotides and purine catabolites were extracted from the in situ frozen brain at the end of the experiment. Striatal tissue adenine nucleotides, nucleosides and purines during control (C) 10 min ischaemia and 45 min reflow are presented in the following table.

Table 1.5

Purine	C	10' I	45' R
ATP	3.1 ±0.1	0.3 ± 0.1	1.84 ±0.05
ADP	0.34 ±0.07	0.42± 0.06	0.43 ±0.04
AMP	0.03 ±0.003	1.07± 0.15	0.079±0.03
Adenosine	0.0031±0.00043	0.49± 0.12	0.02 ±0.0003
Inosine	0.0039±0.00035	0.138±0.015	0.062±0.0001
Hypoxanthine	0.0019±0.0003	0.22 ±0.005	0.008±0.0003
Xanthine	0.0034±0.00024	N.D.	0.024±0.007

All values are expressed as μmol/g wet weight

The concentrations of adenosine in the E.C. space were estimated to be 40μM (15 min ischaemia) and 500μM in the I.C. space (10 min ischaemia). These concentrations of adenosine are more than adequate to exert its effects at either A_1 or A_2 receptors. Analysis of typical levels of nucleotides and adenosine in normoxic and ischaemic guinea pig hearts compare favourably with that of brain, (normoxic concentration range of ATP = 4-6mM) (Collinson et al., 1987). The percentage increase in ADP and AMP in the brain is somewhat greater than in the heart; this may be due to the fact that a substantial amount of ADP in the heart is bound to actin and thus the increase in free ADP is greater than is indicated by measurement of total ADP (Collinson et al., 1987).

1.7 METABOLIC REGULATION OF INTRACELLULAR ADENOSINE FORMATION

A mechanism for the elevation in adenosine concentration during ATP catabolism was proposed by Arch & Newsholme, (1978a,b). They suggested that a substrate cycle may exist between AMP and adenosine; the role of this substrate cycle being to amplify the increase in adenosine concentration resulting from increased AMP hydrolysis by the ectoenzyme 5'-nucleotidase. It was postulated since adenosine kinase has a low Km for adenosine (<0.4 - 5.8μM) that in the basal state the kinase may approach saturation with substrate. The Km of adenosine deaminase for adenosine is some 50 fold greater, it was proposed therefore that the rate of

adenosine formation in the basal state would approximate to the maximal rate of adenosine kinase, little adenosine being available for deamination.

Any increase in 5'-nucleotidase activity cannot be accompanied by an increase in the kinase, since it is already saturated. Almost all the additional adenosine would be deaminated by adenosine deaminase. Having a relatively high Km this enzyme could only increase its activity after a proportional rise in adenosine concentration. In this way, the change in the activity of the nucleotidase would be amplified resulting in a greater change in the concentration of adenosine. It was also suggested that since adenosine kinase from some species is inhibited by concentrations of adenosine that are only slightly higher than the Km value, adenosine kinase activity may actually decrease as adenosine levels increase due to a greater rate of hydrolysis of AMP by the 5'-nucleotidase.

Contrary to the proposals put forward by Arch & Newsholme, (1978a,b), Newby et al., (1983) did not find that the rate of adenosine formation, as determined in polymorphonuclear leucocytes or in cultured neonatal heart cells approximated to the in situ activity of adenosine kinase. In fact they deduced that at steady state, the rates of adenosine formation and metabolism being equal, the kinase reaction must be far from saturation. Using similar methodology Bontemps et al., (1983) reached a similar conclusion for isolated hepatocytes.

The role for the ectoenzyme 5'-nucleotidase in the regulation of intracellular adenosine, as proposed by Arch & Newsholme, is inconsistent with the fact that this enzyme is powerfully inhibited by ADP. ADP concentrations would rise in response to a decrease in the energy charge of the cell.

Of the two cytosolic 5'-nucleotidases previously described, the one that is most likely to be responsible for ischaemia-induced adenosine formation is the one which has a higher affinity for AMP compared to IMP. This deduction was made by Newby, (1988) and Truong et al., (1988), since the latter group compared the ratios of Vmax/Kapp for AMP/Vmax/Kapp for IMP for the two nucleotidases isolated from rat heart. In the presence of 5mM ATP these ratios are 2.8 for 5'-nucleotidase I (Km IMP > AMP) and 0.026 for 5'-nucleotidase II (Km AMP > IMP). In the absence of ATP, these ratios are 16 and 0.015 respectively. Furthermore, Itoh et al., (1986) showed that 5'-nucleotidase II from rat heart is inhibited by over 90% by 5mM Pi, by contrast 5'-nucleotidase I is inhibited by less than 10% by 5mM Pi (Truong et al., 1988). The Pi concentration rises during ATP and phosphocreatine depletion, as occurs for instance during hypoxia. The strong inhibition of 5'-nucleotidase II by Pi is therefore at variance with its role in adenosine production. Also 5'-nucleotidase II has not been shown to be sufficiently active to explain observed rates of ischaemia-induced

adenosine formation in rat or pigeon heart (Newby et al., 1987).

5'-nucleotidase I from pigeon heart ventricle was assayed at concentrations of ATP, ADP and AMP that mimicked tissue concentrations between 0 and 2 min of normothermic ischaemia (Newby, 1988). At 0.66 mM AMP a mean homogenate 5'-nucleotidase activity of 4μmol min^{-1} g wet $weight^{-1}$ was obtained. Given a Km value of 4.9mM this implies a Vmax of the order of 40μmol min^{-1} g wet $weight^{-1}$, a level of activity more than sufficient to account for the initial rate of adenosine formation (0.41 ± 0.04 μmol min^{-1} g wet $weight^{-1}$) in ischaemic pigeon ventricles (Meghji et al., 1988).

The activity of this 5'-nucleotidase decreases sharply with pH values below 7.0. Also the presence of IMP (0.1 - 0.2mM) and Pi (20 - 30 mM) decrease the activity by approximately 50%. Taken together these factors may contribute to the decreased adenosine formation observed after long periods of ischaemia (Meghji et al., 1988).

Homogenates of rats hearts were found to contain a 5'-nucleotidase with similar characteristics to the pigeon enzyme, (Newby, 1988). The activity was 0.15 - 0.17 μmol min^{-1} g wet $weight^{-1}$ somewhat less than previously measured rates of ischaemia induced adenosine formation (0.47 ± 0.06 μmol min^{-1} g wet $weight^{-1}$ (Meghji et al., 1988).

Newby (1988) has suggested that other phosphatases may contribute to adenosine formation in rat heart or that full activity of the 5'-nucleotidase is not preserved during extraction. However in the study

performed by Truong et al., (1988) on the rat heart 5'-nucleotidase I the rate of anaerobic production of adenosine was taken as 0.071μmol min^{-1} g fresh $weight^{-1}$ (Frick & Lowenstein, 1976). According to the data of Frick & Lowenstein, (1976) the AMP content of rat heart perfused aerobically in about 0.24mM whilst that for an anaerobic heart is 1.4mM. The equivalent rates of adenosine production for 5'-nucleotidase I at these concentrations of AMP were found to be 24 and 138 nmol of AMP hydrolysed min^{-1} g fresh $weight^{-1}$ respectively (Truong et al., 1988). Comparing this with a rate of purine production of 71 nmol min^{-1} g fresh $weight^{-1}$ (Frick & Lowenstein, 1976) Truong et al., (1988) deduced that 5'-nucleotidase I activity is sufficient to account for the rate of nucleoside plus hypoxanthine production.

However, the estimates of anaerobic adenosine production made by Frick & Lowenstein (1976) were made in the absence of inhibitors of adenosine metabolism and are thus generally considered to be underestimates of the 'true' value (Newby, 1988). If one is to compare the anaerobic rate of adenosine production in rat hearts obtained by Meghji et al., (1988) to the value obtained for 5'-nucleotidase I by Truong et al., (1988) then the conclusions made by Newby, (1988) may also apply to the enzyme purified by Truong et al., (1988).

Even though a brain cytoplasmic 5'-nucleotidase responsible for ischaemia induced adenosine formation has not been characterised it seems likely from data presently

available (see text) that adenosine originates both intra and extracellularly. From data obtained in the heart it seems likely that the production of adenosine in the brain in situations where the energy charge of the cell is reduced is catalysed by a cytosolic AMP preferring 5'-nucleotidase.

An additional or alternative pathway for adenosine formation to that of ATP catabolism is the transmethylation pathway (Schrader et al., 1981). S-adenosylhomocysteine (SAH), formed from S-adenosyl-methionime (SAM) after the transfer of the methyl group of SAM to a variety of methyl acceptors, is hydrolysed by SAH hydrolase, to adenosine and homocysteine (cf. Lloyd & Schrader, 1987).

Although the equilibrium of the reaction catalysed by SAH hydrolase favours synthesis, physiologically the reaction proceeds in the direction of hydrolysis because both adenosine and homocysteine are further metabolised (see fig. 1.2).

It is unlikely that this pathway contributes to adenosine production in ischaemia, as the flux through the transmethylation pathway is not accelerated and cannot explain the massive release of adenosine during hypoxic perfusion in e.g. the guinea pig heart (Lloyd et al., 1988). However, the rate of adenosine formation by the transmethylation pathway during normoxic perfusion of guinea pig heart (in the presence of the adenosine deaminase inhibitor EHNA) exceeded the basal release 15-

fold; strongly suggesting that this pathway is an important intracellular source of adenosine (Lloyd et al., 1988). Most of the adenosine formed by this pathway is reincorporated into the ATP pool, most likely by adenosine kinase (Lloyd et al., 1988).

It would seem that the relative importance of the 5'-nucleotidase compared with the transmethylation pathway depends on the energy state of the cell. It has been suggested (Lloyd & Schrader, 1987) that the major source of adenosine (in the heart) under normoxic conditions is the transmethylation pathway and that under such conditions the activity of the intracellular 5'-nucleotidase is very low. In hypoxia increased AMP concentration triggers production of adenosine by activation (or disinhibition) of the I.C. 5'-nucleotidase.

SAH hydrolase activity has been shown to be located in myelin and synaptosomes (Miyake and Innami, 1987). Incorporation of adenosine into SAH was studied by Reddington and Pusch (1983) in a hippocampal slice preparation. Their results have indicated that at least part of the free adenosine pool in brain tissue is in dynamic equilibrium with SAH. No studies, as such, have been undertaken to examine the flux through or the importance of the transmethylation pathway in producing adenosine in normoxic or ischaemic conditions in brain. The possibility however exists that this pathway may be very important in normoxia in the brain as it is in the heart.

1.8 THE CONTROL OF ADENYLATE CYCLAYSE

1.8.1 THE DUAL REGULATION OF ADENYLATE CYCLASE

Adenylate cyclase is a multicomponent enzyme system situated in the plasma membrane that is subject to regulation by hormones, neurotransmitters and guanine nucleotides.

The adenylate cyclase enzyme appears to be composed of at least three distinct components inserted in the lipid bilayer of the plasma membrane. The receptor component (R), which has specific binding sites for hormone or neurotransmitter, is located on the external face of the plasma membrane. In any given tissue there is only a limited number of types of hormone/neurotransmitter receptor present, and hence the hormone sensitivity of the enzyme is dependent on this. The catalytic unit (C) and the regulatory guanine nucleotide binding proteins (G proteins) are present on the inner face of the plasma membrane. The G proteins have the properties of binding as well as hydrolysing GTP and of regulating the hormone affinity of the receptor. They therefore mediate the effects of GTP and various hormones on the activity of C.

In the adenylate cyclase system two types of G protein have been identified, one mediates the stimulation of the cyclase enzyme (Gs) and the other its inhibition (Gi) (Rodbell, 1980). The pattern of dual regulation of adenylate cyclase was initially observed in fat cells when the enzyme was assayed at increasing concentrations of GTP. An initial stimulatory phase was followed at higher

concentrations of GTP by an inhibitory phase. The phenomenon can currently be explained in terms of differing requirements of Gs and Gi for GTP. Half maximal stimulation has been observed at 0.01 - 0.03μm (Limbird 1981, Cooper 1982, Jakobs et al., 1985).

1.8.2 STRUCTURE OF GUANINE NUCLEOTIDE REGULATORY PROTEINS

The first G proteins to be classified were Gs and Gi, initially by their respective abilities to stimulate and inhibit adenylate cyclase, (Rodbell, 1980 and Gilman, 1984). They were found to be heterotrimers composed of α β and γ subunits. Though similar in some respects several experiments have shown that Gs and Gi are in fact different. The α subunit of Gs can be ADP-ribosylated by cholera toxin whilst pertussis toxin acts upon Giα.

Initial molecular weight studies (for review see Birnbaumer, 1985) revealed an Mr of 42,000 for Gsα with lesser amounts of 51,000 - 52,000 Da and 41,000 for Giα. However they are similar in that both α subunits bind guanine nucleotides and possess an intrinsic GTPase activity, also both the $\alpha\beta\gamma$ hetrotrimers are dissociated by guanine nucleotides (Sternweiss et al., 1981 and Bokoch et al., 1983).

The β subunits of Gs and Gi are very similar and are of mol wt 35,000, likewise the subunit which has been found to have a mol wt of 5,000 - 8,000 Da. The $\beta\gamma$ component forms a single functional unit, as demonstrated

by the fact that native β and γ subunits do not dissociate (Neer & Clapham, 1988). The $\beta\gamma$ complexes released from Gs and Gi on subunit dissociation are therefore functionally interchangeable.

A third member of the family was discovered independently in rod photoreceptor cells of the vertebrate retina. Transducin (G_T) is activated by rhodopsin in response to light rather than by hormones, it acts as a substrate for both Pertussis and Cholera toxins, αG_T has a Mr of 40,000 (Gilman, 1987). Two forms of this G protein have in fact been identified by cloning of cDNA's, one in rod outer segments, G_{T1}, and one in cones G_{T2} (Tanabe et al., 1985 and Yatsunami & Khorana, 1985).

A fourth member of the family Go was identified in bovine brain (Sternweiss & Robishaw, 1984; Neer et al., 1984). It is a substrate for pertussis toxin, though it is immunologically distinct from Gi the α subunit of Go also has a Mr of 39,000 whilst that of Gi is 41,000. The $\beta\gamma$ subunits of Go are interchangeable with those of Gi or Gs. Although Go is five times as plentiful as Gi its function has only been speculated upon. It has been proposed that Go may be involved in the functional coupling of cardiac muscarinic receptors to K^+ channels (Pfaffinger et al., 1985) and of opiate receptors to neuronal voltage-dependent Ca^{2+} channels (Heschler et al., 1987).

The similarities found between Gs and Gi also apply to Go and G_T.

As mentioned, the α subunits vary in size from

39,000 to 52,000 Da (as found by denaturing gel electrophoresis) and respond individually to ADP ribosylation by bacterial toxins, it is this subunit therefore which defines the role of the individual.

The β subunits on the other hand are more highly conserved and have molecular masses of 35-36,000 Da depending on the source of G protein. Although the β subunit is very similar amongst the G protein family, the presence of two genes encoding β subunits has been identified (Sugimoto et al., 1985 and Fong et al., 1987) and these β subunits are immunologically distinct (Roof et al., 1985).

However, no information is currently available as to whether each subtype of the β subunit is able to interact exclusively with a particular subset of α subunits.

Immunological evidence also suggests the presence of a number of forms of the γ subunit (Hildebrandt et al., 1985; Gierschik et al., 1985 and Evans et al., 1987). They possess a molecular mass ranging from 5-10 k Da. It has been suggested that the hydrophobic $\beta\gamma$ subunit serves to anchor the more hydrophillic α subunit to the membrane, although α subunits probably also interact with the membrane directly (Neer & Clapham, 1988).

The existence of multiple forms of individual G proteins has been outlined by a combination of immunological and cloning techniques (for review, see Milligan, 1988). The two forms of transducin have already

been mentioned. Three genes coding for 'Gi-like' proteins namely Gi_1, Gi_2 and Gi_3 have been identified. Molecular cloning has also revealed at least four variants of αs that are derived from alternative mRNA splicing, probably from a single gene, these variants have only modest functional differences (Robishaw et al., 1986 and Bray et al., 1986).

1.8.3 HORMONAL REGULATION OF ADENYLATE CYCLASE

In spite of abundant evidence that G proteins mediate activation and inhibition of adenylate cyclase, the precise mechanism by which agonist occupied receptors activate G proteins is unknown. The hypothesis that has generated the most attention is the subunit dissociation model (Gilman 1984; Katada et al., 1984 a, b and c). Both Gs and Gi have been shown to dissociate in detergent containing solutions when exposed to non-hydrolysable GTP analogues as well as other effectors such as Mg^{2+} and Al^{3+} generating "activated" α units and βγ units (Codina et al., 1983; Smiegal 1986; Katada et al., 1984, a, b and c). This model has been challenged primarily on the basis of kinetic analyses of hormonal activation of adenylate cyclase requiring that the catalytic unit (C) remain associated with either Gs or αs at all times (Levitzki, 1984; Levitzki, 1987; Marbach et al., 1988).

Recent evidence provided by Ransas & Insel, (1988) has shown unequivocally that subunit dissociation occurs in intact membranes. They were able to detect αs in membrane extracts and αs from lymphoma cell membranes

treated with GTPγS or isoproterenol by using an antibody that detects dissociated αs but not the heterotrimer αsβγ.

The subunit dissociation model has been reviewed extensively by Gilman (1987) and Birnbaumer et al., (1985). In the absence of agonist i.e., in the basal state in the presence of both Mg^{2+} and GTP, Gs and Gi undergo a unidirectional kinetic cycle, whereby they bind GTP and become deactivated with GDP remaining bound to it and finally exchange GDP for GTP to reinitiate the cycle. The rate at which G proteins traverse this cycle is greatly increased by interaction with its relevant hormone-receptor (HR) complex. On interacting with G protein HR stimulates the dissociation of GDP presumably as a result of a conformational change that results in the 'opening' of the guanine nucleotide binding site (Brandt & Ross, 1986; Stryer, 1985). In the absence of GTP the H.R.G. 'ternary complex' as named by De Lean at al., (1980) is a relatively stable intermediate. Even though Gs and Gi have different requirements for GTP, half maximal stimulation by hormones has been found to occur at approx. 0.01 - 0.03 μM GTP for Gs and 0.1 - 0.3 μM for Gi (Limbird, 1981; Cooper, 1982; Jakobs et al., 1985). At ambient physiological concentrations of 1μM GTP the formation of the HRG complex is quite transient and the open guanine nucleotide site of both proteins is rapidly filled.

This exchange reaction is highly dependent on the presence of Mg^{2+}. Gi possesses a high (μ molar) affinity for Mg^{2+} both in the presence and absence of HR; therefore

at ambient physiological concentrations of n 0.5mM Mg^{2+} , Gi is always saturated with Mg^{2+}. Gs on the other hand would require approximately 10-100 mM to facilitate guanine nucleotide exchange, interation of Gs with HR lowers this requirement to 10μM Mg^{2+}. The HR complex acts as a Mg^{2+} 'switch' for Gs. It can be seen therefore that the dissociation of GDP in the presence of Mg^{2+} is closely linked to the rate limiting step which is controlled by the HR complex.

The binding of GTP to H.R.G has two important consequences. The first is that the affinity of H for R (and R for G) is decreased. Dissociation of H from R and, therefore R from G.GTP allows R to recycle and thus function catalytically in the activation of G (Pedersen and Ross, 1982). The second is the activation of G protein such that it interacts with its effector, in this case adenylate cyclase. As mentioned a bulk of evidence has shown that activation greatly reduces the affinity of the 'active' GTP - liganded α subunit for the $\beta\gamma$ complex and that the resulting dissociation of subunits is an important component of their mechanism.

The α-GTP is deactivated primarily as a result of the Mg^{2+} dependent GTPase activity of the α subunit itself. In the absence of the HR i.e., during steady state hydrolysis the majority of the protein exists as the GDP - bound form, since Kcat exceeds Koff for GDP (0.3 min^{-1}) by an order of magnitude. The rate of dissociation of GDP, which is controlled by HR and facilitated by Mg^{2+} thus

limits basal GTPase activity. The rate of hydrolysis of GTP by αGs or αGi is about 2-3 mins. Thus the lifetime of Gα-GTP is many seconds, sufficient for substantial amplification due to prolonged activation of effector.

Numerous lines of evidence suggest that the Gsα-GTP complex is the actual regulator of adenylate cyclase. It interacts with C at a specific site to greatly increase its Kcat for MgATP (by approximately 200 fold, Birnbaumer, 1985). Once deactivated by its own GTPase to form Gα-GDP it dissociates from C and may recombine with βɣ to form the inactive heterotrimer αs. GDP.β, (see Fig. 1.3).

In contrast, activation of Gi to its respective α-GTP and βɣ units has revealed that the bulk of the capacity to inhibit adenylate cyclase is in the βɣ subunit and that this inhibition is largely dependent on Gs (Katada et al., 1984a; 1984b). This hypothesis proposed by the aforementioned authors states that an increase in βɣ within the lipid bilayer would attenuate the activation of C by Gs by scavenging αs-GDP forming the inactive heterotrimer.

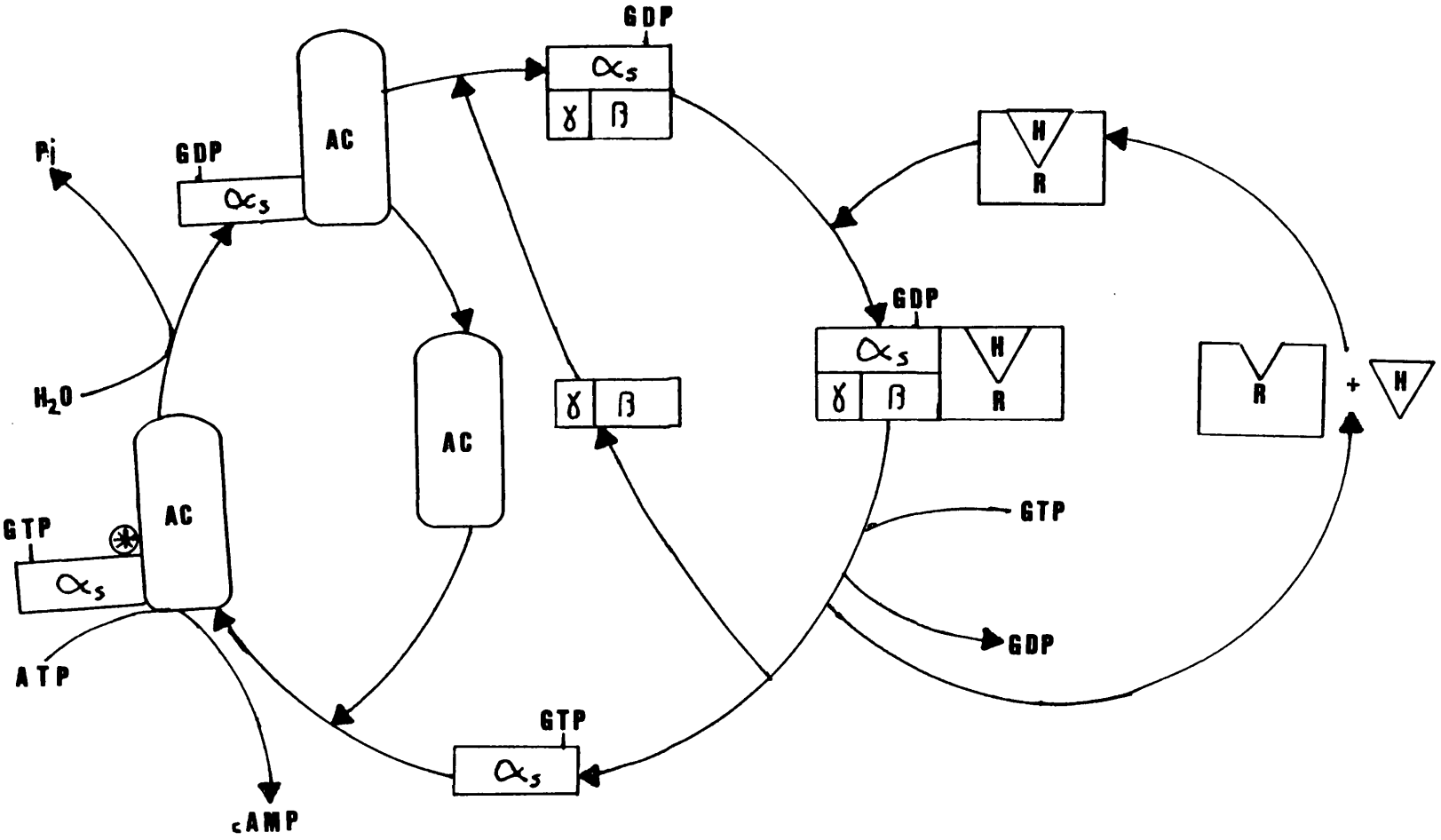
Two further mechanisms have been outlined by which Gi may inhibit C (Katada et al., 1986). Firstly βɣ units may interact directly with C to inhibit its activity. Secondly kinetic experiments performed in Cyć S49 cell membranes (lacking a functional Gs) have revealed that Giα-GTP may also interact with C by competing with Gsα-GTP for its binding site on C, though it does so with relatively low apparent affinity (Katada et al., 1986; Hildebrandt et

Fig 1.3 The subunit dissociation model for the regulation of adenylate cyclase

KEY

H	-	Stimulatory hormone/agonist
R	-	Stimulatory receptor
AC	-	Adenylate cyclase
αs	-	α - subunit of Gs
β	-	β - subunit of Gs
γ	-	γ - subunit of Gs
αsβ	-	inactive heterotrimer Gs
αs-GTP	-	active α - subunit of Gs
*	-	activated AC

(Note: this activation inactivation of Gs may likewise be applied to Gi)


GDP
α_s
γ β
AC
Pi
GDP
α_s
H
R
GDP
α_s
γ β
H
R
R
+ H
H_2O
AC
γ β
AC
GTP
α_s
GTP
ATP
GDP
GTP
α_s
cAMP

al., 1984; Jakobs et al., 1984), (see Fig. 1.4).

The subunit dissociation model treats adenylate cyclase as a five component system where all the functional units: Rs, Ri, Gs, Gi and C are physically separate but dynamically interact with one another. Such a model yields complex kinetics of activation, in direct contrast with the experimental observation that the kinetics of activation by hormones and guanine nucleotides, both in native membranes and systems reconstituted from resolved components are first order (Levitzki, 1987). In order to accommodate these findings a modified dissociation model was proposed by Levitzki, 1987), which assumes that αs is always associated with C and the $\beta\gamma$ subunits dissociate from the Gs.C complex, leaving behind αs.C. However more recent findings by Levitzki, (1988) seem to eliminate even this option as Levitzki found that the $\beta\gamma$ subunit co-purifies with Gpp(NH)p activated adenylate cyclase suggesting that no subunit dissociation occurs during Gs activation. This phenomenon contrasts directly with the findings of Ransnas & Insel (1988). Levitzki, (1988) rather controversially suggests that the only role for $\beta\gamma$ is to anchor the α-subunit to the membrane. Alternatives to the dissociation model have been proposed by Marbach et al., (1988), they propose that the effects of Gi require the presence of Gs, suggesting a direct Gs-Gi interaction or a Gs dependent Gi to C interaction.

Nevertheless, the subunit dissociation has gained widespread acceptance not only because activitated subunits

Fig. 1.4 Possible mechanisms for the inhibition of adenylate cyclase activity

KEY

AC - Adenylate cyclase

αs - α - subunit of Gs

αi - α - subunit of Gi

β - β - subunit of Gs or Gi

γ - γ - subunit of Gs or Gi

\+ - Stimulation

\- - Inhibition

α_i
β
γ
α_i
α_s
AC
β
γ
α_s
β
γ

of Gi and Gs may regulate adenylate cyclase but also because activated α and $\beta\gamma$ subunits from other G proteins have been shown to regulate effectors such as: potassium channels, calcium channels and phospholipase A_2 (see Table 1.6). It should be noted that much debate has centred upon discerning the roles of α and $\beta\gamma$ subunits of a PT sensitive G protein (named Gk) in the control of muscarinic/adenosine receptor linked K^+ channels in the heart (for detailed discussion see Neer & Clapham, 1988).

TABLE 1.6 SUMMARY OF FUNCTIONS OF ISOLATED α or β SUBUNITS

	Functions of isolated α or β subunits
Subunit	Target
αs	Adenylate cyclase catalytic unit Ca^{2+} channel
αi	Rhodopsin Adenylate cyclase catalytic unit cAMP phosphodiesterase
αK	K^+ channel
αo	Rhodopsin Muscarinic cholinergic receptor K^+ channel
β	K^+ channel Phospholipase A_2 cAMP phosphodiesterase Gs-stimulated adenylate cyclase catalytic unit Unstimulated adenylate cyclase catalytic unit

Taken from Neer & Clapham, (1988)

1.8.4 ADENYLATE CYCLASE, ACTIVATION BY FORSKOLIN

At least two forms of adenylate cyclase exist in the nervous system of higher organisms, one that is stimulated by Ca^{2+}/calmodulin and the other, which is independent of Ca^{2+}/calmodulin (Brostrom et al., 1975).

These two forms of adenylate cyclase have also been identified by differences in physico-chemical parameters. Sucrose density gradient centrifugation of lubrol soluble brain adenosine cyclases showed the calmodulin insensitive form to have a sedimentation coefficient of 8.8s whereas that of the calmodulin sensitive form was 10s (Coussen et al., 1986). However discrepancies exist at present as to the molecular mass of the enzymes, both appear to be a single polypeptide of 150,000 kDa (Coussen et al., 1986; Yeager et al., 1986).

Nevertheless both of these forms of adenylate cyclase are stimulated by forskolin, a diterpene from the root Coleus forskohlii (Coussen et al., 1986, Daly, 1984). The mechanism by which forskolin is able to activate adenylate cyclase in membranes or intact preparations in a reversible fashion remains unclear. Forskolin can interact directly with adenylate cyclase, although for full expression of enzyme activity the presence of Gs is required (Barovsky & Brooker, 1985). Combinations of forskolin and hormones that activate adenylate cyclase via Gs are synergistic with respect to adenylate cyclase stimulation (Seamon & Daly, 1986). Barovsky & Brooker (1985) have proposed that this effect is due to enhanced

Gs-C coupling. Support for this hypothesis has been given by a study which showed that the attenuation of Gs function by removal of Mg^{2+} or addition of $\beta\gamma$ subunits to rat erythrocyte membranes was prevented by forskolin suggesting that it stabilises the Gs activated state of adenylate cyclase (Yamashita et al., 1985). Moreover, the concentration of forskolin that is required for the synergestic activation of adenylate cyclase (<100nM) is 10-100 fold lower than that required for the direct activation of the enzyme in the absence of activated Gs (Nelson & Seamon, 1988).

To further elucidate the mechanism of forskolin action binding of [^{3}H] forskolin has been performed on various membrane preparations including rat brain (Seamon et al., 1984 and Seamon & Daly, 1985) and human platelet membranes (Nelson & Seamon, 1986). In brain membranes forskolin was shown to have two types of interactions with adenylate cyclase, one with high and one with low affinity. The high affinity binding sites in brain membranes (in the presence of 5mM Mg^{2+}) have a Bmax of 220 fmol/mg protein and a K_D of 16nM, (very similar results were obtained using platelet membranes). Agents such as NaF, Mg^{2+}, Mn^{2+} and Gpp(NH)p increase the Bmax for forskolin at the high affinity site without affecting the K_D (Seamon & Daly, 1985; Nelson & Seamon, 1986). All of these agents are known activators of Gs, it has been proposed therefore that the high affinity binding sites are associated with an activated Gs-C complex (Nelson & Seamon, 1986). In support

of this proposal it has been demonstrated that forskolin as well as Gpp(NH)p can increase the size of adenylate cyclase in solubilised preparations by a 42,000 mol wt component which is thought to be Gsα, (Bouhelal et al., 1985).

Furthermore high affinity sites are not detected in the Gs-lacking S49 cyc membranes, whereas kinetic studies using wild type S49 lymphoma cells suggest that forskolin may have high and low affinity sites of action (Green & Clark, 1982). Binding of forskolin to a low affinity site in brain membranes revealed a Bmax of 5 pmol/mg protein and a K_D of 1.1μM (Seamon et al., 1984).

Overall, therefore, it has been proposed that the high affinity interaction requires Gs whereas the one with low affinity occurs in the absence of Gs (Nelson & Seamon, 1988). This type of binding is compatible with either a one site or two site model i.e. the diterpene may act at two independent sites or at one site which changes in affinity depending on the presence or absence of activated Gs. Such a change in affinity would be expected for co-operative binding between activated Gs and forskolin at the catalytic protein (Seamon & Daly, 1985).

1.8.5 INHIBITION OF ADENYLATE CYCLASE, THE INFLUENCE OF SODIUM IONS

In the normal mammalian cell or the cell membrane derived from it containing both Gs and Gi, the activity of adenylate cyclase is presumed to be regulated by the relative extent of dissociation of the two guanine

nucleotide binding proteins which in turn are under the control of stimulatory and inhibitory hormone receptor complexes. This pattern of dual regulation was initially observed in fat cell membranes when adenylate cyclase was assayed at increasing concentrations of GTP. An initial stimulatory phase was followed, at increasing concentrations of GTP by an inhibitory phase (Londos et al., 1978; Londos et al., 1981). This phenomenon can currently be explained in terms of the differing requirements of Gs and Gi for activation by GTP. Half maximal stimulation is observed at 0.02 - 0.03 μM GTP whereas inhibition is observed at 0.1 - 0.3 μM. (Limbird, 1981; Cooper, 1982; Jakobs et al., 1985). It should be noted that these values for GTP vary somewhat depending on the system in question (Jakobs et al., 1981).

Membrane preparations from, for example, adipocytes or brain still contain enough GTP required for the in vitro study of hormonal stimulation of adenylate cyclase. In contrast, in order to study in vitro hormonal stimulation of adenylate cyclase, GTP has to be added to the system, Jakobs et al., 1981). The GTP-dependent hormone-induced inhibition is most evident under conditions where GTP itself is able to lower adenylate cyclase activity (Jakobs et al., 1981). However, in order to fully observe the inhibition of adenylate cyclase by hormones, the presence of monovalent cations as well as GTP is required (Blume et al., 1979; Lichtshtein et al., 1979a,b; Jakobs et al., 1981).

This requirement has been shown to be particularly evident in adipocyte membranes (Cooper, 1982; Jakobs et al., 1984a). The fact that adenylate cyclase is inhibited in the presence of GTP (at concentrations > 0.1 μM) means that the further addition of inhibitory hormones to the assay system would cause only a slight or even no further inhibition. Monovalent cations in the potency order $Na^+ > Li^+ > K^+$ are able to counteract the GTP induced inhibition. Under these conditions inhibitory hormones are able to decrease adenylate cyclase activity via Gi in a GTP dependent manner by overcoming the monovalent ion effect (Lichtshtein et al., 1979; Cooper et al., 1982; Jakobs et al., 1985).

Jakobs et al., (1984b) demonstrated that Na^+ ions are inhibitory at both Gs and Gi proteins. Evidence for this has come from studies on platelets, adipocytes, S49 lymphoma wild type cells and their cyć variants. The inhibitory effect of Na^+ can be overcome by high concentrations of stiulatory or inhibitory hormones. The mechanism by which Na^+ inhibits Gs and Gi is rather unclear. It is known that Na^+ decreases the basal and high affinity GTPases (Jakobs et al., 1984b; Koski et al., 1982). Also Na^+ is able to increase the lag phase of GTP S action at Gs and Gi without causing a final change in activity induced by the stable GTP analogue (Jakobs et al., 1984b).

The data suggests therefore that the activation process of Gs and Gi with the consequent stimulation or inhibition is inhibited by Na^+. It is presently unclear as

to whether this Na^+ inhibition, (which is reversible) is at the binding step of GTP or at a reaction distal to that in the activation process (Jakobs et al., 1984b).

1.9 THYROID HORMONE MODULATION

Thyroid hormones have been shown to have a profound effect on the ability of cells to respond to other hormones such as catecholamines and hormone-like substances such as adenosine. In general it has been shown that tissue response to stimulatory hormones is accentuated by hyperthyroidism and blunted by hypothyroidism (Stiles et al., 1984). However, the mechanisms by which thyroid hormones elicit their "permissive effects" on cellular responsiveness is not entirely clear.

One phenomenon that has been widely investigated is the ability of thyroid hormones to regulate β-adrenergic receptor number and function (for review see Stiles et al., 1984). In general β-adrenergic receptor density has been shown to be elevated in hyperthyroidism in e.g. the heart (Stiles & Lefkowitz, 1981; Tsai & Chen, 1978); brain (Fox et al., 1985; Perumal et al., 1984; Smith et al., 1980) and rat reticulocytes (Stiles et al., 1981).

An exception to this 'general rule' is the liver where hyperthyroidism is associated with a decrease whilst hypothyroidism is associated with an increase in β-adrenergic receptor density. Nevertheless in the liver as in all other tissues, the changes in receptor levels agree well with the responses mediated by those receptors.

Consequently it has been suggested that these changes in receptor number may be responsible, in part, for the altered responses of these tissues to β-adrenergic receptor agonists (Stiles et al., 1984). In heart Tsai et al., (1978) found that in hyperthryoidism not only was there an increase in β-adrenergic receptor density, in sensitivity and maximal stimulation of adenylate cyclase by isoproterenol, but also a decreased PDE activity, suggesting that changes in sensitivity were not limited to changes in β-adrenergic number.

This conclusion was further substantiated by Stiles & Lefkowitz (1981) where an increase in heart β-adrenergic receptor density in the hyperthyroid state was associated with altered coupling of G protein to receptor as determined by analysis of isoproterenol competition curves. These curves were shifted to the left suggesting an increased affinity of isoproterenol for the β-adrenergic receptor, which should result in a better coupling of the receptor (R) with adenylate cyclase in the hyperthyroid state.

In hypothyroidism a decrease in β-adrenergic receptor number in the heart is associated with decreases in the basal, isoproterenol-stimulated and NaF-stimulated adenylate cyclase activities. Results compatible with alterations of multiple sites within the β-adrenergic receptor adenylate cyclase system, (Brodde et al., 1980). Stiles & Lefkowitz, (1981) however found no alteration in the coupling of R to G protein in the hypothyroid state, so

perhaps a decrease in β-adrenergic receptor number may be predominantly responsible for a decreased responsiveness in the hypothyroid state.

In adipose tissue rendered hypothyroid, sensitivity to catecholamines or other hormones in producing cAMP accumulation or a lipolytic response appears to be reduced (Goswami & Rosenberg 1978; Malbon et al., 1978., Ohisalo & Stouffer 1979; Malbon & Graziano 1983; Saggerson 1986). This reduction is accompanied either by a reduction in β-adrenergic receptor number (Guidicelli, 1978) or no change in β-adrenergic receptor number as found in adipocyte membranes prepared from hypothyroid rats (Goswami & Rosenberg; Malbon et al., 1978). Nor was the affinity of the β-adrenergic receptors found to be altered (Malbon et al., 1978), a result later contradicted since a reduction in receptor affinity for agonist was found by Malbon, (1980). Nevertheless Malbon et al., (1978) found that the maximum catalytic activity of adipocyte membrane adenylate cyclase in response to stimulation by forskolin, Gpp(NH)p, fluoride and cholera toxin was unaffected by hypothyroidism.

Based on this data and on data showing that neither the amount nor the structure of fat cell α-Gs appears to be affected in the hypothyroid state; as determined by the extent of cholera toxin catalysed ADP-ribosylation of α-Gs subunits, the map of partial proleolytic digests of the toxin substrates on two dimensional gel electrophoresis and functional

reconstitution of mouse lymphoma S49 cyc cell adenylate cyclase (Malbon et al., 1984), it has been suggested that Gs levels as well as the interaction between Gs and the catalytic subunit are unaltered.

Nevertheless, since guanine nucleotides are unable to induce a shift in the affinity of R for β-adrenergic agonists in fat cell membranes from hypothyroid rates (Malbon, 1980), it was suggested that unlike Gs-C interactions, the interactions between R and Gs are impaired in the hypothyroid state i.e. that the locus of the defect resides in R and not Gs.

Reticulocytes from hypothyroid rats also display altered R-Gs interactions, they possess a reduced ability to stabilise the high affinity state of the receptor, as shown by computer analysis of isoproterenol competition curves. This suggests that agonists should be less effective in activating adenylate cyclase in reticulocyte membranes from hypothyroid rats (Stiles et al., 1981). Nevertheless the reticulocyte system differs from the fat cell system in that β-adrenergic density is decreased by 50% in the hypothyroid state, this is accompanied by a decrease of approximately 40% in the quantity of Gs as assessed by the number of 42,000 Mr substrates for cholera toxin catalysed ADP-ribosylation and SDS-PAGE compared with controls (Stiles et al., 1981).

Hypothyroidism not only impairs the β-adrenergic receptor mediated responses in adipose tissue, but also potentiates the inhibitory regulation of adenylate cyclase

(Ohisalo & Stouffer, 1978; Malbon et al., 1985; Saggerson, 1986). This potentiation is accompanied by a small though not significant decrease in the inhibitory A_1 adenosine receptor by 15% for [^{3}H] PIA binding (Chohan et al., 1984) and 14% for [^{3}H] CHA binding (Malbon et al., 1985). These decreases were not accompanied by any change in affinity. However, Malbon et al., (1985) showed that GTP-induced inhibition of forskolin-stimulated adenylate cyclase was markedly enhanced in the hypothyroid state, suggesting an alteration in the inhibitory regulatory component (Gi) mediated control of adenylate cyclase. Polyacrylamide gel analysis of radioactive products resulting from pertussis toxin catalysed ADP-ribosylation of membranes from euthyroid and hypothyroid rats revealed that the amount of label incorporated by pertussis toxin into the α subunit of Gi per mg protein is increased 2-3 fold in the hypothyroid state.

However, it is now appreciated that Gi is not the sole substrate for pertussis toxin and that transducin and Go as well as other less characterised G proteins can be modified (Milligan et al., 1987). A more conclusive means of identifying G proteins either in their holomeric forms or as subunits involves the use of anti-peptide antisera. Such antisera which recognise the α subunit of Gi but not Go were used to demonstrate conclusively that amounts of the α subunit of Gi were substantially increased in rat adipocyte membranes after treatments which induce a hypothyroid state (Milligan et al., 1987). A similar

result was obtained by Ros et al., (1988) who utilised specific antisera to αGo and αGi. Both of these subunits were found to be markedly increased in comparison to the control. Although it seems likely that the increase in Go detected in the hypothyroid state by Ros et al., (1988) is in fact a form of Gi rather than Go itself (Hinsch et al., 1988, Mitchell et al., 1989).

Whether the increased amounts of G protein α-subunits measured in the hypothyroid state represent an increase in synthesis de novo or a decreased rate of turnover remains to be determined.

In addition, alterations in sensitivity to adenosine in the hypothyroid state may be caused by altering tissue production or disposal of adenosine. For instance in adipocytes from hypothyroid rat ecto-5'-nucleotidase activity was decreased by approximately 50%, adenosine deaminase by 60% and adenosine kinase unchanged (Jamal & Saggerson, 1987). If membrane bound 5'-nucleotidase in adipose tissue is important in determining the extracellular concentration of adenosine then it can be predicted that adenosine levels would be decreased in hypothyroidism.

Studies using homogenates or crude membrane preparations from developing rat or mouse brain have also shown a decrease in brain 5'-nucleotidase activity in hypothyroidism (Smith et al., 1980; King et al., 1983; Shanker et al., 1984). In the adult rat brain a significant proportion of 5'-nucleotidase is associated

with myelin (Camer et al., 1980; Heymann et al., 1984; Casado et al., 1988). However, in the developing rat brain decreases in 5'-nucleotidase could not be accounted for in terms of delayed myelination and consequent absence of myelin associated enzyme. It was not possible to identify the cells and structures responsible for the development related decrease in 5'-nucleotidase (King et al., 1983).

1.10 ADENOSINE AND NEUROMODULATION

The role of adenosine as a modulator in the CNS has been well established, this modulation is achieved primarily through inhibition of release of certain excitatory neurotransmitters such as: dopamine, noradrenaline, serotonin, acetylcholine and excitatory amino acids from several regions of the brain (Fredholm et al., 1986; Phillis & Wu, 1981; Williams, 1984; Snyder, 1985). Consequences of such modulation are for instance sedation, decreased motor activity, inhibition of breathing and anticonvulsant actions (Katims et al., 1983; Williams, 1984; Eldridge & Milhorn, 1987).

Although this modulation has been convincingly demonstrated and its effects widely investigated the mechanism of adenosine action is less clear.

Pharmacological evidence points to the A_1 receptor subtype being the mediator of adenosine action (Reddington, et al., 1982), this subtype is accordingly widely disributed throughout the central nervous system (Lewis et al., 1981; Goodman & Snyder, 1982; Daly, 1985; Weber et

al., 1988). At present it seems unlikely that a single mechanism is responsible for adenosine's neuromodulatory effects, rather a combination of mechanisms would seem a more likely explanation. These mechanisms have both a pre and a post-synaptic locus. Importantly A_1 receptors have been shown to be located both pre- and post-synaptically in the rat hippocampus (Deckert & Jorgensen, 1988).

The relative importance of these pre and post-synaptic actions was determined in the in vitro rat hippocampus by Proctor & Dunwiddie, (1987) who showed that the principal effect of adenosine is at the pre-synaptic locus, but that the post-synaptic effects of adenosine may also contribute to synaptic depression.

The postsynaptic actions of adenosine appear to be accounted for primarily by the action of a G-protein coupled potassium channel (Trussel & Jackson, 1985; Trussell & Jackson, 1987). The opening of the K^+ channels serves to shift the membrane potential closer to the K^+ equilibrium potential (E_k) causing hyperpolarisation and a reduced firing frequency.

The mechanisms underlying the presynaptic effect are more obscure. The activation of a presynaptic K^+ channel would diminish transmitter release, but there is no direct evidence for such a channel, however K^+ channel blockers such as 4AP can reduce the effects of adenosine, which might be taken to support the involvement of a potassium channel in synaptic modulation (Scott & Dolphin, 1987).

The inhibition of action potential induced influx of Ca^{2+} ions into the axon terminal has also been implicated as a possible mechanism. However the data reported so far on the effect of adenosine on neuronal calcium fluxes are not conclusive. The K^{+} evoked uptake of radioactive Ca^{2+} ions into brain synaptasomes has been reported to be depressed (Ribeiro, et al., 1979; Wu et al., 1982) or unaffected by adenosine (Barr et al., 1985; Garritsen et al., 1988).

Stimulus evoked calcium fluxes were found to be reduced by adenosine in the cerebellum (Ten Bruggencate et al., 1977) and a depression of calcium spikes has been described in ganglionic (Henon & McAfee, 1979) and rat hippocampal neurones (Proctor & Dunwiddie, 1983). Whereas calcium currents in guinea pig hippocampal neurones have been described as unaffected (Halliwell & Scholdfield, 1984).

With the voltage clamp technique a depression of barium currents by chloroadenosine has been seen in dorsal root ganglion cells (Dolphin et al., 1986). The reason for these discrepancies is unclear, nevertheless it has been suggested that this current may be carried via the N-channel (Maddison et al., 1987) which is the ω-conotoxin sensitive channel that is suggested to be responsible for the Ca^{2+} influx that triggers neurotransmitter release. The relationship of the N-channel to G-proteins is unclear even though a similar current in neuroblastoma X glioma cells that is inhibitable by opiates was found to be linked to

the G protein Go (Heschler et al., 1987).

The existence of an adenosine sensitive N-channel on nerve terminals has not been demonstrated, though clearly such a demonstration would provide a very direct mechanism by which adenosine could limit transmitter release.

The role of adenylate cyclase in neuromodulation is also unclear. It is highly unlikely that such a stimulation of cAMP production as suggested by Silinski, (1986) is in any part responsible for an inhibition of neurotransmitter release, since the receptors mediating the former and A_2 receptors are very clearly different entities (Reddington et al., 1982).

However it is possible that inhibition of cAMP formation via A_1 receptors may contribute to the pre-synaptic effects of adenosine, particularly under conditions where cAMP has been raised in the nerve terminal (Fredholm & Dunwiddie, 1988). A tentative model has been proposed by Fredholm and Dunwiddie (1988) that seeks to explain the way by which the multiple mechanisms act at the pre-synaptic terminal. They suggest that only one type of adenosine receptor (similar if not identical to the A_1 receptor) can couple with different types of G protein that have specificities for three types of effector systems, (see Fig. 1.5).

It is envisaged that adenosine also plays an important neuroprotective role within the CNS. Following a metabolic insult such as ischaemia or hypoxia, excitatory

Fig. 1.5 A model for the coupling of adenosine receptors to multiple effectors

KEY

A_1 - A_1 adenosine receptor

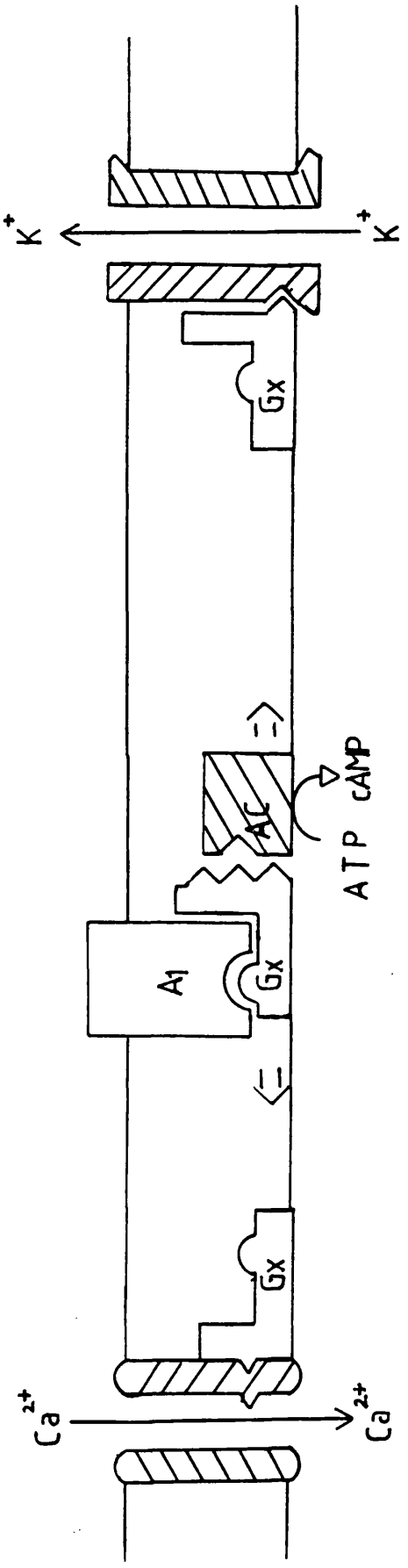
$Ca^{2+}\downarrow$ - Ca^{2+} ion channel

$K^+\uparrow$ - K^+ ion channel

AC - Adenylate cyclase

G_x - One type of G protein which interacts with several types of effector

(Taken from Fredholm & Dunwiddie, 1988)


K+
K+
Gx
AC
ATP cAMP
A1
Gx
Gx
Ca2+
Ca2+

amino acids are released which act to depolarise neurones sufficiently to overcome the voltage-dependent Mg^{2+} block of NMDA receptors. This leads to NMDA receptor activation and to an influx of Ca^{2+} ions into cells causing bursting. Adenosine which is released alongside the excitatory amino acids acts in a multicomponent fashion to limit cell death. These actions include hyperpolarisation of neurones, which would lead to Mg^{2+} block of NMDA receptors, inhibition of excitatory amino acid release, inhibition of neuronal Ca^{2+} uptake, block of Na^{+} uptake, increased astrocytic glycogenolysis and increased cerebral blood flow to the affected brain region and inhibition of blood clots (for review see Dragunow & Faull, 1988).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Routinely used reagents were of the highest possible grade and were obtained from BDH Ltd., Poole, Dorset, U.K., FSA Laboratory Supplies Ltd., Loughborough, Leics., U.K. and May and Baker Ltd., Dagenham, Essex, U.K.

(-)N^6-R-[G-^{3}H] phenylisopropyladenosine, ([^{3}H] PIA) [2-^{3}H] adenosine 5' monophosphate (ammonium salt), [5, 8'-^{3}H] adenosine 3', 5'-cyclic monophosphate (ammonium salt), [2-^{3}H] adenosine, [α-^{32}P]2'-deoxy adenosine triphosphate (ammonium salt), [8-^{3}H]2'-deoxy adenosine triphosphate (ammonium salt), 1,3-diethyl-8-[4-^{3}H] phenylxanthine, ([^{3}H] DPX) were obtained from Amersham International, Amersham, Bucks., U.K.

[8-^{3}H] 2'-deoxy adenosine 3',5'-cyclic monophosphate (ammonium salt) was ICN Radiochemicals, Division of ICN Biomedicals Inc., Irvine, California, U.S.A.

Adenosine deaminase (from calf intestine), bovine serum albumin (fraction V), BBOT, creatine phosphate (disodium), creatine kinase (from rabbit muscle), guanosine-5'-triphosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from yeast), (-)-N^6-R-phenylisopropyladenosine (PIA), pyruvate (sodium), NADH (disodium) were obtained from BCL Ltd., Lewes, Sussex, U.K. Adenosine, hypoxanthine, inosine, adenine, adenosine 5'-triphosphate (disodium), adenosine 5'-diphosphate (sodium), adenosine 5'-monophosphate (sodium), 5,5' dithiobis-(2-nitro-benzoic acid) (DTNB), forskolin, EDTA, N-glycylglycine, papaverine, 6-n-propyl-2-thiouracil (PTU),

Tris-HCl, β-glycerophosphate, imidazole, succinate (disodium), 2-[N-morpholino] ethane sulfonic acid (MES), 2',3'-cyclic $NADP^+$, 2'-deoxy adenosine monophosphate (sodium), 2'-deoxy adenosine triphosphate (disodium), acetylthiocholine iodide, triton-X-100, chromatographic alumina neutral type WN3, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) were from Sigma (London) Chemical Co., Poole, Dorset, U.K.

EDTA was from FSA Laboratory Supplies Ltd., Loughborough, Leics., U.K.

Ficoll was from Pharmacia Fire Chemicals, Uppsala, Sweden.

Dowex cation exchange resin AG 50W-X4 200-400 mesh hydrogen form was from BIORAD Labs Ltd., Watford, Herts., U.K.

2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) was from BDH Ltd., Poole, Dorset, U.K.

2.2 ANIMALS

Fed, male Sprague-Dawley rats (bred in the animal colony at University College London) weighing 140-170g (hypothyroid) and 200-280g (euthyroid) at time of death were used throughout. Rats were rendered hypothyroid by maintenance on an iodine-deficient diet and treatment with 6-n-propyl-2-thio-uracil (PTU). Normal diet and drinking water were replaced by standard cube diet depleted of iodine (from SDS Ltd., Witham, Essex) and drinking water

containing 0.01% (w/v) PTU, 0.3% ethanol (v/v) for four weeks. Euthyroid control rats were of the same weight as the experimental animals at the beginning of the treatment period (80-90g) and were maintained on a diet containing normal levels of iodine (from SDS Ltd.) and tap water ad libitum.

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

The method by which synaptosomal, myelin and supernatant fractions were isolated from rat brain was essentially that of Booth & Clark (1978), with some minor modifications.

Rats were killed by cervical dislocation. Brains were rapidly dissected and transected behind the occipital cortex and rostral portion to give the forebrain. The forebrains of either one or four rats was used in each preparation. The following procedure described is that for four forebrains. All homogenisation and fractionation procedures were performed at 4°C. The forebrains were dropped into isolation medium (0.32M sucrose, 1mM potassium EDTA, 10mM Tris-HCl, pH 7.4) and weighed on a pretared balance. They were subsequently chopped into small pieces with scissors. The blood and other debris were washed off the brain tissue by adding more isolation medium and decanting supernatant from the top of the minced tissue. This washing procedure was repeated. The chopped tissue was then homogenised in a glass Potter-Elvehjem homogeniser using a teflon pestle (6 up and down strokes at 500 rpm

with radial clearance of 0.2mm). This homogenate was then diluted with isolation medium to 50-60ml to give a final 11-15% (w/v) homogenate.

The homogenate was transferred to plastic centrifuge tubes and centrifuged using a SS-34 rotor (r_{av} 8.26 cm) in a Sorvall RC5-B Superspeed centrifuge at 1,500 gav for 3 minutes. The supernatant was carefully decanted, preventing any contamination by the white portion of the pellet. This supernatant was centrifuged at 18,000 g_{av} for 10 minutes using the same rotor and centrifuge, producing the crude mitochondrial/ synaptosomal pellet. This pellet was resuspended in isolation medium to give a final volume of 5ml and mixed with 25ml of 12% Ficoll/sucrose media [12% Ficoll (w/w), 0.32M sucrose, 50μM potassium EDTA, 10mM Tris-HCl, pH 7.4] giving a final concentration of 10% Ficoll. This was gently homogenised by hand in a Potter type homogeniser in order to fully disperse the pellet. 5ml of the crude mitochondrial suspension was pipetted into 6 separate 14ml capacity polycarbonate centrifuge tubes. Subsequent layers added were 2.5ml of 7% Ficoll [7% Ficoll (w/w), 0.32M sucrose, 50μM potassium EDTA, 10mM Tris-HCl, pH 7.4] followed by 2.5ml of isolation medium. When required the supernatant from the 18,000 g_{av} spin was decanted into centrifuge tubes and centrifuged in a Beckman L-250 ultracentrifuge fitted with a 50Ti rotor for 45 minutes at 105,000 g_{av}. This supernatant was then preportioned into 1.5ml Eppendorf tubes and frozen at -80°C. The gradients were centrifuged in an MSE 6 x 14ml

Titanium swing out rotor (r_{av} 11.0cm) of either a MSE 'Pegasus' AP65 or 'Prepspin' centrifuge at 110,000 g_{av} for 1 hour.

The myelin and synaptosomal membranes banded at the first and second interfaces respectively, with the mitochondria being pelleted at the bottom. The myelin layer was gently sucked off from the first interface with a Pasteur pipette. When required the myelin fraction was resuspended in 50mM Tris-HCl, pH7.4 and transferred to plastic centrifuge tubes. The suspension was centrifuged in a Beckman L-250 ultracentrifuge fitted with a 50Ti rotor for 45 minutes at 105,000 g_{av}. The myelin pellets were resuspended with the aid of a glass homogeniser and teflon pestle in a final volume of 2-3ml, frozen and stored in approximately 300μl aliquots at -80°C.

The synaptosomal layers were removed from the second interface, pooled and then resuspended to 50-60ml with 5mM Tris-HCl, pH 8.0. After sonication for 30 seconds the suspension was left on ice for 30-45 minutes. The combination of sonication and osmotic shock ensured maximum lysis of synaptosomes to give synaptosomal membranes. The synaptosomal suspension was then decanted into centrifuge tubes and centrifuged at 105,000 g_{av} for 45 minutes in a 6 x 14ml swing out rotor of a MSE 'Pegasus' or 'Prepspin' centrifuge. The synaptosomal pellet was resuspended in 50mM Tris-HCl, pH 7.4 with the aid of a glass homogeniser and teflon pestle to a final volume of 2-3ml, frozen and stored in 300μl aliquots at -80°C.

When fractions were prepared for assay of 5'-nucleotidase, adenosine deaminase and adenosine kinase, potassium EDTA was omitted from the incubation and density gradient media.

2.3.1 DISSECTION PROCEDURES FOR RAT BRAIN

Rats were stunned and killed by decapitation. Brains were rapidly dissected out from the skull and placed on an ice-cold petri dish covered with a filter paper wetted with 0.32M sucrose Tris-HCl, pH 7.4. Six regions of the brain were separated and these regions were fractionated by the aforementioned method (see 2.3) to give supernatant, myelin and synaptosomal fractions.

The dissection was performed by following the procedure outlined by Glowinski & Iversen (1966). The regions separated were: Cerebellum, Medulla oblongata (comprising medulla oblongata and pons), striatum (comprising the putamen nucleus, candate nucleus and globus pallidus nucleus), cortex (which corresponds to the telencephalon without the striatum and which includes white and grey matter of the cerebral cortex), hippocampus and the hypothalamus.

The brain was dissected firstly by separating the rhombencephalon (A) from the rest of the brain by a transverse section (Fig. 2.1a, section 1). The rhombencephalon was then dissected into two parts; the cerebellum and the medulla oblongata. A transverse section was then made through the rest of the brain at the level of

Fig 2.1 a

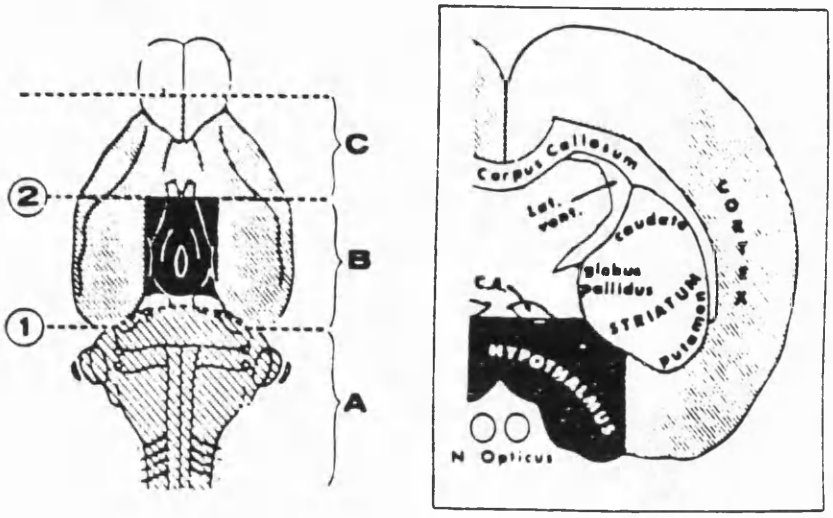
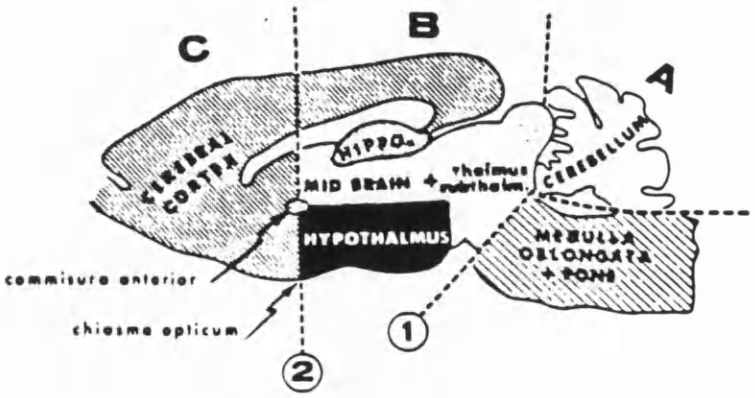


Fig 2.1 b



the 'optic chiasma' which delimits the anterior part of the hypothalamus and passes through the anterior commissure (section 2). This section separated the cerebrum into two parts B and C. Part B is divided into five fractions. The hypothalamus was first to be dissected out by taking the anterior commissure as a horizontal reference and the line between the posterior hypothalamus and the mamilliary bodies as the candal limit (see Fig. 2.1b). The striatum was dissected out with the external walls of the lateral ventricles as internal limits and the corpus callosum as external limits. The frontal parts of the striatum (found in portion C) were dissected separately and combined with posterior parts from portion B. The midbrain was gently teased from the remaining part and discarded. The hippocampus was then dissected. The remainder of part B was combined with the remainder of part C to form the cortex.

2.4 ESTIMATION OF PROTEIN

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

10-20μl of undiluted tissue extract were made up to 1ml with 0.5M NaOH. Bovine serum albumin was used as a protein standard at 0, 6, 12, 30, 60, 90 and 120 μg in 1ml of 0.5M NaOH. 5ml of 2.7% sodium potassium tartrate (tetrahydrate); 1% copper II sulphate (pentahydrate); 2% sodium carbonate in 0.1M NaOH (1:1:98,v/v) were added to samples and standards, vortex mixed and left to stand at

room temperature for at least 10 minutes. 0.5ml of Folin-Ciocalteau reagent, previously diluted 1:1.3(v/v) with distilled water was added to samples and standards, mixed immediately to ensure maximum production of colour and left to stand at room temperature for at least 45 minutes. The absorbance at 750nm was measured against a reagent blank on a Unicam SP8-100 spectrophotometer and protein concentrations of samples determined from the standard curve obtained.

2.5 LIQUID SCINTILLATION COUNTING

Samples were counted in glass scintillation vials (low K^+) in scintillant fluid. Efficiency of counting was determined from the external standard channels ratio and dpm calculated from cpm as programmed with a quench correction curve. All samples were either counted for 10 minutes or until 40,000 cpm had registered. In all cases 2S% (2 x % standard deviation) was less than 2.5.

2.6 RADIOCHEMICAL BINDING ASSSAYS

2.6.1 SATURATION BINDING OF N^6-R-[G^3H) PIA TO RAT BRAIN SYNAPTOSOMAL MEMBRANES

[^{3}H] PIA binding to rat brain synaptosomal membranes was determined by a vacuum filtration technique, the method used was essentially that of Lohse et al., (1984).

Prior to assay synaptosomal membranes were diluted to a concentration of 1mg/ml using 50mM Tris-HCl,

pH 7.4 and incubated for 30 minutes at 37°C with adenosine deaminase (3U/ml) to remove endogenous adenosine. Membranes, 100μg protein, per tube were incubated with the required concentration of [^{3}H] PIA (0.25nM - 50nM) in 50mM Tris-HCl, pH 7.4 for 45 minutes at 37°C in a total volume of 1ml. After incubation the membranes were separated from unbound radioactivity by filtration of a 0.9ml aliquot of incubation medium through a Whatman GF/B filter, using a Millipore vacuum filtration drum and pump. The filters were immediately washed twice with 5ml aliquots of ice-cold 50mM Tris-HCl, pH 7.4.

Filters were counted for radioactivity in a scintillation fluid consisting of 4g/l BBOT, 80g/l napthalene in toluene, 2-methoxyethanol (1.5:11, w/v), after being allowed to equilibrate for at least 12 hours. Non-specific binding was determined in the presence of 50μM unlabelled (-)PIA. It amounted to 30-50% of total binding.

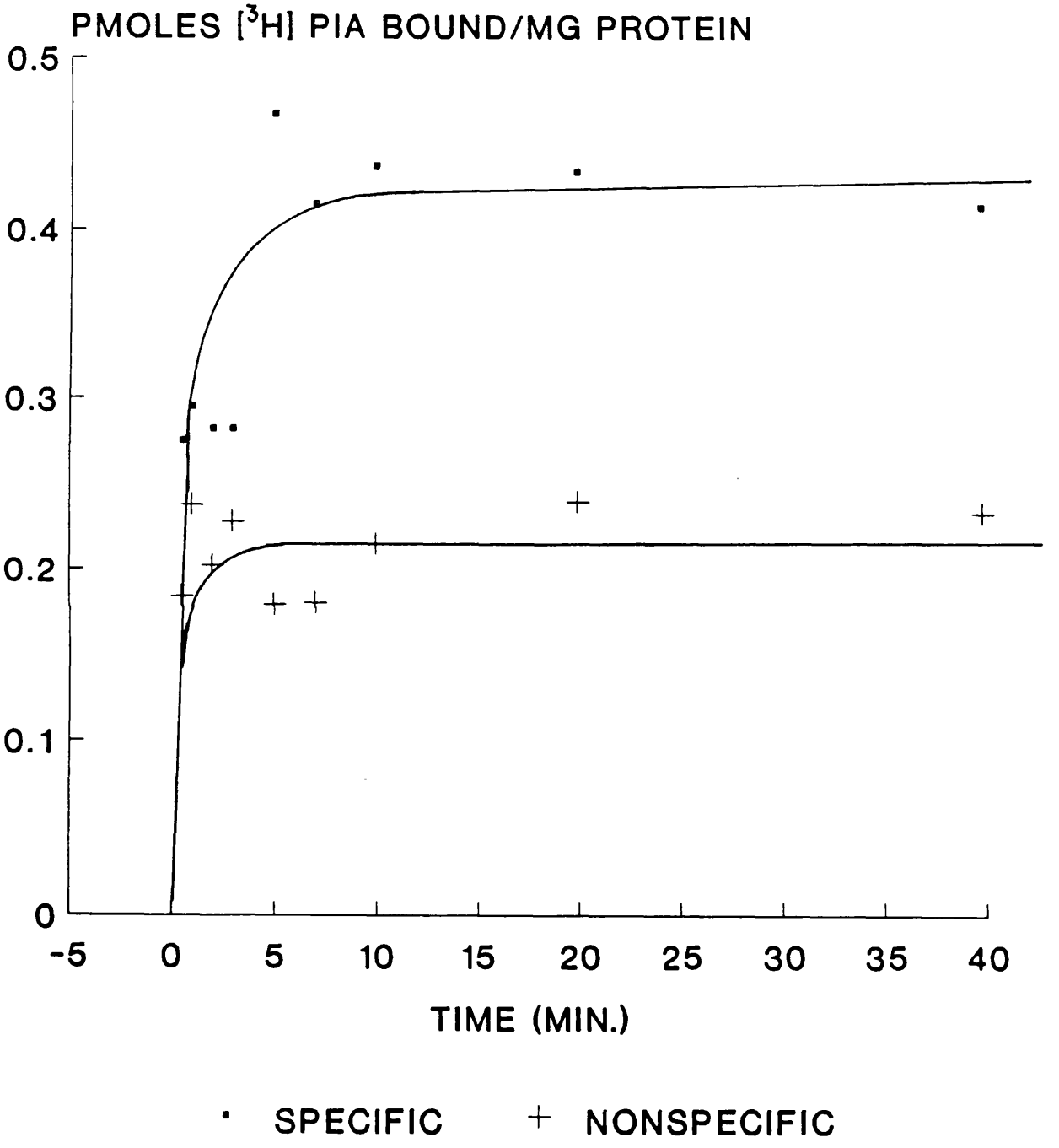
The specific binding occurred rapidly being 70% complete after 2 minutes (see Fig. 2.2). Equilibrium was reached at approximately 10 minutes. Non-specific binding measured in the presence of 50μM unlabelled (-)PIA reached a plateau after 5 min of incubation and did not change significantly for the rest of the incubation period. Since neither specific or non-specific binding varied after reaching equilibrium the incubation time that Lohse et al., (1984) set for their assay was deemed acceptable.

Fig. 2.2 Time course of specific and non-specific binding of [^{3}H] PIA to euthyroid rat brain synaptosomal membranes.

[^{3}H] PIA (10nM) was incubated with euthyroid synaptosomal membranes. At the times indicated 900μl aliquots were removed from the incubation and binding determined as described in section 2.6.1 (n = 1).

Specific binding, ■

Non-specific binding, +


PMOLES [3H] PIA BOUND/MG PROTEIN
0.5
0.4
0.3
0.2
0.1
0
-5
0
5
10
15
20
25
30
35
40
TIME (MIN.)
SPECIFIC
NONSPECIFIC

2.6.2 COMPETITION FOR [^{3}H] DPX BINDING TO RAT SYNAPTOSOMAL MEMBRANES

Binding of [^{3}H] DPX was carried out in essentially the same way as for [^{3}H] PIA following the method of Lohse et al., (1984) except that the final volume was reduced to 0.25ml and the labelled ligand was present in a final concentration of 10nM while that of unlabelled PIA was varied over a logarithmic scale (10^{-10} - 10^{-3}M). Only 3ml of 50mM Tris-HCl pH 7.4 was used to wash the filter each of two times. Incubation was performed for 15 minutes at 37°C. Non-specific binding was not subtracted for data analysis of competition curves.

2.6.3 BINDING DATA ANALYSIS

Saturation and competitive binding data were analysed by a non-linear weighted least squares curve fitting procedure using a search type minimisation method written by D. Colquhoun (1971) and adapted by N. Castle.

The method provided estimates of parameters for different states of the receptor with their respective S.D. values. Parameters estimated were: affinity of ligands, (K_H high affinity dissociation constant, K_L low affinity dissociation constant); Bmax and in the case of competition curves non-specific binding. Fitted estimates were provided for the data assuming either one or two affinity states. Slope factors were calculated from indirect Hill plots.

Where appropriate statistical analysis comparing "goodness of fit" between one and two affinity state models was determined using a partial F test.

$$F = [(SS_1 - SS_2)/df_1 - df_2/SS_2/df_2]$$

Where SS_1 and SS_2 are the sum of squares of residuals with models one and two and df_1 and df_2 are the corresponding degrees of freedom (number of data points minus number of parameters estimated). The more complex model was retained only when it significantly improved the fit ($p<0.05$). Statistical testing for differences between estimates of affinity constants and Bmax values in euthyroid and hypothyroid states was performed using an unpaired Student's t-test.

2.7 SPECTROPHOTOMETRIC ENZYME ASSAYS

2.7.1 ADENOSINE DEAMINASE (EC 3.5.4.4)

The commercial preparation of adenosine deaminase (suspension, from calf intestine) was assayed by a method described by Kalckar (1947).

100μl of the enzyme suspension was pipetted into a 0.4ml plastic Eppendorf tube and centifruged for 2-3 minutes in an Eppendorf 5412 centrifuge. The supernatant was then aspirated off and the pellet resuspended in 1ml of 0.9% NaCl (w/v). The assay mixture contained 0.1M glycylglycine, pH 7.4 and 0.15mM adenosine in a volume of 3ml. The reaction was initiated by the addition of 20μl of the resuspended pellet and adenosine utilisation at 25°C followed by the decrease in absorbance at 265nm in a Unicam

SP8-100 spectrophotometer. Adenosine deaminase activity was calculated from the extinction coefficient of 8.1 $\mu mol^{-1}ml$.

2.7.2 LACTATE DEHYDROGENASE (EC 1.1.1.27)

The spectrophotometric assay of lactate dehydrogenase (LDH) was performed by the method of Saggerson (1974).

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

2.7.3 SUCCINATE DEHYDROGENASE (EC 1.3.99.1)

The mitochondrial marker succinate dehydrogenase (SDH) was assayed spectrophotemetrically by the method of Prospero (1974), using 'INT' as a synthetic acceptor.

The reaction was performed in glass test tubes containing 4mg/ml INT (a saturated solution at 37°C) in 0.1ml of 0.3M phosphate buffer, pH 7.4 and 0.1ml 0.3M sodium succinate. The assay was initiated by addition of sample (diluted where necessary). After vortex mixing and incubation at room temperature for 15 minutes the reaction was terminated by the addition of 2.4ml of a mixture of 86% ethanol (v/v): ethylacetate: 10% trichloroacetic acid (w/v)

[6.5: 10:1 by vol]. After vortex mixing the tubes were centrifuged in a MSE bench centrifuge at approximately 1,000 g_{av} for 10 minutes, to remove denatured protein. The formation of the red, water insoluble formazan produced from the reduction of INT was measured by measuring the absorbance of the supernatant at 490 nm in a Unicam SP8-100 spectrophotometer against a tissue blank containing all assay constituents with the exception of tissue sample. SDH activity was calculated from the extinction coefficient for INT of 20.1 μmol^{-1} ml at 490nm.

[INT = 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride.]

2.7.4 2' - 3' CYCLIC NUCLEOTIDE 3' - PHOSPHOHYDROLASE (CNP) (EC 3.1.4.37)

The myelin marker enzyme CNP was assayed spectrophotometrically using a coupled enzyme assay as described by Sogin (1976).

In this assay the hydrolysis of 2'-3' cyclic NADP proceeds in the presence of glucose-6-phosphate dehydrogenase and its substrate, and the resulting NADPH formed is measured by an increase in absorbance at 340 nm.

The enzyme was assayed at 25°C, in 1ml plastic cuvettes, in a final volume of 1ml containing 1mM 2'-3' cyclic NADP, 0.2M MES, pH 6.0, 5mM glucose-6-phosphate dehydrogenase. The reaction was initiated with 20-50 μl of tissue extract. Prior to assay all tissue extracts had been diluted approximately 30 fold with 1% Triton-X-100

(w/v), 0.2% bovine serum albumin (w/v), such that the change in absorbance at 340nm did not exceed 0.1/min. The increase in absorbance at 340nm was followed against a reagent blank (omitting tissue extract) in a Unicam SP8-100 spectrophotometer. CNP activity was calculated from the extinction coefficient of 6.22 μmol^{-1} ml for NADPH at 340nm.

2.7.5 ACETYLCHOLINESTERASE (EC 3.1.1.7)

The synaptosomal marker enzyme acetylcholinesterase was assayed spectrophotometrically by the method of Ellman et al., (1961).

Enzyme activity is measured by following the increase of the yellow anion of 5-thio-2-nitrobenzoic acid from thiocholine when it reacts with 5:5-dithio-2-nitrobenzoate ion. The assay mixture, in a 1ml plastic cuvette, consisted of 0.2M potassium phosphate buffer, pH 8.0, 10mM dithiobisnitrobenzoic acid (DTNB), 10% Triton-X-100 (w/v), 10-50μl tissue sample in a final volume of 1 ml. Reaction was initiated by the addition of 10μl of 75mM acetylthiocholine iodide and the increase in absorbance at 412nm followed at 25°C in a Unicam SP8-100 spectrophotometer. Absorbance change was measured continuously against a reagent blank containing all assay constituents with the exception of acetylcholine iodide. Acetylcholinesterase activity was calculated from the extinction coefficient of the yellow anion at 412nm of 13.6 μmol^{-1} ml.

2.8 RADIOCHEMICAL ENZYME ASSAYS

2.8.1 ADENOSINE DEAMINASE (EC 3.5.4.4)

The method employed was essentially that as described by Arch and Newsholme (1978b) with some modifications. The method involves measuring the conversion of adenosine to inosine and hypoxanthine using tracer amounts of 2-[^{3}H] adenosine.

The incubation medium contained, (final concentrations) 3.3mM citric acid, 77mM Na_2HPO_4,pH 7.5, 4mM $MgCl_2$, 0.4mM EDTA, 200μM adenosine and 0.4μCi [^{3}H] adenosine in a final volume of 40μl. The reaction was initiated by the addition of 10μl of rat brain 105,000 g_{av} supernatant (prepared as described in Section 2.3). Enzyme activity appeared to be linear with respect to both protein concentration and time in euthyroid and hypothyroid rats (see Figs. 2.3 and 2.4).

The reaction was terminated after 6 minutes by the addition of 7μl of 2M $HClO_4$, containing adenine, adenosine, hypoxanthine, inosine, adenosine, diphosphate and adenosine monophosphate (all approximately 5mM). The reaction products, inosine and hypoxanthine were separated by t.l.c. (see Section 2.8.4) and radioactivity of the appropriate regions of the chromatogram quantified by liquid scintillation counting.

2.8.2 ADENOSINE KINASE (EC 2.7.1.20)

The assay procedure followed for adenosine kinase was also a modification of the method of Arch & Newsholme (1978b).

Fig 2.3 Adenosine deaminase as a function of protein concentration in euthyroid and hypothyroid rat brain supernatant

Adenosine deaminase activity was assayed as described in section 2.8.1 at the concentrations of protein indicated in:

euthyroid rat supernatant, ■

hypothyroid rat supernatant, +

Protein was determined as described in section 2.4 and rat brain supernatant was prepared as described in section 2.3 (n=1)

ADENOSINE DEAMINASE ACTIVITY AS A
FUNCTION OF PROTEIN CONCENTRATION

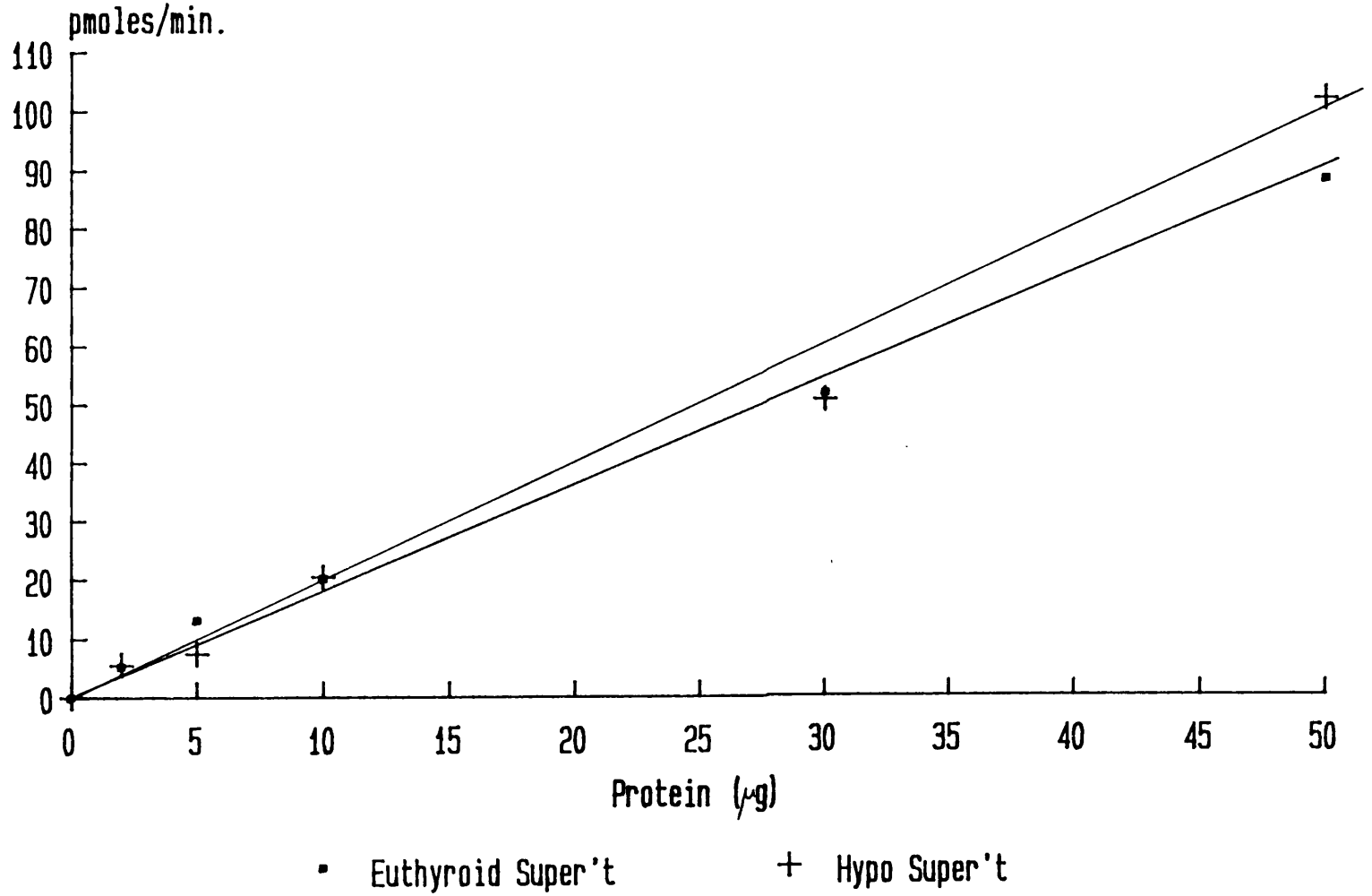


Fig 2.4 Time course of adenosine deaminase activity in rat brain supernatant derived from hypothyroid and euthyroid rats

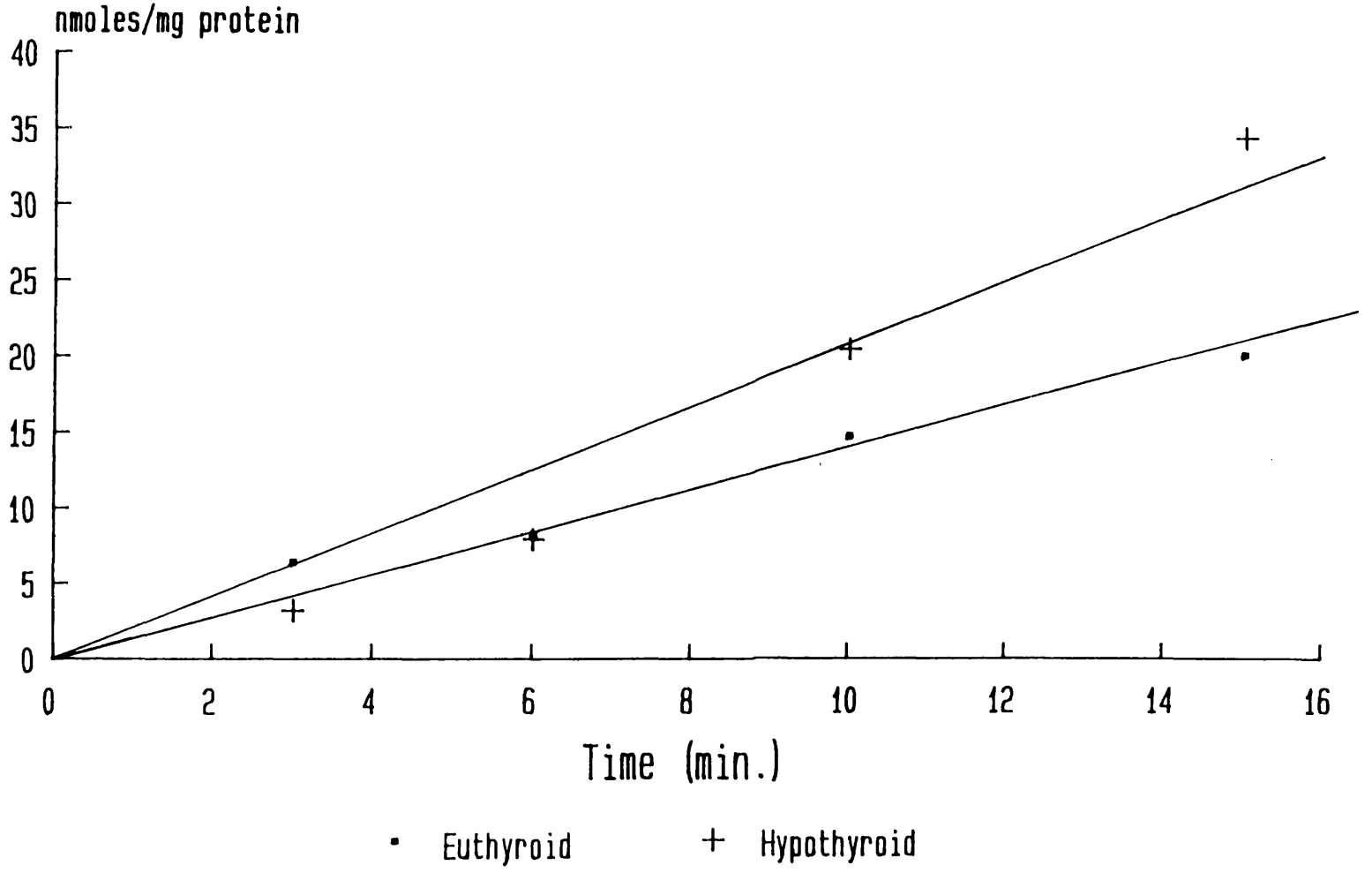
Adenosine deaminase was assayed as described in section 2.8.1 at the times indicated in:-

euthyroid supernatant, ■

hypothyroid supernatant, +

Rat brain supernatant was prepared and protein determined as described in Sections 2.3 and 2.4 respectively (n=1).

Time Course Of
Adenosine Deaminase Activity



The incubation medium was similar except that the final concentration of adenosine was 10μM, 0.7μCi [^{3}H] adenosine and 4mM ATP were also present. In addition 10μM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was also added. EHNA inhibited the deaminase completely, its inclusion in the assay was deemed necessary since in studies performed by Jamal & Jaggerson (1987) it was found that rapid loss of substrate via the deaminase assay caused significant interference in the kinase assay. EHNA had no effect on the assay of adenosine kinase itself.

The reaction was initiated by the addition of 10μl of 105,000 g_{av} rat brain supernatant (prepared as described in section 2.3). Enzyme activity was found to be linear with respect to time though nonlinear with protein concentration (See Figs. 2.5 and 2.6). In fact the specific activity increased appreciably with protein concentration. Since thyroid status did not appreciably alter the nature of this dependence, a comparison of adenosine kinase activities in the two states was still a valid exercise. As a precaution all assays were performed with 5.0μg of soluble protein (125μg/ml in the assays). The reason for this phenomenon is not known. Possibly it reflects enzyme dissociation/dissaggregation or loss of some necessary activator on dilution of soluble extract. The reaction was terminated after 6 minutes of incubation in the same manner as for adenosine deaminase. Again the products were separated by t.l.c. (see section 2.8.4) and quantified by liquid scintillation counting.

Fig 2.5 Time course of adenosine kinase activity in rat brain supernatant derived from euthyroid and hypothyroid rats.

Adenosine kinase was assayed as outlined in section 2.8.2 at the times indicated:

euthyroid supernatant, ■

hypothyroid supernatant, +

Protein was determined and rat brain supernatant prepared as described in sections 2.4 and 2.3 respectively (n=1).

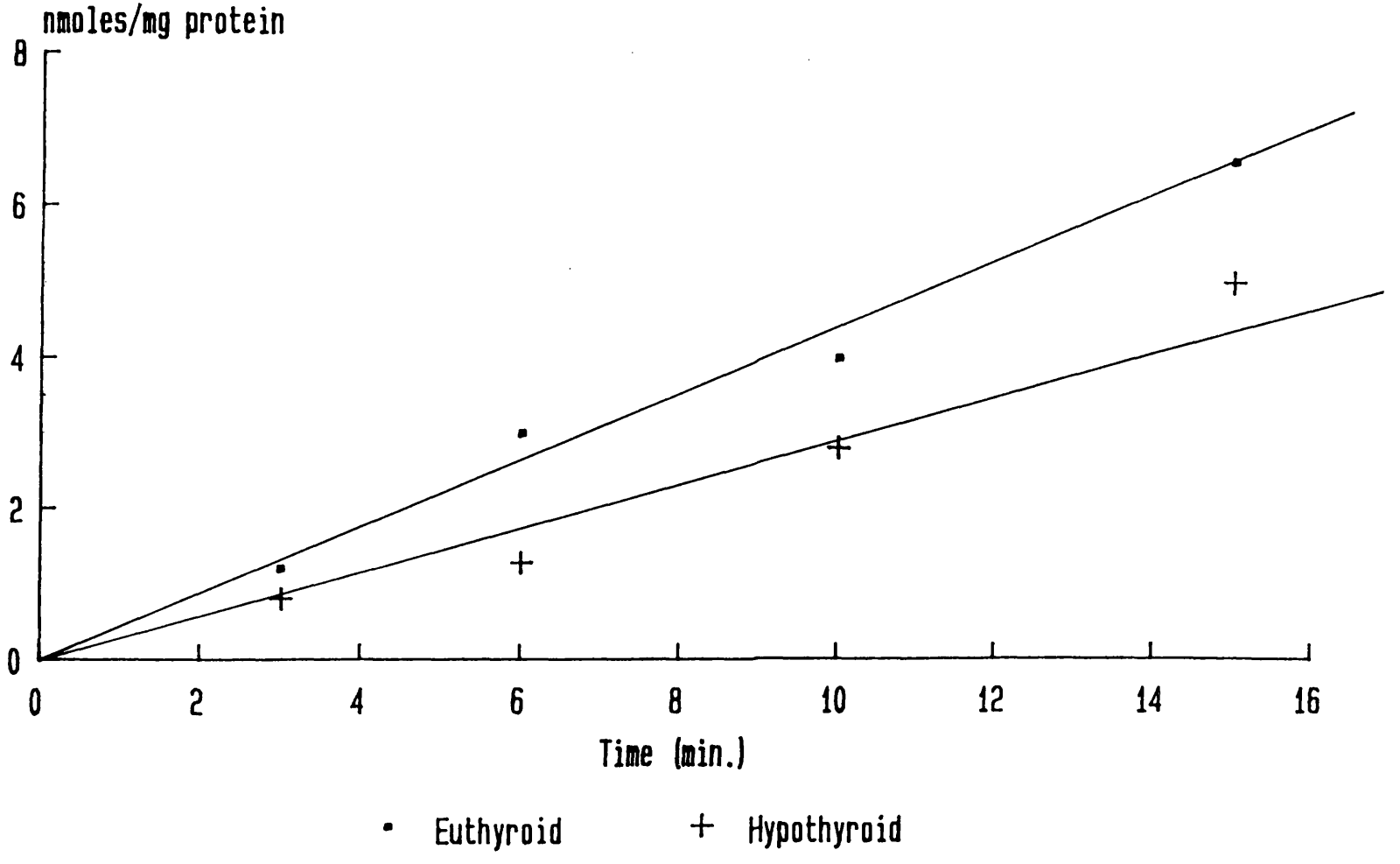

Time Course Of
Adenosine Kinase Activity
nmoles/mg protein
8
6
4
2
0
0
2
4
6
8
10
12
14
16
Time (min.)
Euthyroid
Hypothyroid

Fig 2.6 Adenosine kinase activity as a function of protein concentration in euthyroid and hypothyroid rat brain supernatant

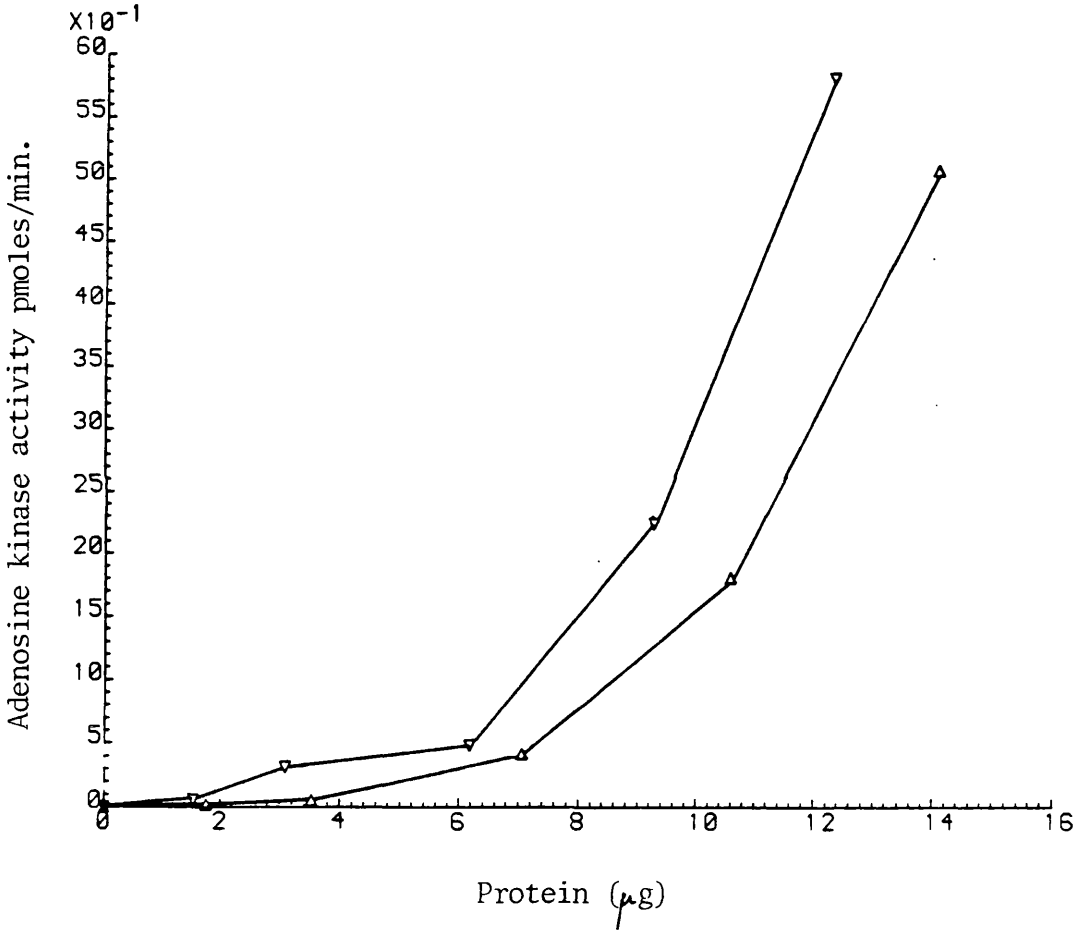
Adenosine kinase was assayed as described in section 2.8.2 at the concentrations of protein indicated, in:

euthyroid rat supernatant, ∇

hypothyroid rat supernatant, △

(n=1)

Protein was determined and rat brain supernatant prepared as described in sections 2.4 and 2.3 respectively


X10⁻¹
60
55
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Adenosine kinase activity pmoles/min.
Protein (μg)

2.8.3 5'-NUCLEOTIDASE (EC 3.1.3.5)

5'-Nucleotidase was assayed by measuring the release of adenosine from AMP using [2-^{3}H] AMP as a tracer by a minor modification of the method of Newby et al., 1975).

The assay was performed at 37°C in a shaking waterbath at 100 strokes/minute in 1.5ml Eppendorf tubes. Assays were in a final volume of 0.5ml containing 200μM AMP, 0.06μCi [2-^{3}H] AMP, 50mM Tris-HCl, pH 8.0 and 4.5mM β-glycerophosphate. The β-glycerophosphate was present in excess of AMP as a substrate for all the non-specific phosphatases that may be present in the membrane fractions.

The reaction was initiated by the addition of 20-50μg of membrane protein. The enzyme activity appeared to be linear with respect to protein concentration and time for both myelin and synaptosomal fractions in euthyroid and hypothyroid states (see Figs. 2.7 and 2.8). The reaction was terminated after 10 minutes by the addition of 100μl of ice-cold 0.15M zinc sulphate immediately followed by 100μl of 0.15M barium hydroxide, vortex mixed and left over ice for 10 minutes. The tubes were then centrifuged in an Eppendorf 5412 centrifuge for 10 minutes. This procedure preferentially precipitates AMP leaving the produced adenosine in the supernatant. 0.5ml of the supernatant was counted for tritium in 13ml of BBOT/toluene (0.4% w/v): Triton-X-100 (2:1, v/v) in a Phillips PW4700 liquid scintillation counter.

Fig 2.7 5'-Nucleotidase activity as a function of protein concentration in myelin and synaptosomal membranes of euthyroid and hypothyroid rats

Myelin and synaptosomal membranes were assayed for 5'-nucleotidase activity at the concentration of protein indicated as outlined in section 2.8.3, in:-

Euthyroid myelin, +
Euthyroid synaptosomal membranes, □
Hypothyroid myelin, ■
Hypothyroid synaptosomal membranes, ✳

Protein was determined and membranes prepared as described in sections 2.4 and 2.3 respectively, (n=1).

5'-NUCLEOTIDASE ACTIVITY AS A FUNCTION
OF PROTEIN IN MYELIN AND MEMBRANES

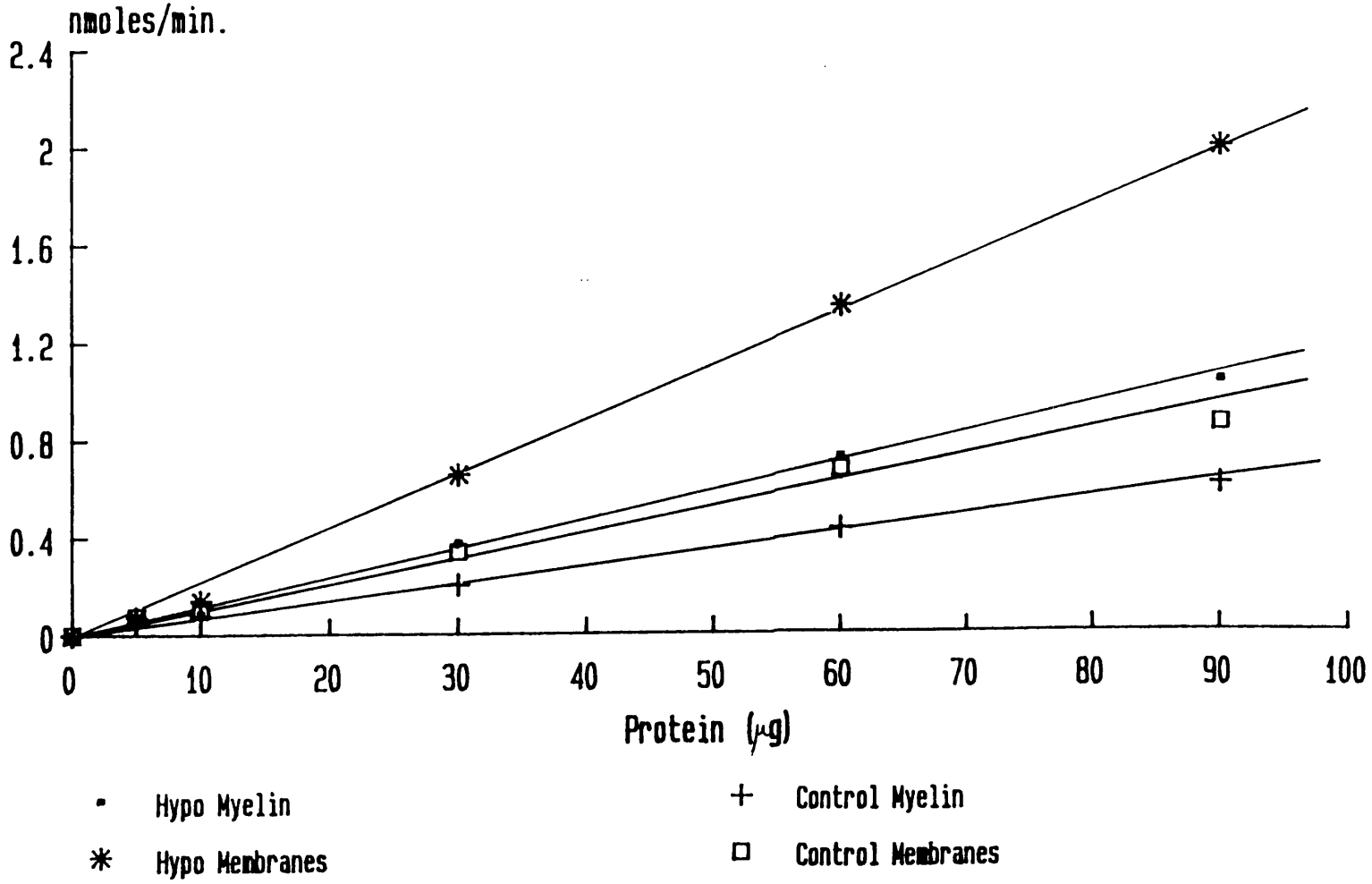


Fig 2.8 Time course of 5'-nucleotidase activity in myelin and synaptosomal membranes from euthyroid and hypothyroid rats

5'-nucleotidase was assayed as described in section 2.8.3 at the times indicated in:

hypothyroid myelin, +

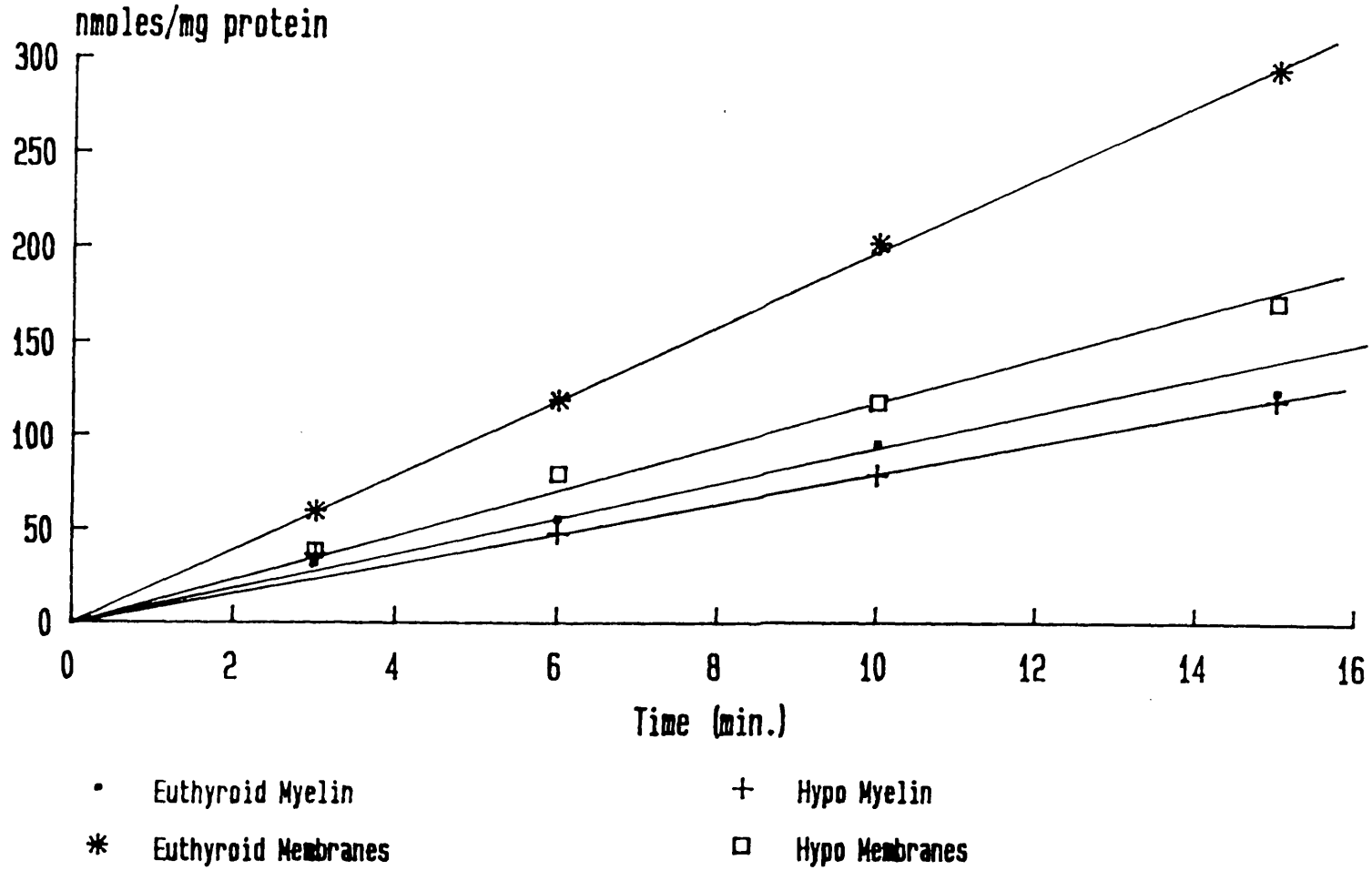
hypothyroid synaptosomal membranes, □

euthyroid myelin, ■

euthyroid synaptosomal membranes, *

Synaptosomal and myelin membranes were prepared, and protein determined, as indicated sections 2.3 and 2.4 respectively (n=1).

Time Course Of
5'-Nucleotidase Activity



2.8.4 SEPARATION OF NUCLEOTIDES AND NUCLEOSIDES BY THIN LAYER CHROMATOGRAPHY

Nucleotides and nucleosides were separated by t.l.c. in order to isolate substrate from product or to follow the elution of cyclic nucleotides through chromatographic columns.

The method used was that of Arch & Newsholme (1976). A t.l.c. silica coated plastic sheet of 20 x 20cm impregnated with a fluor, absorbing at 254nm was divided into 5 equal parts, a 20μl aliquot of supernatant/eluate was applied to a line of length 13mm, which was 15mm from the base of the chromatogram. The plate was developed in a solvent mixture of propan-2-ol: ethyl acetate: 8M ammonia (9:4:3, by vol). To maximise separation the plates were first developed for approximately 15 minutes, dried in a fume cupboard and then developed for 1 - 1.5 hours. The spots were located under U.V. light and the relevant nucleotide/nucleoside spot cut out and placed facing upwards in a scintillation vial to which 1ml of methanol was added. [BBOT/toluene (0.4%, w/v); Triton X-100 (2:1, v/v)] and radioactivity counted in a Phillips PW 4700 liquid scintillation counter. 10μl of supernatant from enzyme assays was also counted for tritium so that recovery from the t.l.c plastic sheets should be estimated.

Typical Rf values were as follows:- purine nucleotides (except cyclic nucleotides) 0-0.03; cAMP, 0.29; dcAMP, 0.32; inosine, 0.33; hypoxanthine 0.39; adenosine 0.52; adenine, 0.58.

2.9 DEVELOPMENT OF THE ADENYLATE CYCLASE ASSAY

2.9.1 ADENYLATE CYCLASE (EC 2.1.1.41)

A highly sensitive method for assaying adenylate cyclase, by measuring the production of cAMP from ATP, using [d-^{32}P] ATP as a tracer was developed by Saloman *et al.*, (1974). This method was subsequently modified by Cooper & Londos (1979). A high degree of sensitivity was achieved by eliminating background radioactivity. The procedure involved initial elution of the assay mixture through a Dowex cation exchange column to remove the bulk of the ^{32}P-associated radioactivity followed by elution of the cAMP fraction which was immediately eluted through an alumina column in order to purify the cyclic nucleotide with negligible background radioactivity.

The assay procedure employed was a minor modification of the method of Cooper & Londos (1979). Adenylate cyclase was assayed in a medium containing 0.1mM dATP, 1μCi [α-^{32}P] dATP, 0.1mM dcAMP, 4mM $MgCl_2$, 30mM Tris-HCl, pH 7.5, 2mM creatine phosphate, 2.5 units of creatine phosphokinase, 2.5 units/ml of adenosine deaminase, 10μM papaverine and 0.4mg/ml bovine serum albumin. Assays were performed at 24°C for 20 minutes in a volume of 0.1ml and in all cases were initiated by the addition of 10μg of membrane protein.

The enzyme activity appeared to be linear with respect to time at a protein concentration of 10μg under all the conditions studied ie. in the presence and absence of forskolin, GTP and sodium in euthyroid and hypothyroid

states; (see Figs. 2.9, 2.10 and 2.11).

The reaction was terminated by the addition of 0.1ml "stopping solution" consisting of 10% SDS (w/v) and 10mM EDTA. 50μl (c.a. 70,000 dpm) [8-^{3}H] dcAMP was subsequently added to monitor recovery of [α-^{32}P] dcAMP.

2.9.2 CYCLIC NUCLEOTIDE ISOLATION

2.9.2.a DOWEX COLUMNS

Initial preparation of the cationic form of the resin involved successive washes with distilled water and IM HCl. 1ml of Dowex resin (AG 50W-X4 200-400 mesh) was decanted into plastic columns (0.5 x 11cm), 2ml of 1M HCl were added to each Dowex column and the columns stored with no further treatment. Prior to use the columns were washed with 10ml of distilled water.

2.9.2.b ALUMINA COLUMNS

Columns (0.6 x 11 cm) containing 0.6g neutral alumina (type WN3) were washed successively with 0.1M imidazole-HCl, pH 7.5, until the pH of the eluate equalled that of the starting buffer. Prior to use columns were washed with 8ml of the imidazole buffer.

Initial studies performed by Cooper & Londos (1979) with [α-^{32}P] dATP as substrate showed a small amount of contaminant, assumed to be dAMP, eluted with [α-^{32}P] dcAMP giving spuriously high and erratic basal activities. A purification scheme was devised for the cyclic nucleotide based on the co-recovery of [^{32}P] and [^{3}H] dcAMP. This

Fig 2.9 Time course of the effects of forskolin and sodium on euthyroid rat brain synaptosomal membrane adenylate cyclase activity

Adenylate cyclase was assayed as described in section 2.9.1 at the times indicated in:

The absence of forskolin and Na^+, basal activity, ■

With 10μM forskolin, +

With 10μM forskolin and 100mM Na^+, ✳

Synaptosomal membranes were prepared as described in section 2.3 and protein determined as indicated in section 2.4, (n=1).

TIME COURSE OF THE EFFECT OF FORSKOLIN & SODIUM ON EUTHYROID ADENYLATE CYCLASE

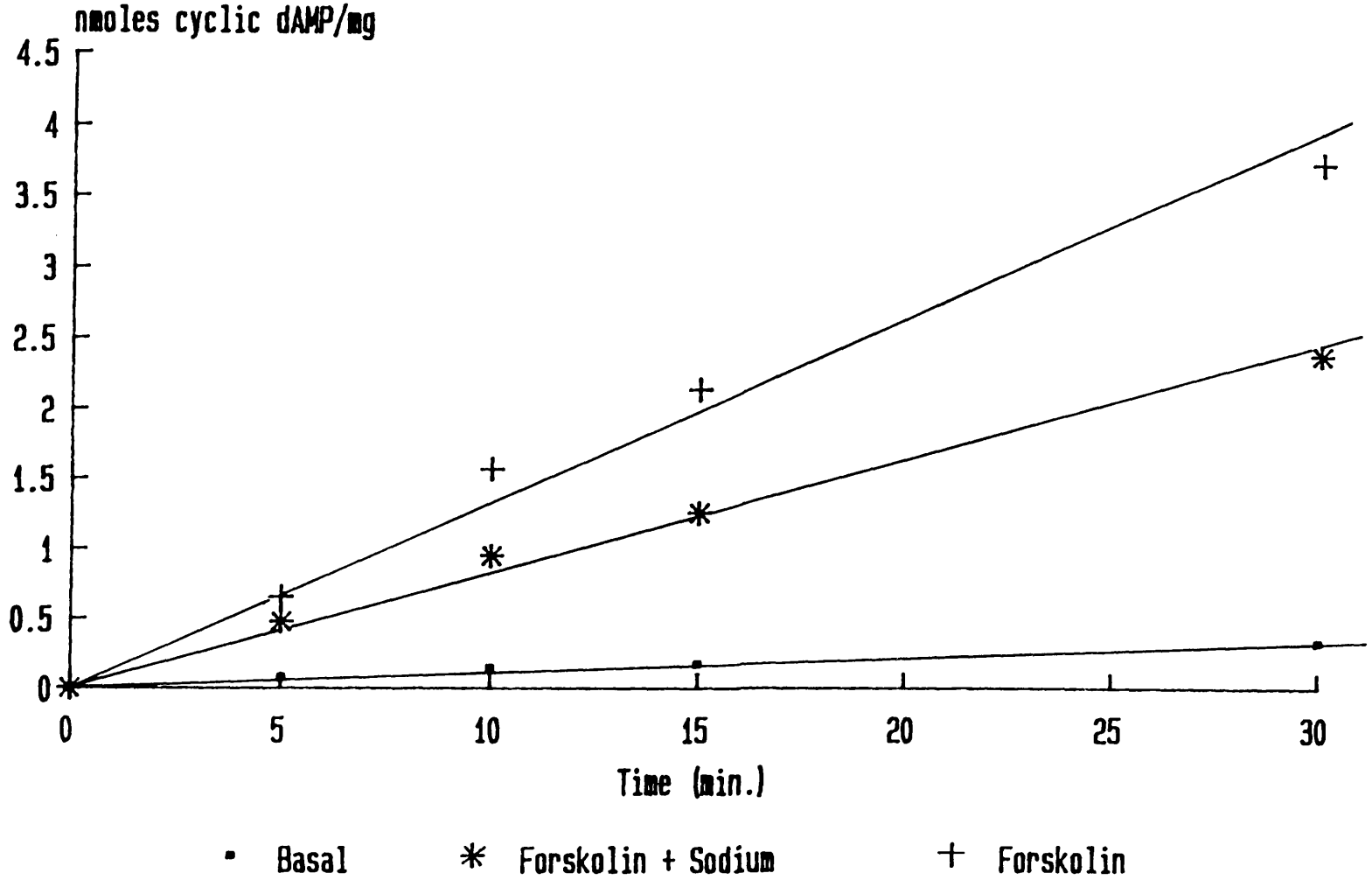


Fig 2.10 Time course of the effect of forskolin and Na^+ on hypothyroid rat brain synaptosomal membrane adenylate cyclase activity

Adenylate cyclase was assayed as described in section 2.9.1 at the times indicated in:
The absence of forskolin and Na^+, basal activity, ■
With 10μM forskolin, *
With 10μM forskolin and 100mM Na^+, +

Rat brain synaptosomal membranes were prepared as described in section 2.3 and protein determined as outlined in section 2.4, (n=1)

TIME COURSE OF THE EFFECT OF FORSKOLIN & SODIUM ON EUTHYROID ADENYLATE CYCLASE

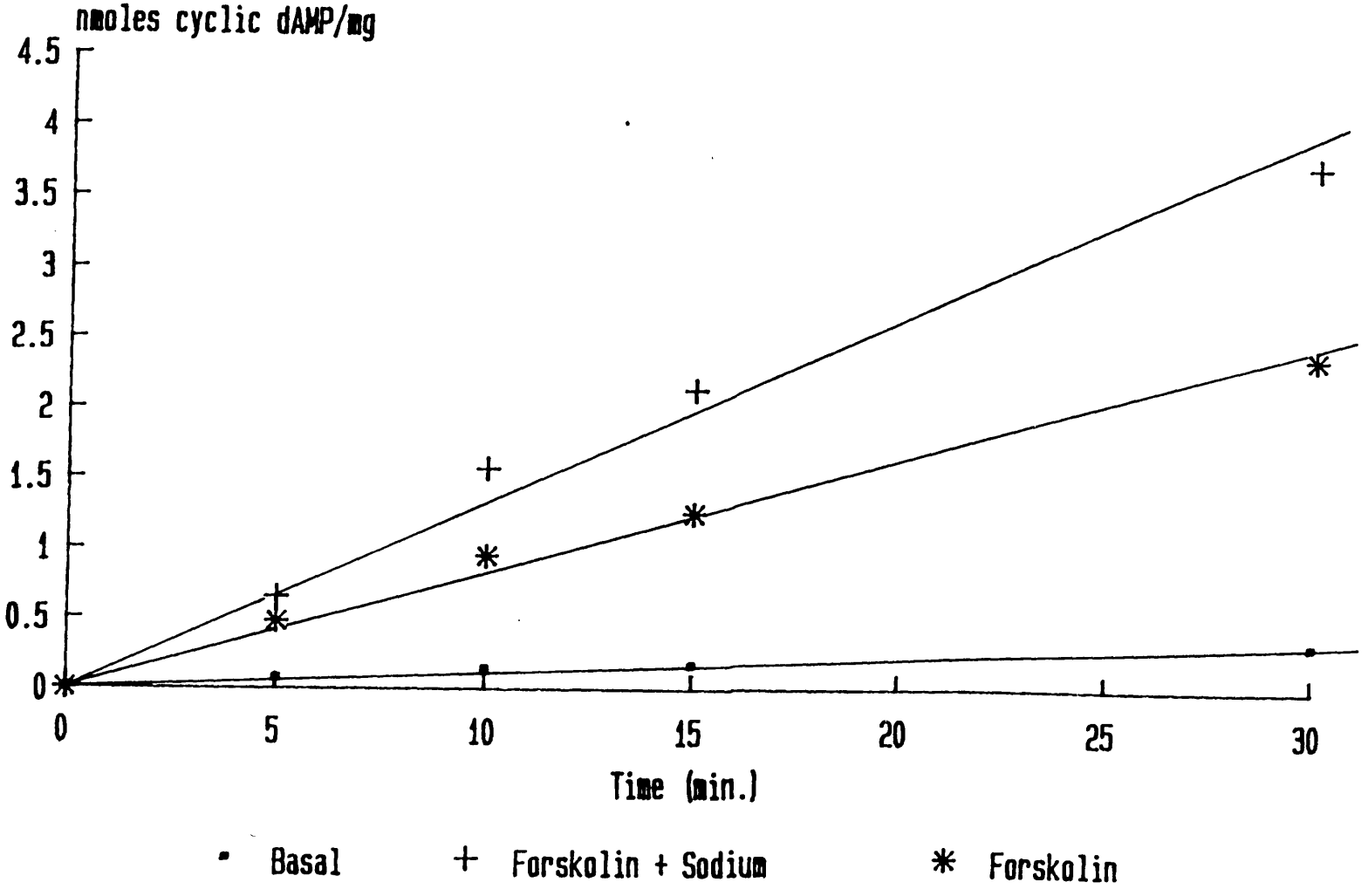


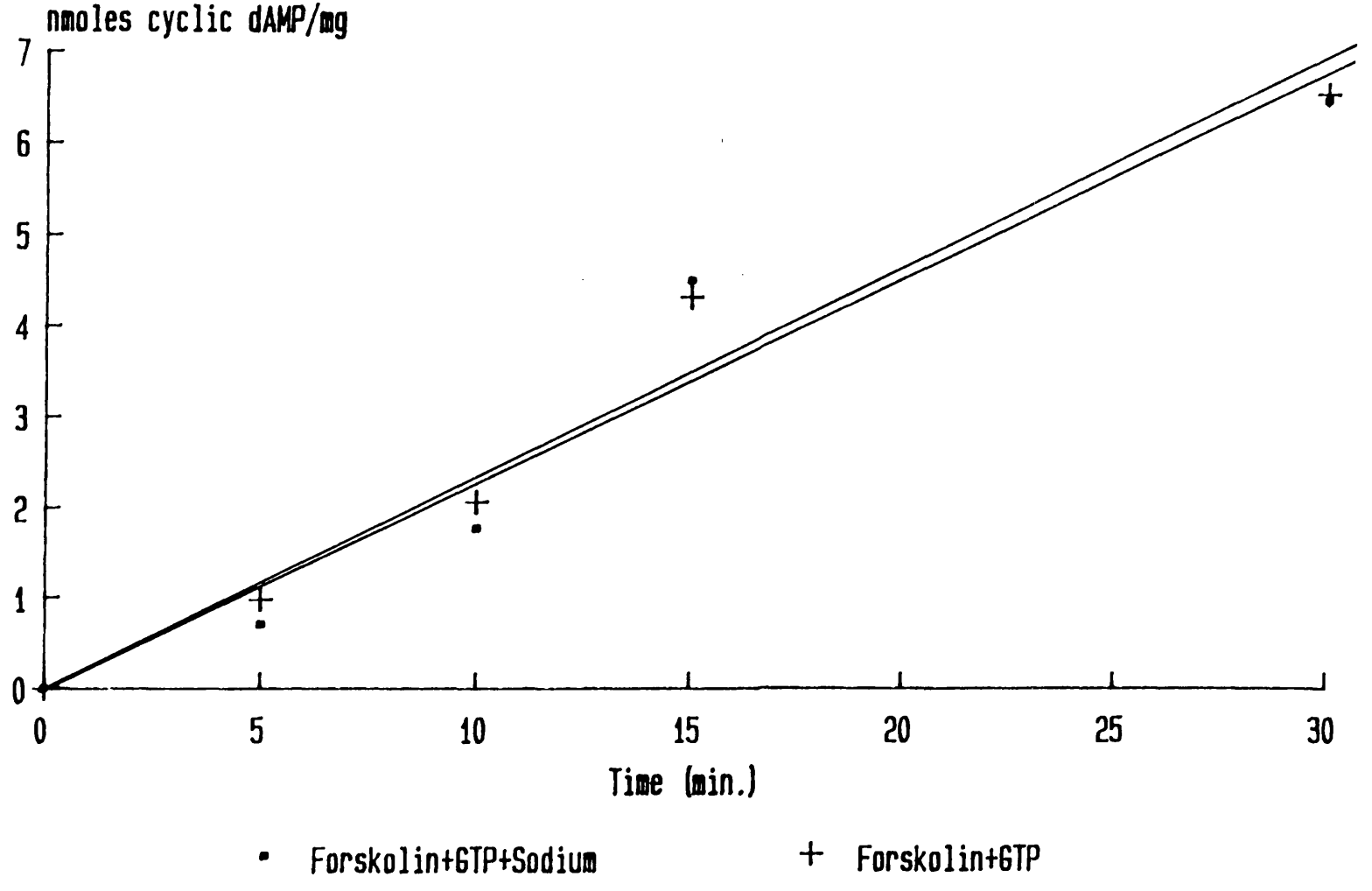
Fig 2.11 Time course of the effect of forskolin, GTP and Na^+ on hypothyroid synaptosomal membrane adenylate cyclase activity.

Adenylate cyclase was assayed as described in section 2.9.1 at the times indicated with:

10μM forskolin, 1μM GTP and 100mM Na^+, ■ 10μM forskolin and 1μM GTP, +

Rat brain synaptosomal membranes were prepared as described in section 2.3 and protein concentrations determined as described in section 2.4, (n=1)

TIME COURSE OF THE EFFECT OF FORSKOLIN
GTP AND SODIUM ON HYPO ADENYLATE CYCLASE



purification scheme was repeated so that parameters for isolation of the cyclic nucleotide could be optimised.

2.9.2.c DOWEX CHROMATOGRAPHY

A 50μl aliquot containing c.a. 100,000 cpm of [5',8-^{3}H]cAMP, [8-^{3}H]dATP and 0.5mM dATP, dcAMP and dAMP separately or in combination were combined with 100μl of "stopping solution" and 1ml distilled water and applied to a Dowex column. Successive fractions were eluted with 1ml distilled water, collected and radioactivity and/or optical density monitored. Radioactivity was monitored by counting the 1ml fractions in 13ml of BBOT/toluene (0.4%, w/v): Triton X-100 (2:1, v/v) in a Phillips PW 4700 liquid scintillation counter. The optical density of the nucleotides was measured at 260nm in a Unicam SP8-100 spectrophotometer.

2.9.2.d ALUMINA CHROMATOGRAPHY

A 5ml aqueous sample containing c.a. 100,000cpm [5',-8-^{3}H] cAMP and 0.5mM of dcAMP and dAMP was applied either separately or in combination to alumina columns, and 1ml fractions collected. Successive 1ml fractions were eluted with 1ml aliquots of 0.1M imidazole, collected and radioactivity and/or optical density monitored as for Dowex chromatography.

2.9.2.e NUCLEOTIDE ELUTION PROFILES FROM DOWEX COLUMNS

Fig. 2.12 compares the elution of dATP, dAMP, cAMP and dATP from the Dowex column. dATP elutes first with 97% coming

Fig. 2.12 Composite nucleotide elution profiles from Dowex columns.

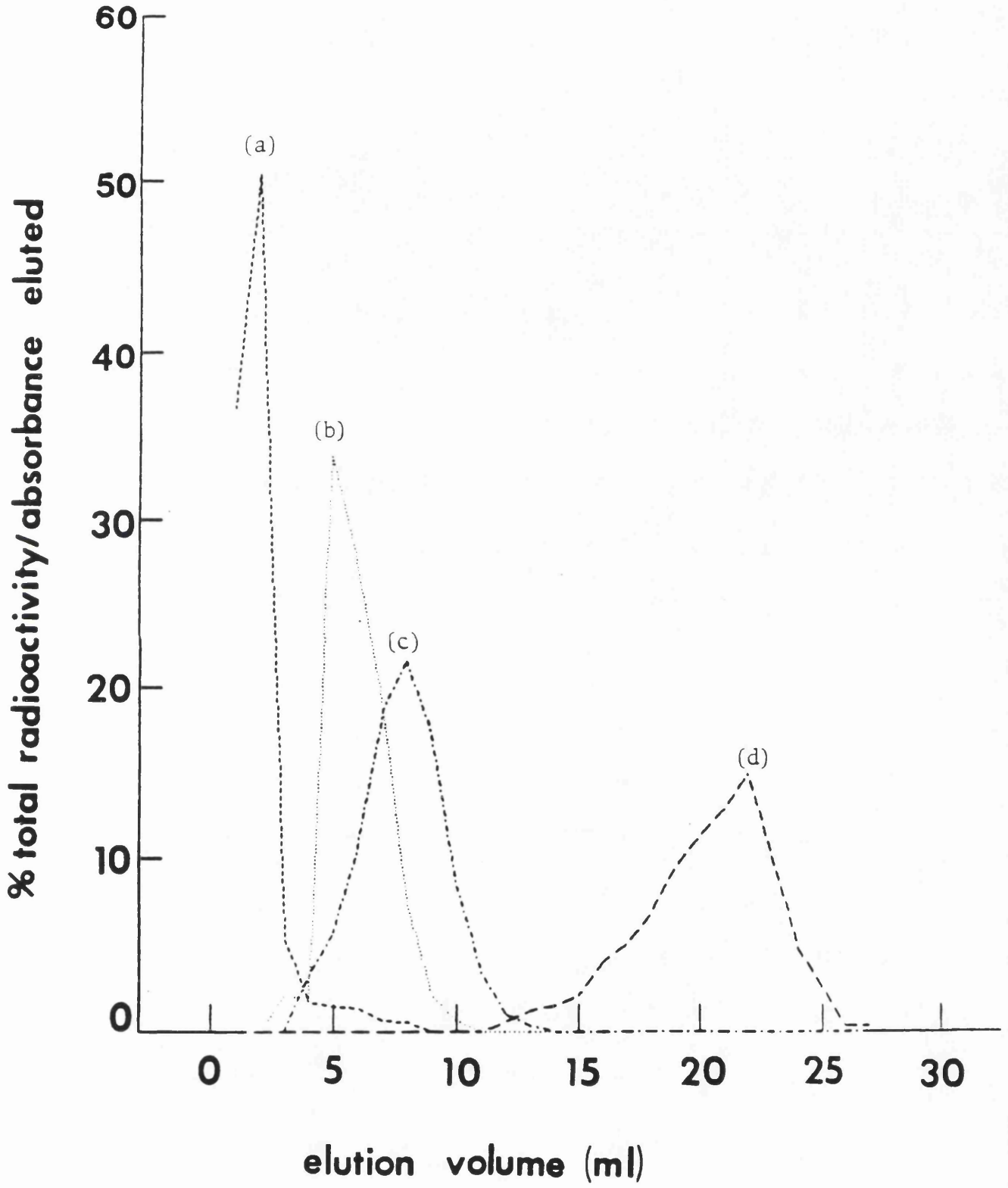
Purification of nucleotides by Dowex chromatography was performed as described in sections 2.9.2.c and 2.9.2.e peaks:

a, dATP

b, cAMP

c, dcAMP

d, dAMP


60
50
40
30
20
10
0
% total radioactivity/absorbance eluted
(a)
(b)
(c)
(d)
0
5
10
15
20
25
30
elution volume (ml)

through in the first 6ml. Cyclic dAMP elutes slightly later than cAMP. This shows that dATP is preferable to ATP as a substrate in assaying adenylate cyclase since the deoxy cyclic nucleotide can be separated from dATP with greater efficacy thus increasing the sensitivity of the assay. Deoxy AMP was seen to elute from the column much later than that found by Cooper & Londos (1979); 75% of the total nucleotide applied was found to elute in the fraction 12-27ml as compared to 5-8ml (Londos & Cooper 1979). This delayed elution of dAMP enabled a more efficient isolation of dcAMP from Dowex columns. Thus, a 5ml fraction (7-11ml) was eluted from the Dowex onto the alumina columns, which provided for maximal cyclic dAMP recovery with minimum deoxy AMP contamination.

2.9.2.f NUCLEOTIDE ELUTION PROFILES FROM ALUMINA COLUMNS

Deoxy AMP was the first to elute from the column with 57% of the total applied coming off in the first 5ml. Addition of imidazole-HCl, pH 7.5 effected the elution of dcAMP (90% in fractions 6-8ml). Cyclic AMP was seen to co-elute with dcAMP (see Fig. 2.13).

The regime chosen for isolation of dcAMP from alumina columns was that the first 5ml of eluate were discarded before collection of the subsequent 3ml into glass scintillation vials. This procedure allowed for approximately 63% recovery of cdAMP. [8-^{3}H] dcAMP was used as a recovery tracer since although cAMP co-elutes on

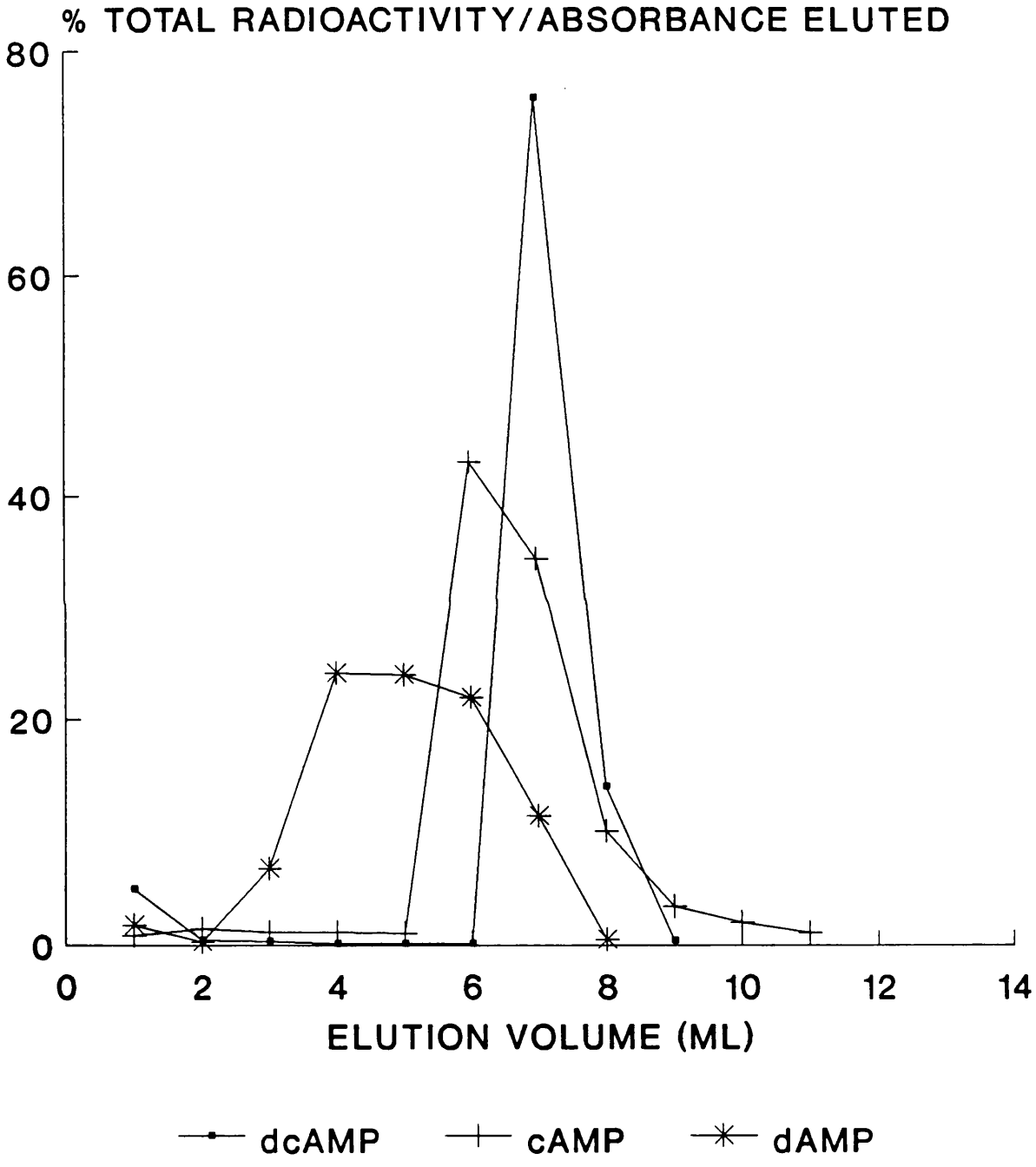
Fig. 2.13 Composite elution diagram for nucleotides on alumina columns.

Purification of nucleotides by alumina chromatography was performed as described in sections 2.9.2.6 and 2.9.2.d

*, dAMP

■, dcAMP

+, cAMP


% TOTAL RADIOACTIVITY/ABSORBANCE ELUTED
80
60
40
20
0
0
2
4
6
8
10
12
14
ELUTION VOLUME (ML)
dcAMP
cAMP
dAMP

alumina columns it does not do so on Dowex columns.

The 3ml fraction was counted for ^{3}H and ^{32}P in 16ml of BBOT/toluene (0.4%, w/v): Triton X-100 (2:1, v/v) in a Phillips scintillation counter.

2.9.3 VERIFICATION OF THE IDENTITY OF CYCLIC dAMP FROM THE dATP ASSAY SYSTEM

In order to verify that all the material collected in the "deoxy cAMP" region was in fact dcAMP, an adenylate cyclase assay was performed as previously described using synaptosomal membranes and the cAMP region analysed by t.l.c.

The assay mixture was applied initially to Dowex followed by alumina columns in order to isolate the "[α - ^{32}P] dcAMP" generated.

In this particular instance [5',8-^{3}H] cAMP (ca 50,000 dpm/column) was used in the verification procedure. It was useful in this since it is known to co-elute with [α - ^{32}P] dcAMP on alumina columns and has an almost identical Rf value to dcAMP on silica t.l.c. plates (0.32 as compared to 0.29 for cAMP). The assay mixture was eluted through alumina columns in the presence and absence of [5',8-^{3}H] cAMP.

10 μl aliquots of the material collected from alumina columns were chromatographed on silica thin layer plates (see section 2.8.4). A 12cm strip was divided into 1cm segments which were cut out and counted for ^{3}H and ^{32}P. 1ml of methanol was added to the thin layer segments, they

were subsequently counted in 13ml of BBOT/toluene (0.4%, w/v): Triton X-100 (2:1, v/v) in a Phillips PW 4700 liquid scintillation counter.

As shown in Fig. 2.14 a single peak of ^{32}P-radioactivity was obtained which coincided exactly with the position of [5',8-^{3}H] cAMP. This suggests that the α-^{32}P material isolated by the Dowex/alumina regime is in fact 2'-deoxy cyclic AMP.

2.10 STATISTICAL METHODS

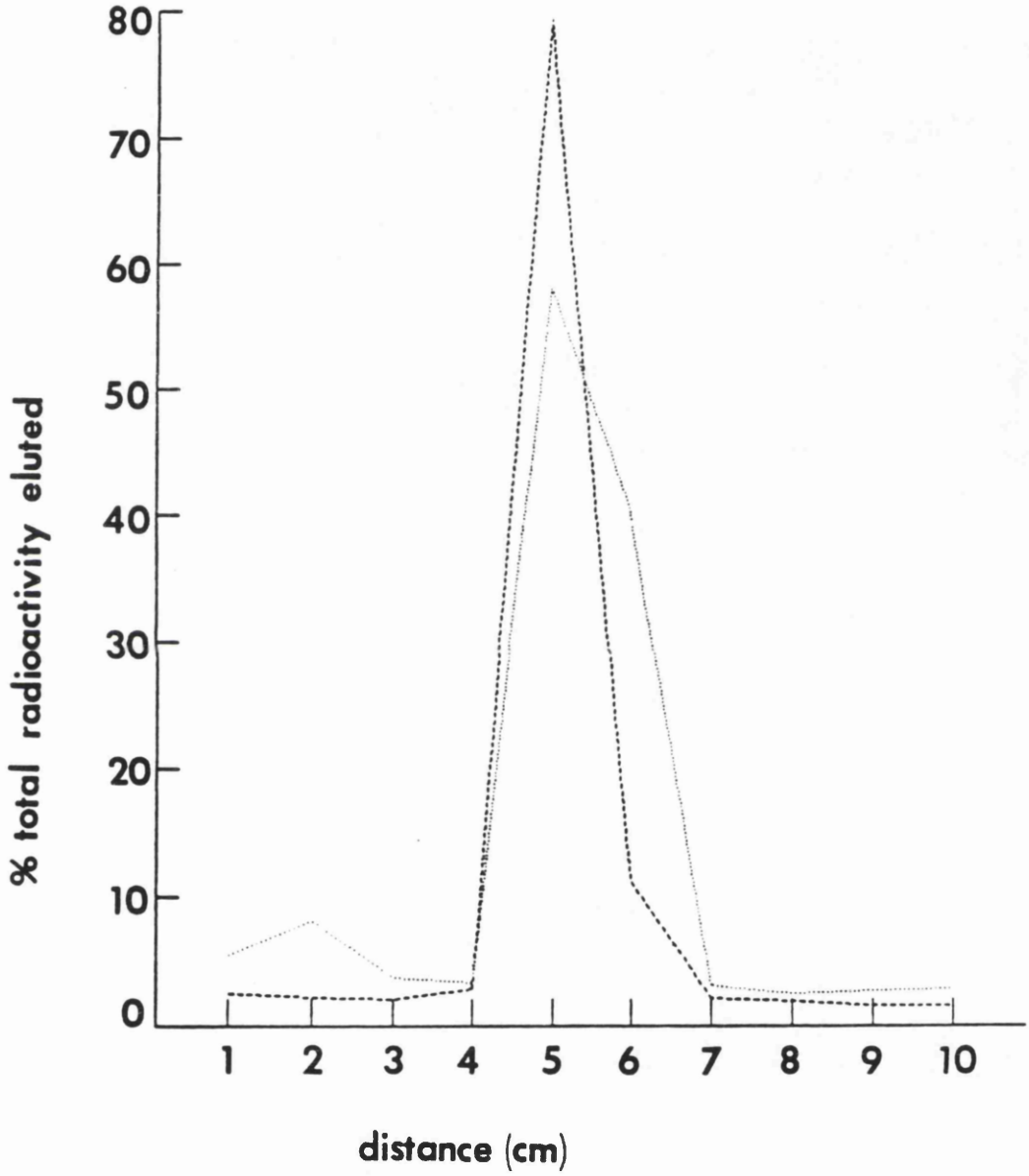
Statistical significance was determined by Student's t test for paired or unpaired samples as appropriate. Bars in figures represent S.E.M. and where these are not shown, they lie within the area of the symbol.

Fig. 2.14 Verification of identity of cyclic dAMP isolated from deoxy ATP assay system.

Adenylate cyclase was assayed as described in section 2.9.1 and the verification procedure carried out as described in section 2.9.3

---, [^{3}H] cAMP (n = 1)

...., [α-^{32}P] dcAMP (n = 1)


80
70
60
50
40
30
20
10
0
% total radioactivity eluted
1
2
3
4
5
6
7
8
9
10
distance (cm)

CHAPTER 3

RESULTS AND DISCUSSION

3.1 THE CHARACTERISATION OF SUBCELLULAR FRACTIONS PREPARED FROM RAT WHOLE FOREBRAIN

The study of the effects of hypothyroidism on the responsiveness of brain to adenosine involved utilising various brain fractions in the assays. It was important therefore to ascertain that the fractions utilised were of a reasonably pure nature. This was done by establishing that marker enzymes for the various fractions were concentrated in the appropriate fractions in both the euthyroid and hypothyroid state. It was necessary to establish the purity of the fractions in both of these states to recognise any changes that hypothyroidism may impose.

The method utilised here involved the initial isolation of a crude synaptosomal pellet and the accompanying supernatant by density centrifugation, followed by the rate separation of the pellet using a discontinuous sucrose/ficoll gradient. The use of ficoll avoided the hyperosmotic problems of high concentrations of sucrose (Lai et al., 1977; Booth & Clark, 1978). Final fractions were obtained by washing and centrifugation of fractions from the gradients.

2'-3' cyclic nucleotide 3'-phosphohydrolase (CNP) has been reported to be particularly active in the while matter of the central nervous system (Kurihara & Tsukada, 1967) CNP has also been reported to be recovered from myelin fractions of brain homogenates and to develop in parallel with myelin sheaths (Kurihara & Tsukada, 1967).

It was used therefore as the myelin marker enzyme, the physiological function of this enzyme remains to be explained.

Histochemical analysis of acetylcholinesterase has shown this enzyme to be associated with axonal and dendritic membranes and endoplasmic reticulum and to some extent with presynaptic endings (Kurihara & Tsukada (1967). Previous fractionation studies have shown acetylcholinesterase to be an excellent synaptosomal membrane marker enzyme (Cotman & Matthews, 1971; Booth & Clark 1978; Lai et al., 1977), acetylcholinesterase was therefore used as a synaptosomal marker enzyme. Succinate dehydrogenase was used as the inner mitochondrial marker enzyme (Morgan et al., 1971) and lactate dehydrogenase as the cytoplasmic marker enzyme.

Of the total activities of each of the enzyme assays (which represents activity in the crude synaptosomal pellet plus supernatant), approximately 62% of acetylcholinesterase activity was found to be associated with the synaptosomal fraction, approximately 79% of CNP activity was found to be associated with the myelin fraction, approximately 72% of LDH activity with the cytoplasmic fraction and about 77% of SDH activity was with the mitochondrial fraction. These results demonstrate that a good separation of fractions was obtained each with a relatively high degree of purity. It is important to note that even though hypothyroidism did not alter the percentage distribution of the marker enzymes the condition

nevertheless altered the specific activity of two of the enzymes namely CNP in the myelin fraction, effecting a decrease of 30% ($p<0.01$) and also a striking 60% decrease in the specific activity of lactate dehydrogenase in the soluble fraction ($p<0.001$). A major decrease in LDH activity in white adipose tissue from hypothyroid rats as compared with tissue from euthyroid rats has also been noted (Baht & Saggerson, 1988). The reasons for these changes are presently unclear.

3.2 THE EFFECT OF HYPOTHYROIDISM ON THE SATURATION BINDING OF [^{3}H] PIA TO RAT BRAIN SYNAPTOSOMAL MEMBRANES

The modulation of hormonal responsiveness by thyroid hormones involves alterations at several loci of the adenylate cyclase system. Changes in sensitivity to hormonal stimuli have been variously attributed to be due to alterations in receptor number which may or may not be accompanied by an alteration in affinity (Stiles et al., 1984), to alterations in the coupling mechanism of receptor with G protein (Stiles et al., 1981; Malbon, 1980). These alterations in the coupling mechanism may be due to an alteration in the receptor, (Malbon 1980; Malbon et al., 1984) or an alteration in the expression of G protein (Malbon et al., 1985, Milligan et al., 1988; Ros et al., 1988) or both (Stiles et al., 1981).

The expression of one or more of these lesions is dependent on tissue type, though generally it can be said

TABLE 3.1

DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS FROM WHOLE RAT FOREBRAIN

Homogenates were prepared and fractions isolated as described in the Materials and Methods section. The values are means ± S.E.M. for four preparations, except where it is indicated that (n=3).

Enzyme activity (nmol/min per mg of protein)

Fraction	Condition	Acetyl-cholinesterase	2',3',-Cyclic nucleotide phosphodiesterase	Lactate dehydrogenase	Succinate dehydrogenase
Synaptic membranes	Control	172 ± 8	2050 ± 128	1667 ± 49	4.3 ± 0.3
	Hypothyroid	148 ± 28	2250 ± 128	227 ± 15	4.5 ± 0.3
Myelin	Control	28 ± 2	14583 ± 850	463 ± 71	1.5 ± 0.1
	Hypothyroid	21 ± 2	10000 ± 517 (n = 3)	240 ± 36	0.7 ± 0.1 (n = 3)
105,000g supernatant	Control	32 ± 2	275 ± 20	7550 ± 567	1.2 ± 0.1
	Hypothyroid (n=3)	24 ± 3	427 ± 40	2567 ± 195	1.0 ± 0.1
Mitochondria	Control	50 ± 3	829 ± 62	983 ± 168	23 ± 2
	Hypothyroid	39 ± 5	727 ± 66	435 ± 23	21 ± 2

that hypothyroidism impairs the stimulatory β-adrenergic receptor mediated responses and potentiates the inhibitory regulation of adenylate cyclase (Stiles et al., 1984; Ohisalo & Stouffer, 1978; Malbon, 1984; Malbon et al., 1985; Saggerson 1986).

Three of five studies to date have shown the density of β-adrenergic receptors to be reduced in drug, or surgically-induced hypothyroidism in the brain (Fox et al., 1985; Gross et al., 1980; Smith et al., 1980). Surgically induced hypothyroidism was not associated with a significant alteration in β-adrenergic receptors in rat cortex (Joffe et al., 1988; Atterwill et al., 1984). However, other studies reported significant reductions in the β-adrenergic receptor density of hypothyroid rat brain (Gross et al., 1980; Smith et al., 1980; Fox et al., 1985). Differences in the strain of rat used, the use of adult as compared with neonatal rats and differences in time for pharmacological compared to surgical induction of hypothyroidism may account for the difference in findings.

In the present study "adult rats" were rendered hypothyroid i.e. rats at four weeks old were treated with PTU in their drinking water (0.01% w/v PTU) and fed a low iodine diet, then killed four weeks after commencing treatment. This is an important point since in the developing animal thyroid hormones have been shown to promote mitosis of neurones and later to inhibit mitosis by promoting differentiation (Walz & Howlett, 1987) and so studies utilising neonatal rats would encompass the

complications of brain development as well as any modulation of the adenylate cyclase system per se.

It is still not clear, however, quite how thyroid hormone affects the mature brain. Biochemical variables, such as oxygen consumption, α-glycerophosphate dehydrogenase and malic enzyme activities, entities known to be regulated by thyroid hormone in target organs are not affected by this hormone in mature rat brain (Schwartz & Oppenheimer, 1978; Shapiro & Percin, 1966; Hemon, 1968). These results do not necessarily prove the unresponsiveness of the brain to thyroid hormone. Instead, it is likely that the appropriate biochemical variables have not yet been defined. Several investigators have reported the existence of nuclear T_3 receptors both in neonatal and mature rat brain (Schwartz & Oppenheimer, 1978; Valcana & Timiras, 1978; Kolodny et al., 1985; Yokota et al., 1986). Yokota et al., (1986) were able to show the existence of a high level of nuclear T_3 receptors in neuronal nuclei, indistinguishable from nuclear T_3 receptors in the liver. This raises the possibility that thyroid hormone has a direct action via nuclear T_3 receptors in the cerebral cortex of mature rat brain. A result supported by the fact that β-adrenergic receptor density can be altered in the hypothyroid state.

The protocol adopted in determining the extent and affinity of radioligand binding to adenosine A_1 receptors in euthyroid and hypothyroid states involved the use of the radioligand [^{3}H] PIA. Adenosine itself has been

proven to be unsatisfactory since it is liable to uptake by high affinity transport mechanisms and also to extensive metabolism to adenine nucleotides and other purine degradation products. [^{3}H] PIA is resistant to metabolism by adenosine deaminase and so its inclusion in the assay procedure in the presence of adenosine deaminase prevents any contamination by endogenous adenosine and its consequent competitive binding to receptors.

Because much of the radioligand may be non-specifically bound to non-receptor proteins at each concentration of radioligand a duplicate assay was performed in which a large amount (approximately 100 fold) excess of unlabelled ligand was added, thereby presumably saturating the specific receptor. Any bound radioactivity in this instance is assumed to represent binding to non-specific or non-saturable proteins. Subtraction of this non-specific element from the total binding measured results in an estimate of the amount of radioligand bound to receptor.

The saturation binding hyperbolae may be analysed by the construction of a Scatchard plot i.e., the plot of the ratio of bound to free ligand versus bound ligand. From this plot can be determined the affinity of binding by fitting a straight line to the data using the relationship K = -1 x slope. Furthermore, the intercept with the x axis is an estimate of binding capacity, designated Bmax (or $Bmax_1$ or $Bmax_2$ of there are two classes of binding sites). However, in this case the saturation binding hyperbolae

obtained for hypothyroid and euthyroid rat brain synaptosomal membranes were analysed by a non-linear weighted least squares curve fitting procedure using a search type minimisation method (Colquhoun, 1971). Computerised analysis often results in improved estimates of the parameters and offers protection from the inaccuracies and subjectivity of graphical methods. This same method was used for all subsequent binding analyses.

It was investigated as to whether the data obtained fitted either a one or a two site model. In fact it was not possible to fit either the euthyroid or hypothyroid data to a two site model. Nevertheless, the one site model showed that [^{3}H] PIA bound to synaptosomal membranes from euthyroid rates with a Bmax value of 600 fmol/mg (see Fig. 3.1a). Values for [^{3}H] PIA binding of 810 fmol/mg and 740 fmol/mg have previously been reported by Schwabe and Trost (1980) and Lohse et al., (1984), respectively. All in all Bmax values of 426,230,120 and 416 fmol mg^{-1} protein have been reported for [^{3}H] 2-CADO, [^{3}H] CHA and [^{3}H] CPA binding to rat brain membranes, (Williams & Risely, 1980; Patel et al., 1982; Williams et al., 1986). The euthyroid Bmax value obtained in the present study is therefore comparable with Bmax values previously obtained. The K_D of 3.3nM was not dissimilar to the values of 5.1nM and 1.4nM previously reported for [^{3}H] PIA binding by Schwabe & Trost (1980) and Lohse et al., (1984) respectively. Fig 3.1b shows that fitting the data to a Scatchard plot yields a straight line ($r = - 0.96$,

3.1 Specific binding of [^{3}H] PIA to synaptosomal membranes from euthyroid and hypothyroid rats

[^{3}H] PIA binding was determined as described in the materials and methods section. The curves are computer estimates of best fit to a single-site binding model. Estimates of binding parameters were (means ± S.D.): euthyroid K_D = 3.3 ± 0.4nM, Bmax = 600 ± 33 fmol/mg of protein; hypothyroid, K_D = 3.8 ± 0.5nM, Bmax = 518 ± 28 fmol/mg of protein.

●, euthyroid (n = 3-5)

0, hypothyroid (n = 6-12)

Fig. 3.1 a

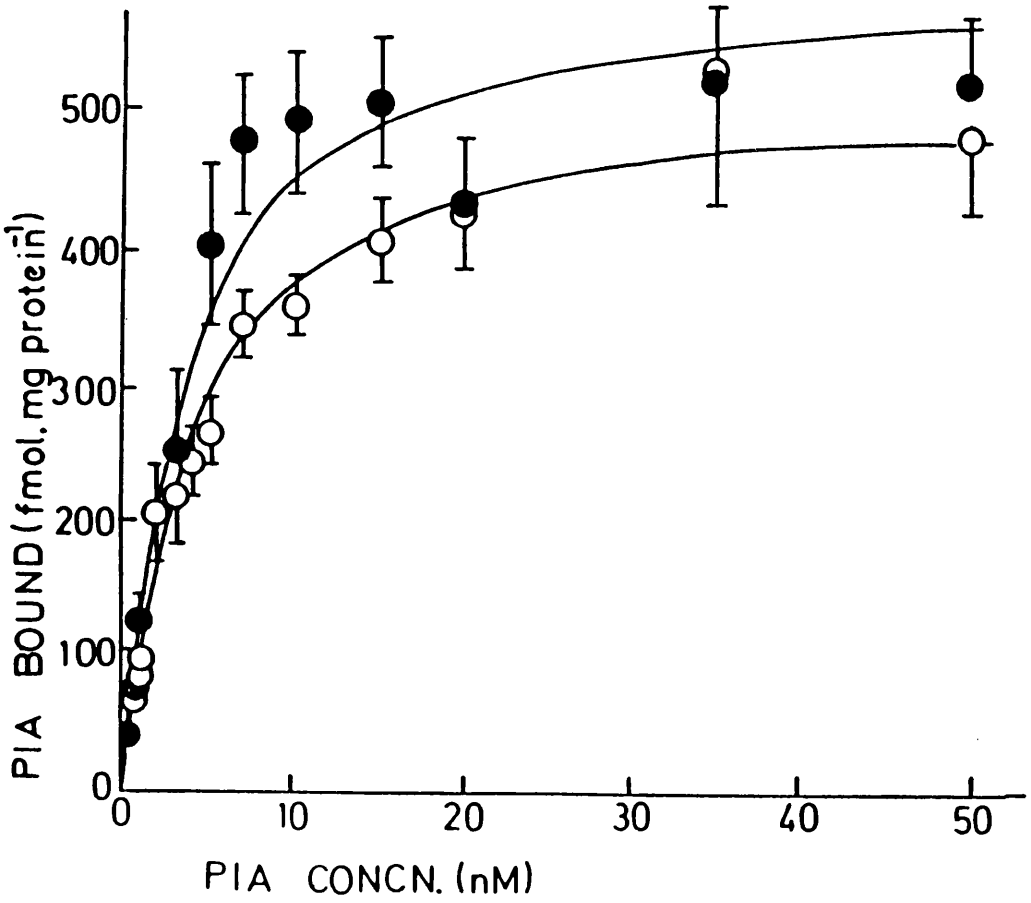
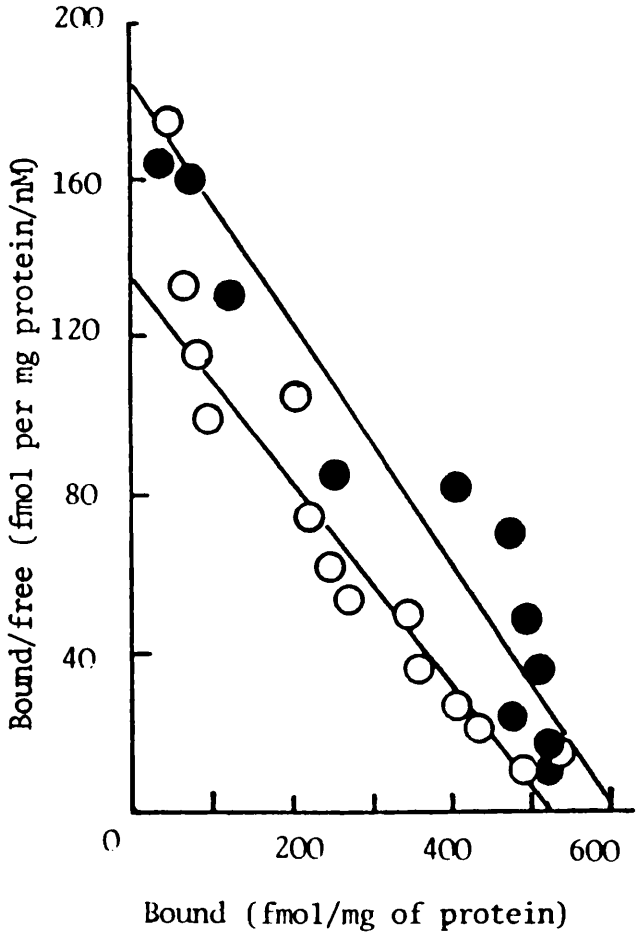


Fig. 3.1 b



$p<0.001$) which further substantiates the one site model derived by computer analysis.

Hypothyroidism slightly decreased the Bmax for [^{3}H] PIA binding by 14% ($p<0.05$) to 518 fmol/mg, although no appreciable change in the K_D was obtained (3.8nM). Again a linear Scatchard plot yielded a straight line ($r = -0.94$, $p<0.001$) in accordance with the one site model derived by computer analysis. A decrease in [^{3}H] PIA binding might suggest a decreased sensitivity to adenosine in the hypothyroid state in contrast to that found by Malbon et al., (1985) and Saggerson (1986) in adipose tissue. Nevertheless, in a study performed by Chohan et al., (1984), hypothyroidism decreased the total amount of specific binding of [^{3}H] PIA to fat cell membranes from 1230 to 1040 fmol/mg (a 15% decrease). Also Malbon et al., (1985) observed a small decrease in [^{3}H] CHA binding to fat cell membranes in hypothroidism. Therefore the fact that a decrease in receptor density is seen does not preclude the possibility that an increase in sensitivity to adenosine may be found in rat brain in the hypothyroid state.

3.3 THE EFFECT OF HYPOTHYROIDISM ON THE COMPETITION FOR [^{3}H] DPX BINDING TO RAT BRAIN SYNAPTOSOMAL MEMBRANES BY PIA

To further explore whether hypothyroidism was bringing about alterations in adenylate cyclase at the level of coupling between receptor and G protein as found in the β-adrenergic system in the heart (Stiles &

Lefkowitz, 1981), in reticulocytes (Stiles et al., 1981) and in adipose tissue (Malbon 1979; 1980) the effect of GTP was investigated in being able to modify the profile of displacement of the antagonist [^{3}H] DPX by unlabelled PIA.

It has been shown that whilst competition curves for antagonist versus radiolabelled antagonists are 'steep' with slope factors ('pseudo Hill coefficients') of 1 competition curves for agonists are shallow with slope factors of <1 (Kent et al., 1980; DeLean et al., 1980). The shallow agonist competition curves are compatible with at least two states of the receptor (in the absence of guanine nucleotides), these states being: a magnesium dependent slowly reversible high affinity state and a lower affinity state which predominates in the presence of guanine nucleotides at high concentrations.

By analogy with the results obtained by Lohse et al., 1984 and Goodman et al., (1982) competition for [^{3}H] DPX binding by PIA in the absence of GTP for both euthyroid and hypothyroid states yielded shallow curves (see Figs. 3.2 and 3.3). These curves were most appropriately described by a two state model $p<0.005$ and $p<0.01$ in the euthyroid and hypothyroid cases respectively for comparison of one and two state models, using the F test. A two state model was also evidenced by the low values of the slope factors which were 0.32 and 0.48 for hypothyroid and euthyroid states respectively. (See Fig. 3.4).

Table 3.2 shows that no significant changes were seen in either Bmax values or the high and low affinity

Fig 3.2 Competition for [^{3}H] DPX binding to euthyroid rat brain synaptosomal membranes by PIA

Euthyroid synaptosomal membranes were incubated with 10nM - [^{3}H] DPX and with 24-26 different concentrations of unlabelled PIA over the range 0.1nM - 100μM, as described in section 2.6.2. The values are means of three to six separate experiments and are displayed as the specific binding of [^{3}H] DPX to membranes ± S.E.M. of the total binding data. It should be noted that kinetic parameters were determined by computer analysis of total and not specific binding curves as described in sections 2.6.2 and 2.6.3.

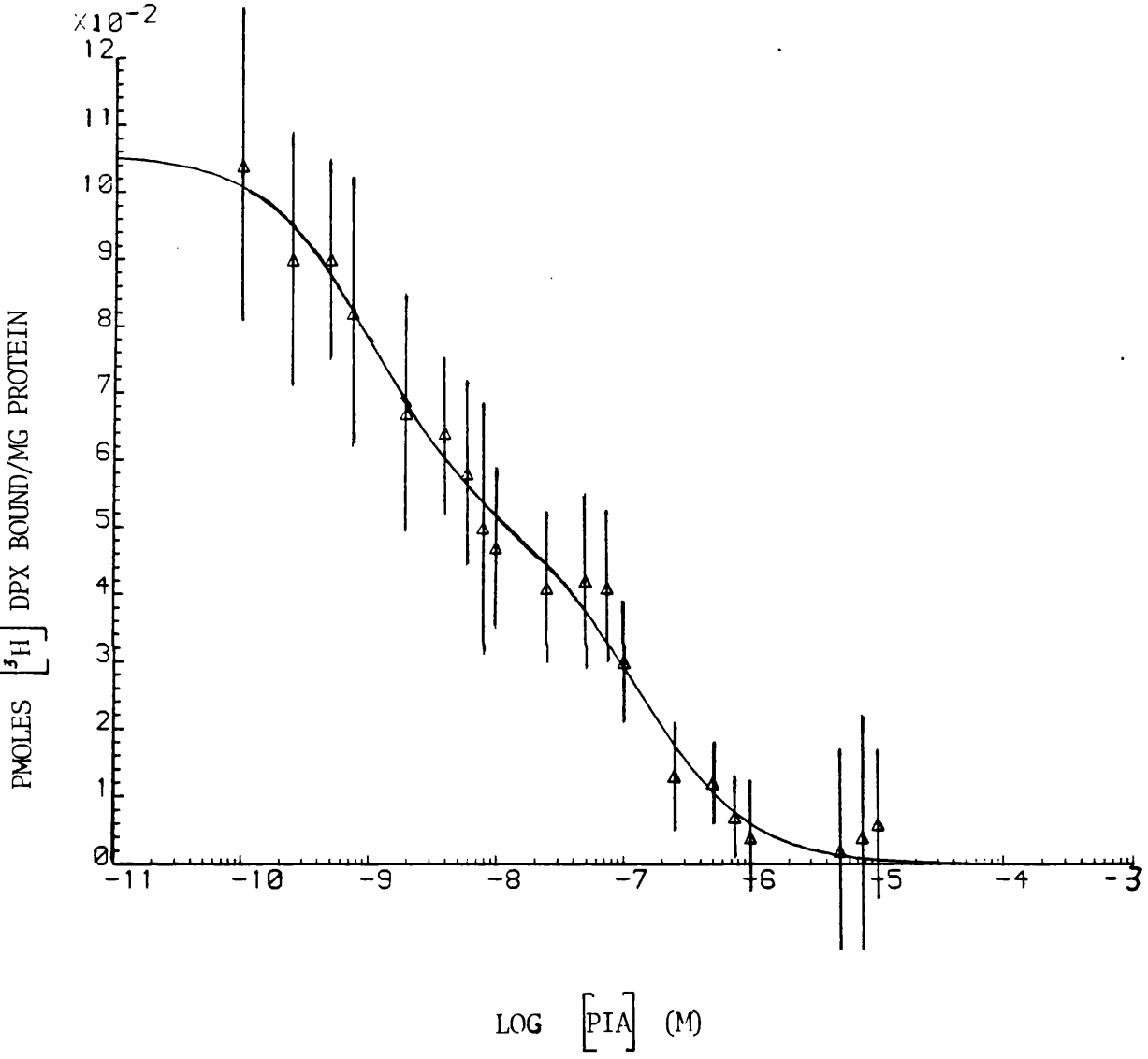

X10-2
12
11
10
9
8
7
6
5
4
3
2
1
0
PMOLES [3H] DPX BOUND/MG PROTEIN
-11
-10
-9
-8
-7
-6
-5
-4
-3
LOG [PIA] (M)

Fig 3.3 Competition for [^{3}H] DPX binding to hypothyroid rat brain synaptosomal membranes by PIA

Hypothyroid synaptosomal membranes were incubated with 10nM -[^{3}H] DPX and with 24-26 different concentrations of unlabelled PIA over the range 0.1nM - 100μM as described in section 2.6.2. The values are means of five to seven separate experiments and are displayed as the specific binding of [^{3}H] DPX to membranes ± S.E.M. of the total binding data. It should be noted that kinetic parameters were determined by computer analysis of total and not specific binding curves as described in sections 2.6.2 and 2.6.3.

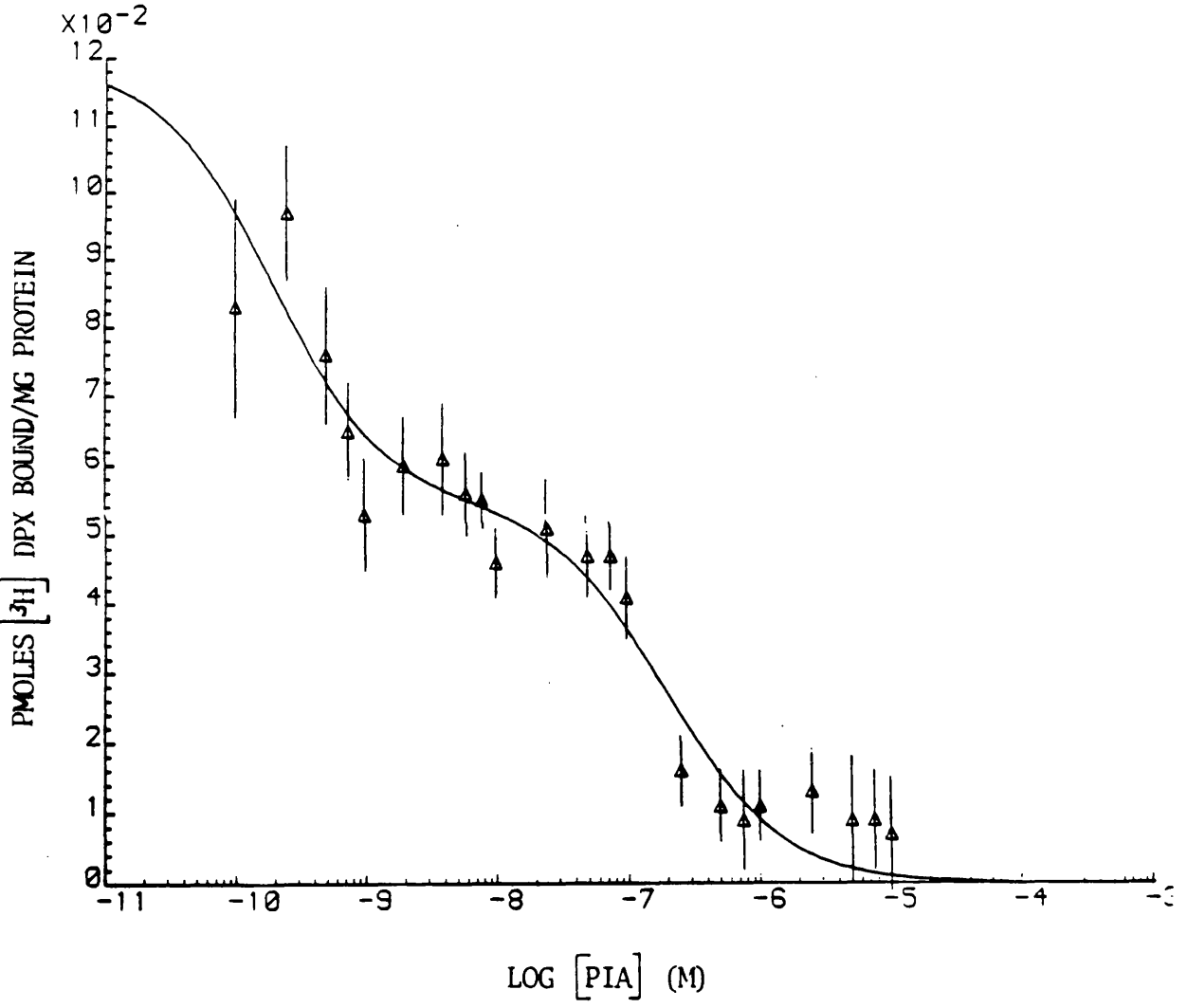

X10-2
12
11
10
9
8
7
6
5
4
3
2
1
0
PMOLES [3H] DPX BOUND/MG PROTEIN
-11
-10
-9
-8
-7
-6
-5
-4
LOG [PIA] (M)

Fig. 3.4 Competition for [^{3}H] DPX binding to rat synaptosomal membranes by PIA in the presence and absence of GTP

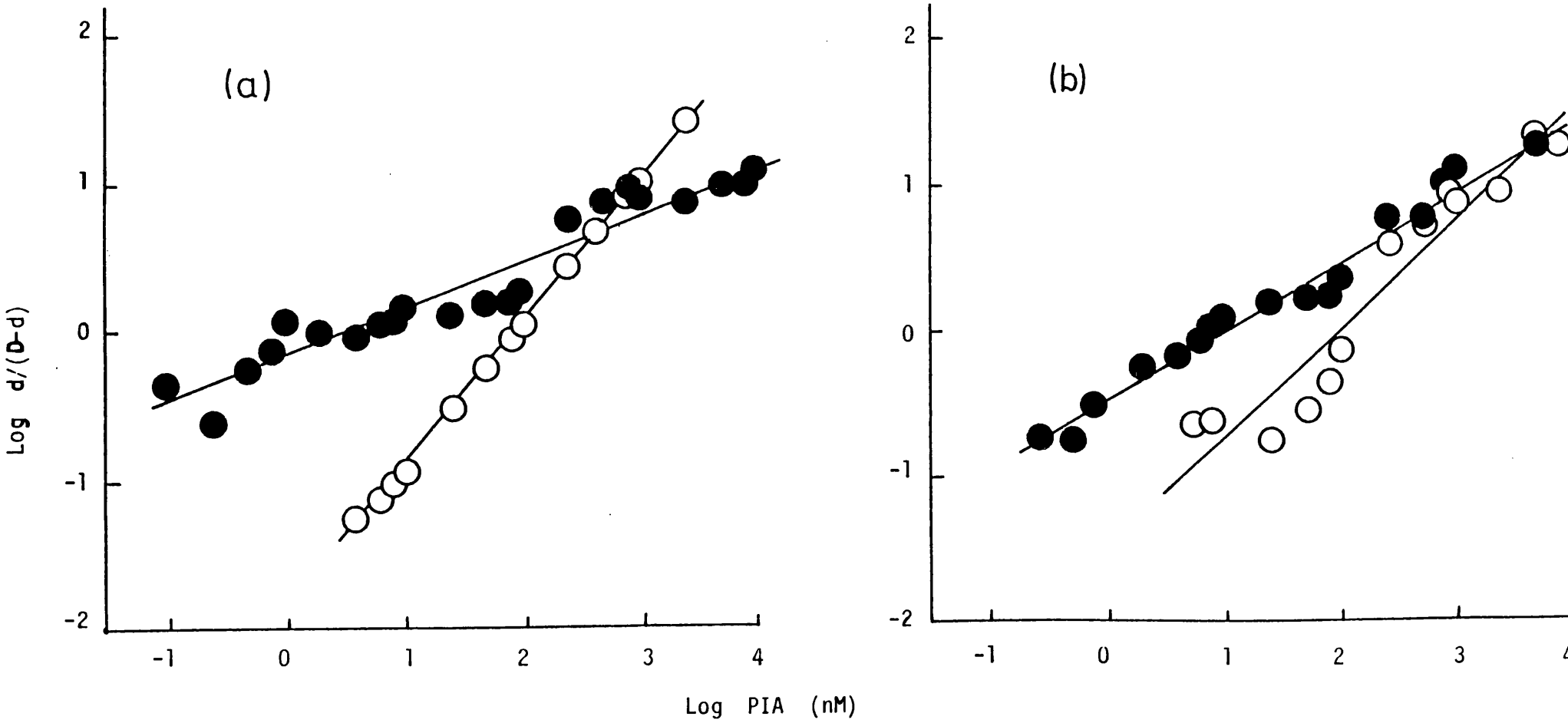
Synaptosomal membranes were incubated with 10nM - [^{3}H] DPX in the absence or presence of 100μM - GTP and with 24-26 different concentrations of unlabelled PIA over the range 0.1nM - 100μM, as described in the materials and methods section. The values are means of three to seven separate experiments and are presented in the form of Hill plots. Displacement at a given concentration of PIA is represented by d and the maximum displacement by D.

(a) Hypothyroid: ●, without GTP (Hill coefficient = 0.32, r = 0.95

O, with GTP (Hill coefficient = 0.98, r = 0.99).

(b) Euthyroid: ●, without GTP (Hill coefficient = 0.48, r = 0.98)

0, with GTP (Hill coefficient = 0.75, r = 0.95)


(a)
(b)
Log d/(D-d)
Log PIA (nM)
2
1
0
-1
-2
-1
0
1
2
3
4

dissociation constants. The proportion of high and low affinity states of the adenosine A_1 receptor for PIA were R_H 54% and R_L 46% for membranes from hypothyroid rats and R_H 56% and R_L 44% for membranes from euthyroid animals. This result is similar to that obtained by Goodman et al., (1982) who found adenosine receptors existing in a 50:50% mixture of their high and low affinity states in brain membranes labelled with [^{3}H] DPX, the competing agent being either CHA or L-PIA. However the results differ slightly from that obtained by Lohse et al., (1984) who demonstrated a 69:31% ratio of the A_1 receptor in states of high and low affinity respectively in rat brain membranes labelled again with [^{3}H] DPX and utilising L-PIA as the displacing agent.

Figs 3.5 and 3.6 show that addition of 100μM GTP steepened the curves and shifted them to the right.

Using the F test the data were fitted to a one site model. In fact since the F test did not reveal any significant differences between the one and two site models, the more complex two site model was therefore rejected. A monocomponent curve with reduced affinity is consistent with results obtained for β-adrenergic receptors (Kent et al., 1980; Lefkowitz et al., 1982) and for adenosine A_1 receptors (Lohse et al., 1984; Goodman et al., 1982).

The present results i.e. the approximate 56:44% and 54:46% mixture of high and low affinity in the euthyroid and hypothyroid states and the conversion to a monocomponent curve, of single low affinity, upon addition

Fig 3.5 Competition for [^{3}H] DPX binding to euthyroid rat brain synaptosomal membranes in the presence of 100μM GTP by PIA

Euthyroid synaptosomal membranes were incubated with 10nM - [^{3}H] DPX, 100μM GTP, and with 24-26 different concentrations of unlabelled PIA over the range 0.1nM - 100μM as described in section 2.6.2. The values are means ± S.E.M. of three to four separate experiments and are displayed as the specific binding of [^{3}H] DPX to membranes ± S.E.M. of the total binding data. It should be noted that kinetic parameters were determined by computer analysis of total and not specific binding curves as described in sections 2.6.2 and 2.6.3.

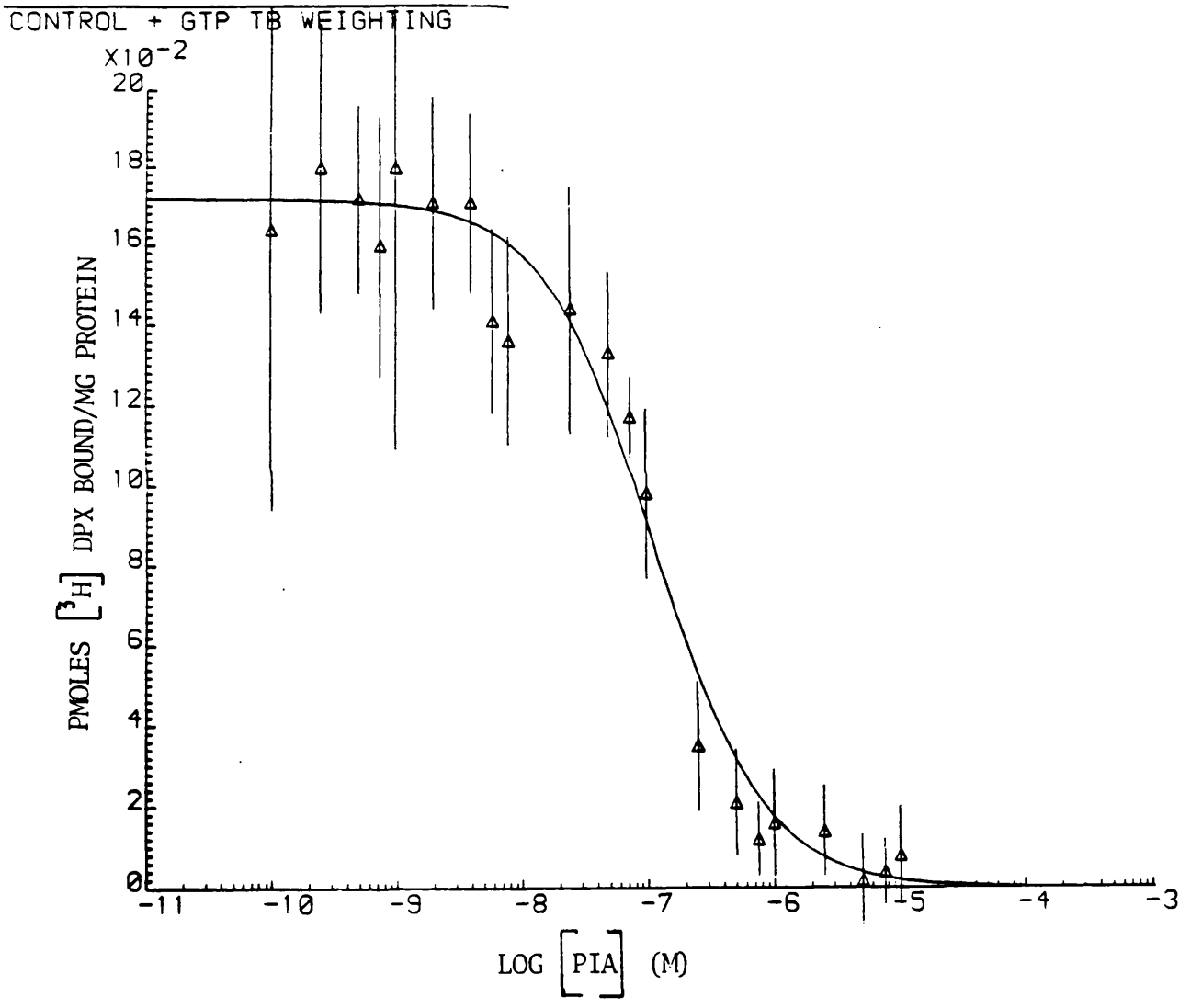
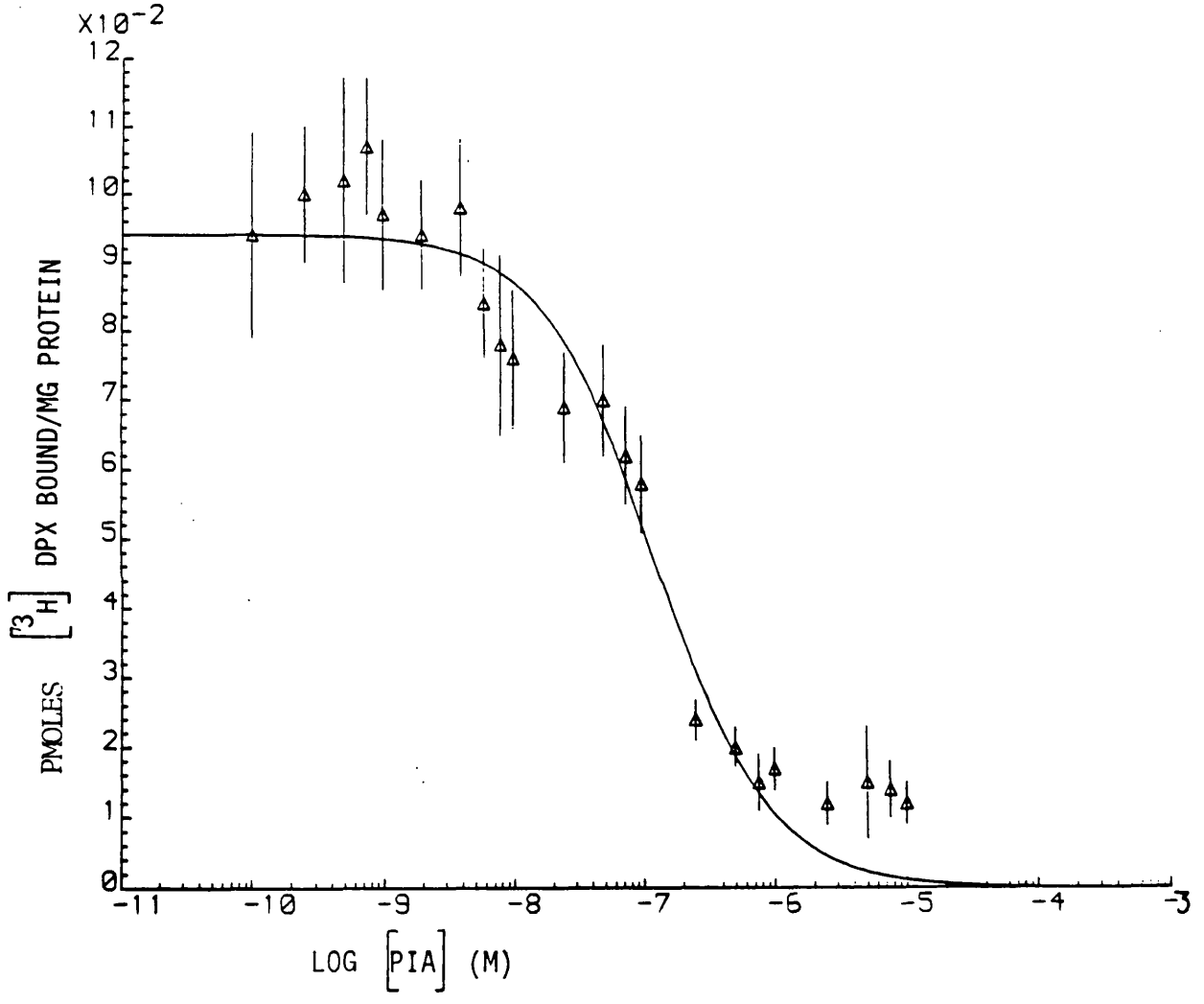

CONTROL + GTP TB WEIGHTING
X10-2
20
18
16
14
12
10
8
6
4
2
0
PMOLES [3H] DPX BOUND/MG PROTEIN
-11
-10
-9
-8
-7
-6
-5
-4
-3
LOG [PIA] (M)

Fig 3.6 Competition for [^{3}H] DPX binding to hypothyroid rat brain synaptosomal membrane in the presence of 100μM GTP by PIA

Hypothyroid synaptosomal membranes were incubated with 10nM -[^{3}H] DPX, 100μM GTP, and with 24-26 different concentrations of unlabelled PIA over the range 0.1nM - 100μM as described in section 2.6.2. The values are means of three to five separate experiments and are displayed as the specific binding of [^{3}H] DPX to membranes ± S.E.M. of the total binding data. It should be noted that kinetic parameters were determined by computer analysis of total and not specific binding curves as described in sections 2.6.2 and 2.6.3.


X10-2
12
11
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8
7
6
5
4
3
2
1
0
PMOLES [3H] DPX BOUND/MG PROTEIN
-11
-10
-9
-8
-7
-6
-5
-4
-3
LOG [PIA] (M)

Table 3.2

Binding parameters for competition of [^{3}H] DPX binding by PIA in the presence and absence of 100μM GTP in euthyroid and hypothyroid state.

Values are means ± S.D. and were obtained by computer analysis of [^{3}H] DPX binding data (Figs. 3.2, 3.3, 3.5, The values in parentheses indicate the number of independent measurements.

	Control (n = 3 - 6)	Hypothyroid (n = 5 - 7)	p value *
$Bmax_1$ [(a)] without GTP	59.1 ± 17.0	64.8 ± 31.6	N.S
$Bmax_2$ [(a)] without GTP	48.4 ± 14.3	55.2 ± 3.45	N.S
$Bmax_1 + Bmax_2$ [(a)]	108	120	N.D.
Bmax (total binding) [(a)] with GTP	252 ± 9.55	154 ± 3.50	$p<0.001$
Bmax with [(a)] GTP	176	90.4	N.D
K_L (nM) without GTP	242 ± 199	194 ± 50	N.S
K_H (nM) without GTP	1.80 ± 2.48	0.18 ± 0.15	N.S
K_L (nM) with GTP	1.22 ± 24.7	80.3 ± 11.7	N.S
$\frac{K_L (GTP)}{K}$	67.8	446	N.D

* = compared to control (a) = fmoles [^{3}H] DPX 1 mg protein (specifically bound)

of 100μM GTP, can be rationalised in terms of the ternary complex model (DeLean et al., 1980; Lefkowitz et al., 1982). According to this model the low affinity state of the receptor appears to represent the interaction of agonist with free receptor. Binding of agonist as determined in the frog erythrocyte β-adrenergic system to receptor induces conformational changes in the receptor leading to the interaction of HR with guanine nucleotide binding protein (in the GDP bound form). Dissociation of GDP from the complex forms the high affinity state HRG. Under normal physiological circumstances or in vitro as in the present case, where GTP is added, the nucleotide binds to the G proteins causing a decrease in affinity of R for G and also a decrease in affinity of H for R ie. it precipitates the formation of the low affinity form of receptor.

Table 3.2 shows that addition of 100μM GTP increased the Bmax for [^{3}H] DPX by 63% in the euthyroid state (from 108 to 176 fmoles mg^{-1} protein), though it lowered it in the hypothroid state by 25% (from 120 to 90 fmoles mg^{-1} protein), resulting in a 49% decrease in Bmax. Unfortunately, due to the way that errors are expressed by the CURVFIT program significance of the decrease expressed in the hypothyroid state could not be tested. However, a significant decrease is seen by comparing the difference in total binding in the presence of GTP. Hypothyroidism resulted in a 39% decrease in Bmax total ($p<0.001$) with no change in the low affinity binding constant.

Previous studies have also shown GTP to cause increases in Bmax for [^{3}H] DPX. Bruns et al., (1980) observed an increase of approximately 8% in rat cortical membranes. Yeung & Green (1983) also observed an increase, all be it in the presence of 4mM MgCl. Conversely, Goodman et al., (1982) and Lohse et al., (1984) found no change in Bmax upon addition of GTP. The overall decrease in Bmax ($p<0.001$) observed in the hypothyroid state compared to control is seen as a consequence of the opposing changes in Bmax effected by GTP in hypothyroid and euthyroid states. Quite why GTP should increase Bmax in euthyroid membranes and decrease it in hypothyroid membranes is presently unknown.

Analysing the data by determining 'pseudo' Hill coefficients showed that 100μM GTP changed the slope factor from 0.32 to 0.98 in the hypothyroid state. By contrast, in the euthyroid state, this concentration of GTP only changed the Hill coefficients from 0.48 to 0.75 (see Fig. 3.3). These qualitative findings imply that this chosen concentration of GTP is more effective at changing the affinity of agonist binding in membranes from hypothyroid animals. Another indicator of the efficiency of coupling of R with G protein is the ratio of the dissociation constants of an agonist for the high and low affinity states of the receptor "K_L/K_H" (Kent et al., 1980). Computer modelling of radioligand binding data in the frog erythrocyte β-adrenergic system showed a correlation of K_L/K_H with intrinsic activity of agonist. An increase in

this ratio would imply an increased ability of agonist to promote the formation and stabilise the high affinity complex HRG. This ratio yielded a value of 446 for the hypothroid state and 67.8 for control, an indication that hypothyroidism results in the more efficient inhibition of adenylate cyclase.

Despite the fact that hypothyroidism resulted in a 90% decrease in the high affinity constant this change was not a significant one, a result similar to that found in rat heart (Stiles et al., 1981) but differs from the reticulocyte β-adrenergic system where changes in affinity were found, hypothyroidism increasing K_H 2.4 fold and K_L 1.2 fold. There was also a concomitant decrease in the K_L/K_H ratio while no difference was seen in the percent R_H (Stiles et al., 1981). It is unclear at present why, in the present study, the addition of GTP should reveal a decrease in Bmax in the hypothyroid state, since no significant difference is seen in the absence of GTP.

The fat cell system seems to be unique in that hypothyroidism impairs the agonist specific shift in affinity of β-adrenergic receptors in the presence of Gpp(NH)p, suggesting altered interaction between R and Gs i.e. that the locus of the defect resides in R and not Gs (Malbon, 1980). The rat brain synaptosomal A_1 receptor system on the other hand, does display agonist specific shifts in affinity in the presence of GTP. Furthermore, qualitative evidence as shown by alterations in 'pseudo' Hill coefficients and K_L/K_H ratios would seem to indicate an

increased ability of agonist to inhibit adenylate cyclase, at the level of R-Gi, despite a decrease in A_1 adenosine receptor Bmax in the hypothyroid state.

3.4 THE EFFECT OF HYPOTHYROIDISM ON THE ACTIVATION OF ADENYLATE CYCLASE BY FORSKOLIN

In order to test the hypothesis that alterations in sensitivity to adenosine could be occurring at the level of Gi protein in the hypothyroid rat brain synaptosomal adenylate cyclase system, the effects of GTP and PIA were investigated. However rather than study these effects upon basal adenylate cyclase, as determined by Cooper et al., (1980) in rat brain cortical membranes, the forskolin-activated enzyme was chosen as the test system. Forskolin is able to stimulate adenylate cyclase in a reversible, rapid and GTP and receptor independent fashion, as shown in a number of membrane preparations and rat cortical slices (Seamon et al., 1981). The diterpene acts directly on the catalytic subunit of adenylate cyclase, since neither Gs or Gi are required for stimulation. However, there is an interdependence between Gs and forskolin resulting in a marked synergism in activation of the catalytic subunit (Daly, 1984).

Moreover forskolin stimulated cAMP synthesis can be inhibited by the action of inhibitory hormones as shown by Jakobs & Schultz (1979) in human platelet membranes and McMahon & Schimmel (1982) in hamster adipocyte membranes.

In order to ensure that adenosine, either endogenously generated or derived from added ATP did not obscure the effects of the inhibitory agents to be investigated a number of precautions were taken, namely: that dATP was utilised as substrate, this not only prevented the formation of adenosine but afforded greater sensitivity to the assay (Cooper et al., 1980). Adenosine deaminase was nevertheless added so as to metabolise endogenous adenosine, papaverine a phosphodesterase inhibitor was added to prevent degradation of dcAMP; creatine phosphate and creatine kinase were present in the assay so as to act as a regenerating system for the substrate dATP, which might otherwise be metabolised by nucleotidases in the membrane preparation.

Since forskolin was to be used as the stimulatory agent it was necessary to ascertain whether hypothyroidism altered the effect of forskolin on the enzyme. This was found not to be so, (see Fig. 3.7). Hill coefficients of less than 1 were observed in all cases, consistent with high affinity states (requiring Gs) and low affinity states (occurring in the absence of Gs) as previously reported (Green & Clark 1982; Seamon et al., 1984; Seamon & Daly 1985). Forskolin dose response curves were also performed in the presence of 100mM NaCl, as it was necessary to evaluate whether hypothroidism altered the interaction between Na^+ and the adenylate cyclase system. Inclusion of Na^+ in the assay was deemed necessary as an overwhelming amount of evidence in various membrane and cell systems has

Fig. 3.7 Effect of forskolin and Na^+ upon adenylate cyclase activity in synaptosomal membranes from euthyroid and hypothyroid rats.

Synaptosomal membranes were assayed for adenylate cyclase, at the indicated concentrations of forskolin, in the presence and absence of 100mM NaCl as described in the materials and methods section. The values are means ± S.E.M. and are expressed relative to those with 100μM forskolin in the absence of Na^+ (100% activity). These were 124±20 and 99±9 pmol/min per mg of protein (euthyroid and hypothyroid states respectively). Maximum activities in the presence of Na^+ were 78±19 and 83±8 pmol/min per mg of protein. Basal activities in the absence of Na^+ were 39±8 and 36±2 and in the presence of Na^+ were 27±3 and 20±4 pmol/min per mg of protein in the euthyroid and hypothyroid states respectively. Significant effects of Na^+ are indicated by : *P<0.01, **P<0.001 (unpaired differences)

(a) Euthyroid + , without Na^+ (Hill coefficient = 0.73, EC_{50} = 0.63μM) (n = 4)
Euthyroid with 100mM-Na^+ (Hill coefficient = 0.62, EC_{50} = 0.83μM) (n = 3)
(b) Hypothyroid: without Na^+ (Hill coefficient = 0.82, EC_{50} = 1.54μM) (n = 4)
Hypothyroid with 100mM Na^+ (Hill coefficient = 0.68, EC_{50} = 0.89μM) (n = 4). Where S.E.M. bars are not shown, these lie within the symbol.

Fig. 3.7 a

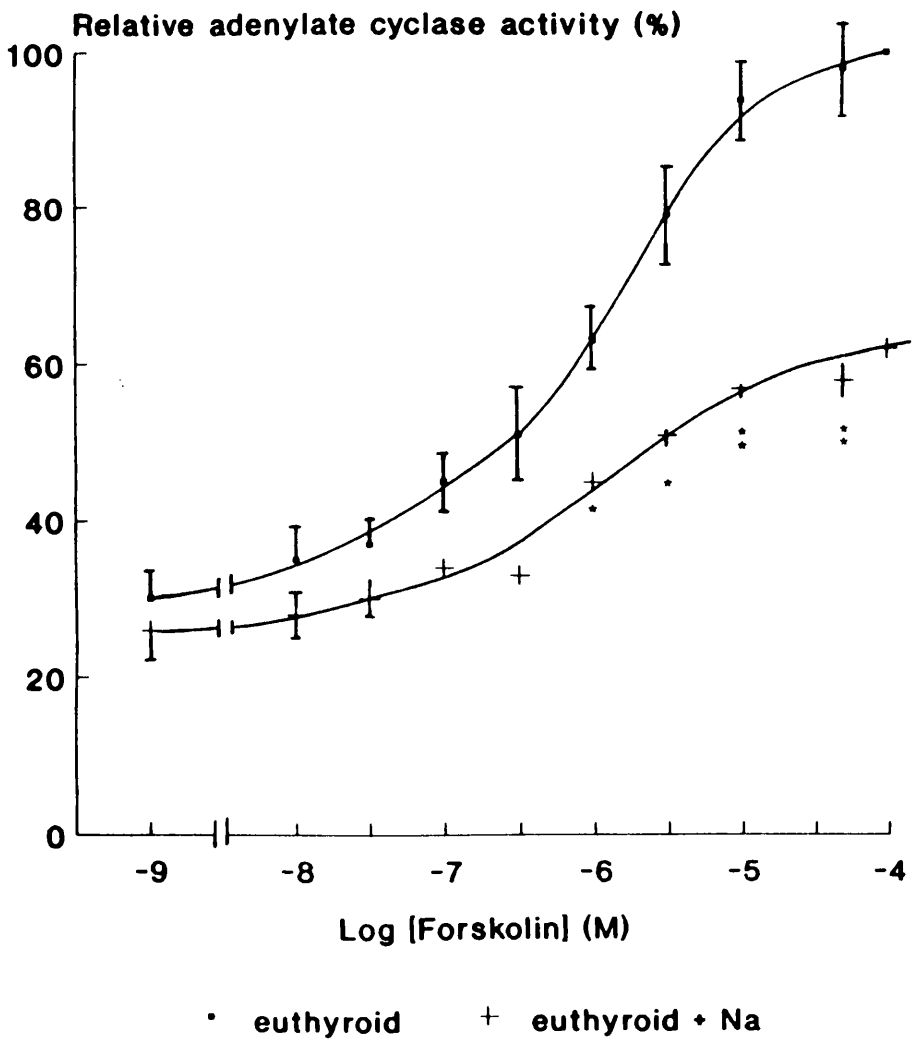
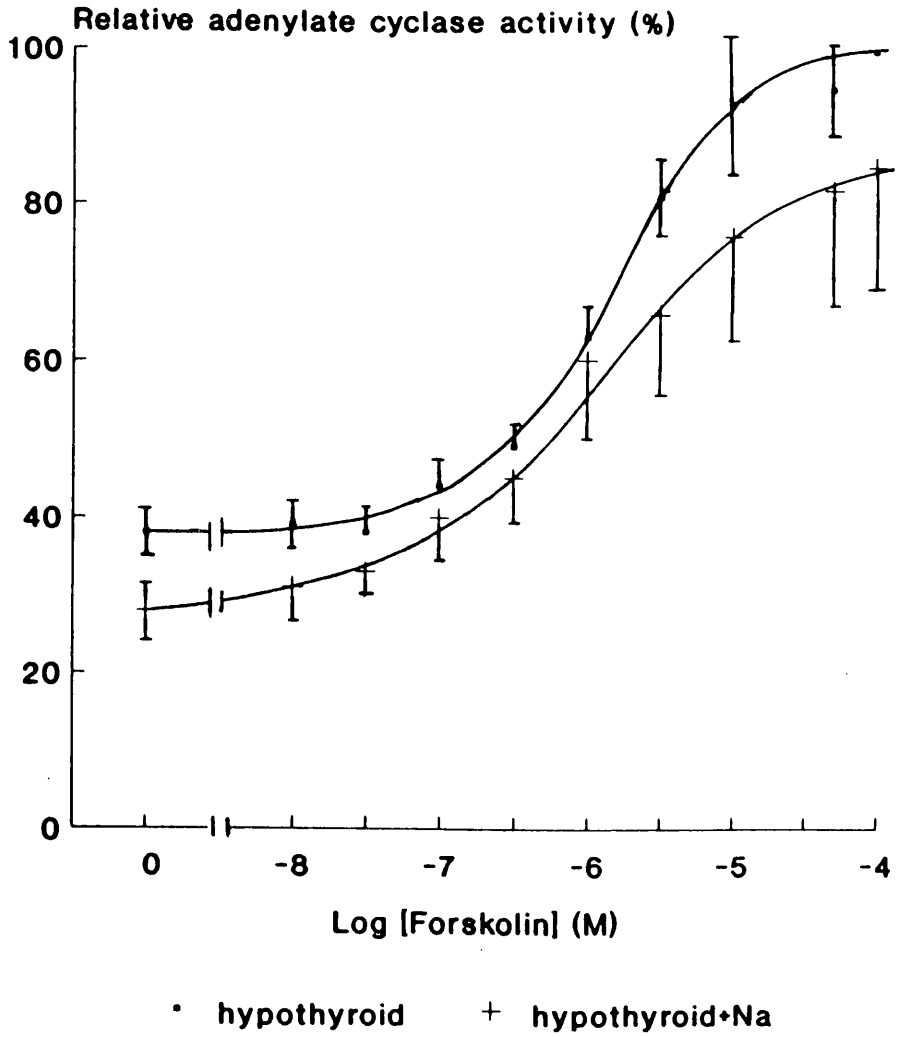


Fig. 3.7 b



shown that when investigating an inhibitory response on the adenylate cyclase system, Na^+ is required to abolish the inhibitory effects of GTP (Aktories et al., 1979; Aktories et al., 1980a,b; Lichshtein et al., 1979a.b; Cooper et al., 1980; Cooper et al., 1982). Fig. 3.7 shows that approximately 3-fold stimulations of adenylate cyclase were obtained in the presence and absence of Na^+ with 100μM forskolin in both euthyroid and hypothyroid membranes.

Comparing the dose response curves in terms of relative adenylate cyclase activity (relative to those with 100μM forskolin in the absence of Na^+), in the hypothyroid state Na^+ caused a slight decrease in adenylate cyclase activity both of basal and forskolin stimulated enzyme, though this decrease was not a significant one. In the euthyroid state, however, 100mM Na^+ caused a significant decrease in adenylate cyclase activity at forskolin concentrations of 1μM and greater. The overall effect of Na^+, therefore, is to reduce the potency of forskolin to stimulate adenylate cyclase. It did not however affect the relative stimulation of the enzyme as in all cases an approximate 3-fold stimulation was achieved. Overall, however, hypothyroidism did not significantly alter adenylate cyclase activity at any forskolin concentration in the presence and absence of Na^+, nor were there any appreciable difference in the Hill coefficients or calculated EC_{50} values.

It is noteworthy that neither basal nor forskolin stimulated activities of adenylate cyclase were altered by

hypothyroidism. This would imply that hypothyroidism does not result in an altered expression of the catalytic unit of adenylate cyclase. Interestingly, Walz et al., (1987) looking at thyroid effects on adenylate cyclase activity in cultured neuroblastoma cells (a model for developing cell neurons) demonstrated an increased forskolin stimulated adenylate cyclase activity in cells supplemented with T_3. Though direct comparisons between the present study and that of Walz et al., (1987) can not be made as the latter study concerned itself with a "developing cell model" it further suggests that if the expression of catalytic unit is altered, this alteration can be demonstrated by means of an adenylate cyclase assay.

Of interest in this respect is that diabetes decreases the basal activity of adenylate cyclase, possibly as a result of altered expression of the catalytic subunit in rat liver cell membranes (Gawler et al., 1987).

Although it is generally accepted that Na^+ has a stimulatory rather than an inhibitory effect on adenylate cyclase the data presently available are still somewhat contradictory. Two and three-fold stimulations of hamster fat cell membrane adenylate cyclase by 100mM NaCl, have been reported by Aktories et al., (1981) and McMahon & Schimmel (1982). A stimulatory effect was also found in rat brain striatal membranes, rat brain cortical membranes and mouse parotid and submandibular gland membranes, though to a lesser extent (Cooper et al., 1980; Cooper et al., 1982; Watson et al., 1989). No effect on basal adenylate

cyclase activity by Na^+ was found in mouse brain membranes (Watson et al., 1989).

In NG 108-15 neuroblastoma x glioma membranes Na^+ at 100mM or greater actually inhibited basal adenylate cyclase by 24 and 44% respectively (Blume et al., 1979; Koski et al., 1982). 100Mm NaCl also inhibited basal adenylate cyclase in hamster adipocyte membranes (Aktories et al., 1980a).

Data on the effect of sodium on stimulated adenylate cyclase is more coherent. Studies on hamster fat cell membranes, NG-108 cells, mouse parotid and submandibular gland membranes showed Na^+ to produce a significant increase in adenylate cyclase activity up and above that produced by the stimulatory agent, be it hormone or forskolin (McMahon & Schimmel 1982; Blume et al., 1979; Watson et al., 1989). In mouse brain membranes, Na^+ had no effect on forskolin stimulated adenylate cyclase activity (Watson et al., 1989).

All further studies of the cyclase either in the presence or absence of 100mM NaCl were performed using 10μM forskolin, since this concentration was found to stimulate adenylate cyclase submaximally in both groups of membranes.

3.5 EFFECTS OF GTP AND SODIUM IONS UPON FORSKOLIN STIMULATED ADENYLATE CYCLASE ACTIVITY IN EUTHYROID AND HYPOTHYROID STATES

The GTP dependent inhibition of forskolin stimulated adenylate cyclase was next investigated. In the fat cell system GTP is stimulatory at approximately 0.05μM whereas

the maximum inhibitory response occurs at 0.1 - 1μM (Jakobs et al., 1984a; Limbird 1981; Cooper 1982). This bimodal pattern of stimulation and inhibition reflects the differing requirements of Gs and Gi for activation by GTP and since, in general terms, adenylate cyclase activity is governed by the relative extent of dissociation of the two guanine nucleotide binding proteins a low concentration results in stimulation and a high concentration of GTP in inhibition of adenylate cyclase.

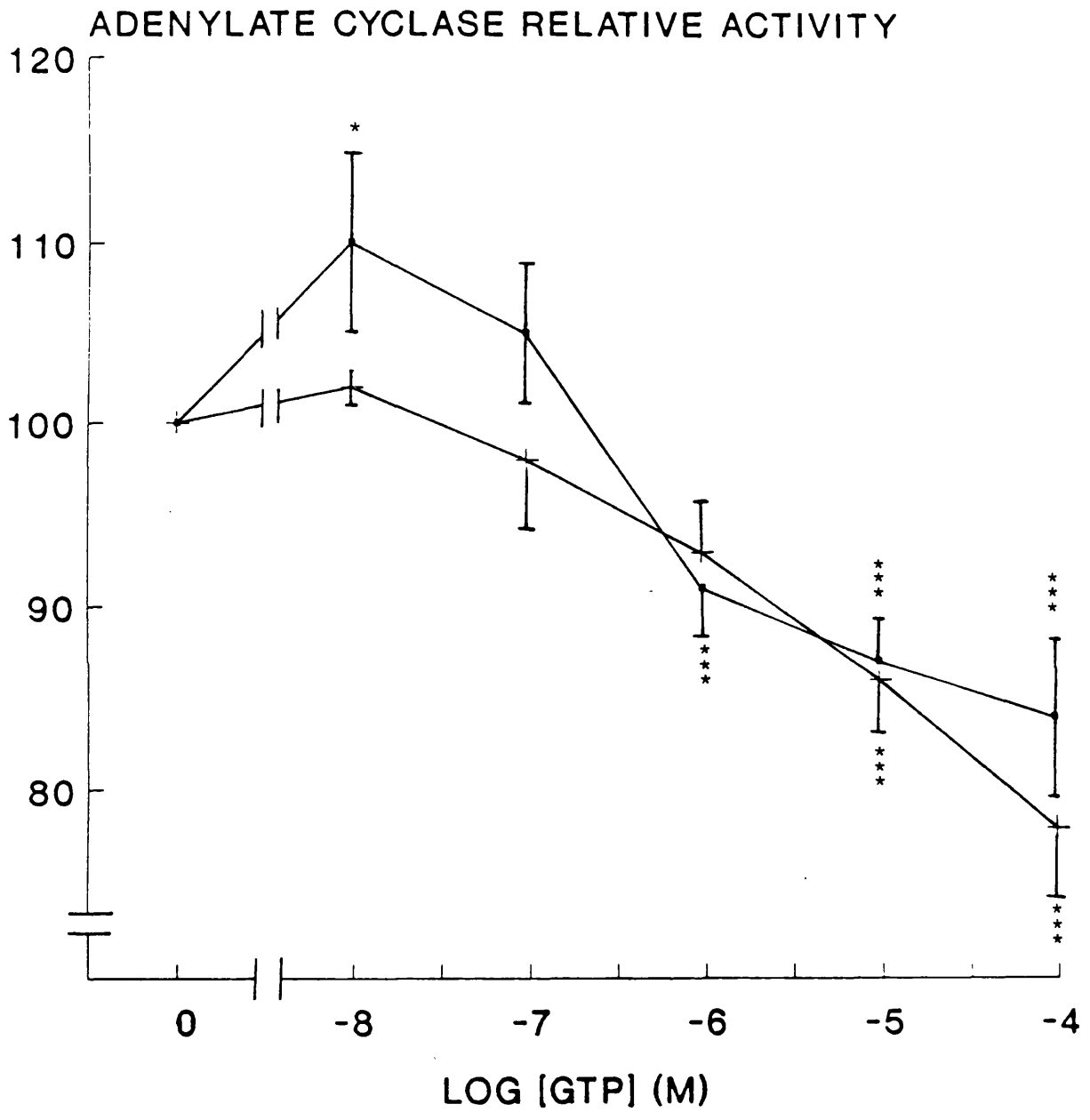
Looking at Fig. 3.8 one can see that the bimodal pattern was more pronounced in the control compared to hypothyroid case. At 0.01μM, GTP in the absence of Na^+ caused a significant stimulation of adenylate cyclase in the euthyroid, but not in the hypothyroid state. The nucleotide also significantly inhibited the enzyme at concentrations of 1, 10 and 100μM GTP in the euthyroid state ($p<0.005$), whereas a ten fold greater concentration of GTP was required to achieve a significant inhibitory response relative to the value in the absence of GTP (10μM, $p<0.005$, 100μM, $p<0.005$). These results are comparable to that achieved by Cooper et al., (1982) where a 10μM concentration of GTP effected a 10% inhibition of adenylate cyclase in rat striatal membranes and this same concentration of GTP, in the present study, effected a 13 and 19% decrease in adenylate cyclase in euthyroid and hypothyroid states respectively.

However, the present study, unlike a study performed in fat cell membranes on the inhibition of

Fig. 3.8 Effects of GTP upon forskolin-activated adenylate cyclase in the absence of Na^+.

Synaptosomal membranes were incubated with 10μM forskolin and the indicated concentrations of GTP are described in the materials and methods section. The values are means ± S.E.M. and are expressed relative to those in the absence of GTP (100%). Significant effects of GTP are indicated by: *P<0.05, ***P<0.005 (paired differences).

■ Euthyroid, (n = 8) basal adenylate cyclase activity was 110 ± 9 pmol/min per mg of protein.
+ hypothyroid (n = 5) basal adenylate cyclase activity was 124 ± 20 pmol/min per mg of protein.


ADENYLATE CYCLASE RELATIVE ACTIVITY
120
110
100
90
80
0
-8
-7
-6
-5
-4
LOG [GTP] (M)

forskolin-stimulated adenylate cyclase activity by GTP, (Malbon et al., 1985) did not show an increased sensitivity to inhibition of adenylate cyclase by GTP in the hypothyroid state. In the aforementioned study 1μM GTP effected a 25% inhibition in the euthyroid state and a 70% inhibition in the hypothyroid state. In the present study the same concentration of nucleotide resulted in a 9 and 10% inhibition in the euthyroid and hypothyroid states respectively, this compares well with that achieved by Cooper et al., (1982) who demonstrated an approximate 6% decrease in striatal membrane adenylate cyclase by 1μM GTP. However, Cooper et al., (1980) showed this concentration to have a stimulatory effect (by approximately 7%) on rat cortical membrane enzyme. It can be concluded therefore, that the brain membrane enzyme is less sensitive to inhibition by GTP than the fat cell enzyme. Moreover since brain membranes used in the present study are heterogeneous in composition the overall result obtained is a combination of individual responses to GTP, as demonstrated by the differences between cortical and striatal membranes, whereas fat cell membranes, being homogenous, will give one representative response.

The increased responsiveness in fat cells correlated with an increase in the Gi protein as determined by polyacrylamide gel analysis of radioactive products, resulting from pertussis toxin catalysed ADP-ribosylation of membranes from euthyroid and hypothyroid rat fall cells (Malbon et al., 1985). In fact the amount of label

incorporated into fat cell membrane Mr = 40,000/41,000 peptides by pertussis toxin was 115% greater in membranes from hypothyroid as compared to euthyroid rats (32 and 13 fmol/mg protein respectively). Consequently a 115% change in label (reflecting the change in amount of G protein) is associated with a 45% difference in inhibition of adenylate cyclase between the two states; overall therefore a 3% difference in the quantity of G protein is associated with a 1% change in inhibition of enzyme by 1μM GTP.

These results were substantiated by Milligan et al., (1987) and Ros et al., (1988) who demonstrated a 110 ± 15% and 50% increase in αG_{41} respectively, by utilising antipeptide antisera to the α subunit of Gi.

Since alterations in the displacement data, as revealed by changes in Hill plots and the K_L/K_H ratio in the hypothyroid compared to the euthyroid state, implied an alteration at a post-receptor locus, namely Gi, then the fact that hypothyroidism did not alter the sensitivity of adenylate cyclase to inhibition by GTP may seem a little surprising. However, unlike adipose tissue synaptosomal membrane preparations are derived from a heterogenous mixture of cells, so if a cell-specific alteration in Gi is effected by hypothyroidism, especially in e.g. hippocampal or striatal membranes which are derived from a relatively small region of the brain, then the overall increase in Gi may be too small to be detected by an adenylate cyclase type assay. Especially when one considers that assays utilising the fat cell membrane only reveal a 1% difference

in inhibition between test and control states for every 3% increase of Gi protein at 1μM GTP.

In Fig 3.9 values expressed relative to those in the absence of GTP and Na^+ clearly show that addition of 100mM NaCl to the assay resulted in a significant inhibition of adenylate cyclase at 0, 0.01, 0.1 and 10μM GTP in the euthyroid state. A 32% inhibition was seen in the absence of GTP which compares well with the 39% inhibition seen at 10μM forskolin in the previous experiment. Consequently it can be concluded that GTP does not alter the degree of inhibition effected by sodium in forskolin stimulated adenylate cyclase.

Although addition of sodium resulted in a decrease in adenylate cyclase activity at all concentrations of GTP in the hypothyroid state this difference was not a significant one (see Fig. 3.10).

The fact that 100mM NaCl inhibits adenylate cyclase at GTP concentrations up to 10μM compares well with the result obtained by Koski et al., (1982) for the most part. However, a stimulation in adenylate cyclase activity is seen at all concentrations of GTP as found in rat cortical and striatal membranes and hamster adipocyte membranes (Cooper et al., 1980; Cooper et al., 1982; Aktories et al., 1979).

Looking at the data, where values are expressed relative to those in the absence of GTP (Fig. 3.11 and 3.12), it can be seen that in the euthyroid state 100mM NaCl largely eliminated the inhibitory effect of GTP. This

Fig. 3.9 Effects of GTP upon forskolin stimulated synaptosomal membrane adenylate cyclase from euthyroid rats in the presence and absence of Na^+.

Synaptosomal membranes from euthyroid rats were incubated with 10μM in the presence and absence of 100mM NaCl and the indicated concentrations of GTP, as described in the materials and methods section. The values are means ± S.E.M. and are expressed relative to those in the absence of GTP and Na^+ (100%). Significant effects of Na^+ at the indicated concentration of GTP are indicated by: (a) $P<0.0005$, (b) $P<0.005$, (c) $P<0.025$, (d) $p<0.01$ (paired differences). Significant effects of GTP are indicated by: *$p<0.05$, ***$P<0.005$ (paired differences).

■ euthyroid, (n = 8) without 100 mM NaCl; basal cyclase activity was 110 ± 9 pmol/min per mg of protein.
* euthyroid, (n = 8) with 100mM Na^+; basal cyclase activity was 75 ± 8 pmol/min per mg of protein.
(Note: the data for the effects of GTP upon euthyroid adenylate cyclase in the absence of Na^+ are the same as for Fig. 3.8).

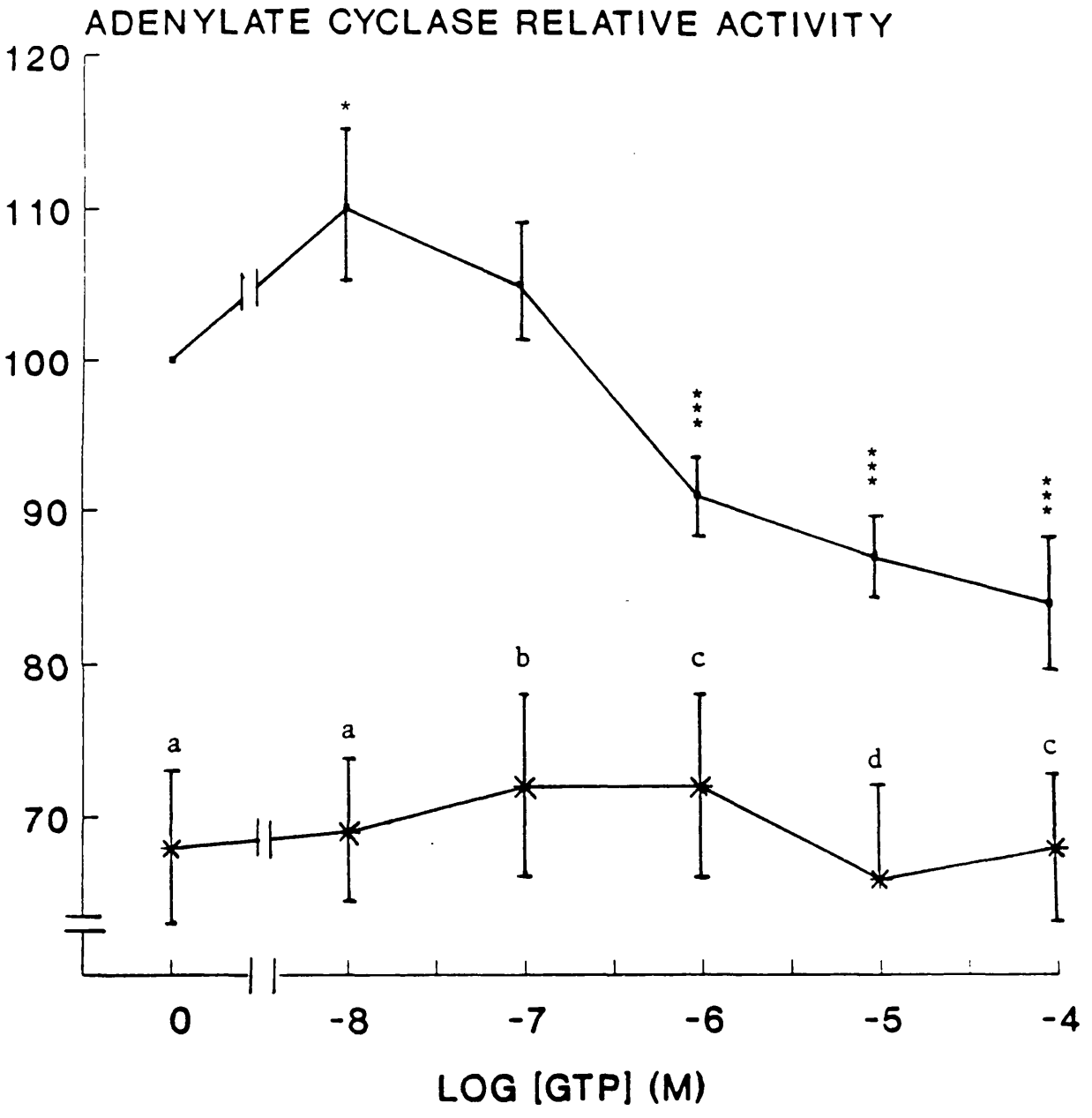

ADENYLATE CYCLASE RELATIVE ACTIVITY
120
110
100
90
80
70
*
***
***
***
a
a
b
c
d
c
0
-8
-7
-6
-5
-4
LOG [GTP] (M)

Fig. 3.10 Effects of GTP upon forskolin activated synaptosomal membrane adenulate cyclase from hypothyroid rats in the presence and absence of Na^+.

Synaptosomal membrances from hypothyroid rats were incubated with 10μM forskolin in the presence and absence of 100mM NaCl and the indicated concentrations of GTP, as described in the materials and methods section. The values are means ± S.E.M. and are expressed relative to those in the absence of GTP and Na^+ (100%). Significant effects of GTP are indicated by: *P<0.05, **P<0.025, ***P<0.005 (paired differences).

■ hypothyroid without Na^+ (n = 5), basal adenylate cyclase activity was 124 ± 20 pmol/min per mg of protein.
* hypothyroid with Na^+ (n = 4), basal adenylate cyclase activity was 83 ± 9 pmol/min per mg of protein.
(Note: the data for the effects of GTP upon hypothyroid adenylate cyclase in the absence of Na^+ are the same as for Fig. 3.8).

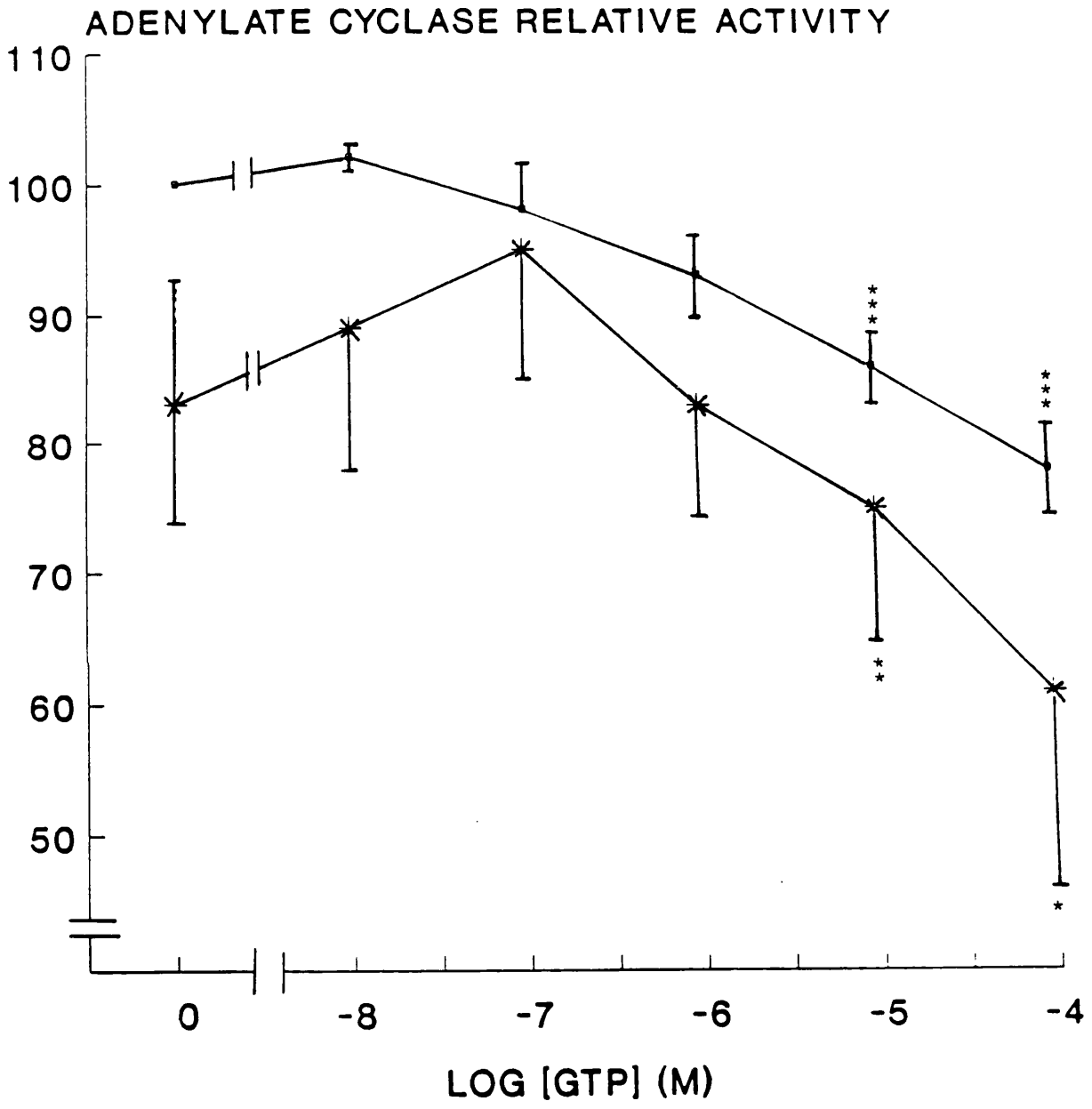

ADENYLATE CYCLASE RELATIVE ACTIVITY
110
100
90
80
70
60
50
0
-8
-7
-6
-5
-4
LOG [GTP] (M)

Fig. 3.11 Effects of GTP and Na^+ upon forskolin activated synaptosomal membrane adenylate cyclase from euthyroid rats.

Synaptosomal membranes from euthyroid rats were incubated with 10μM forskolin in the presence and absence of 100mM Na^+ and the indicated concentrations of GTP, as described in the materials and methods section. The values are means ± S.E.M. and are expressed relative to those in the absence of GTP (100%). Significant effects of GTP are indicated by: *P<0.05, ***P<0.005 (paired differences).

■ euthyroid with Na^+ (n = 8)

* euthyroid without Na^+ (n = 8)

(Note: the data presented in this diagram are the same as for Fig. 3.8)

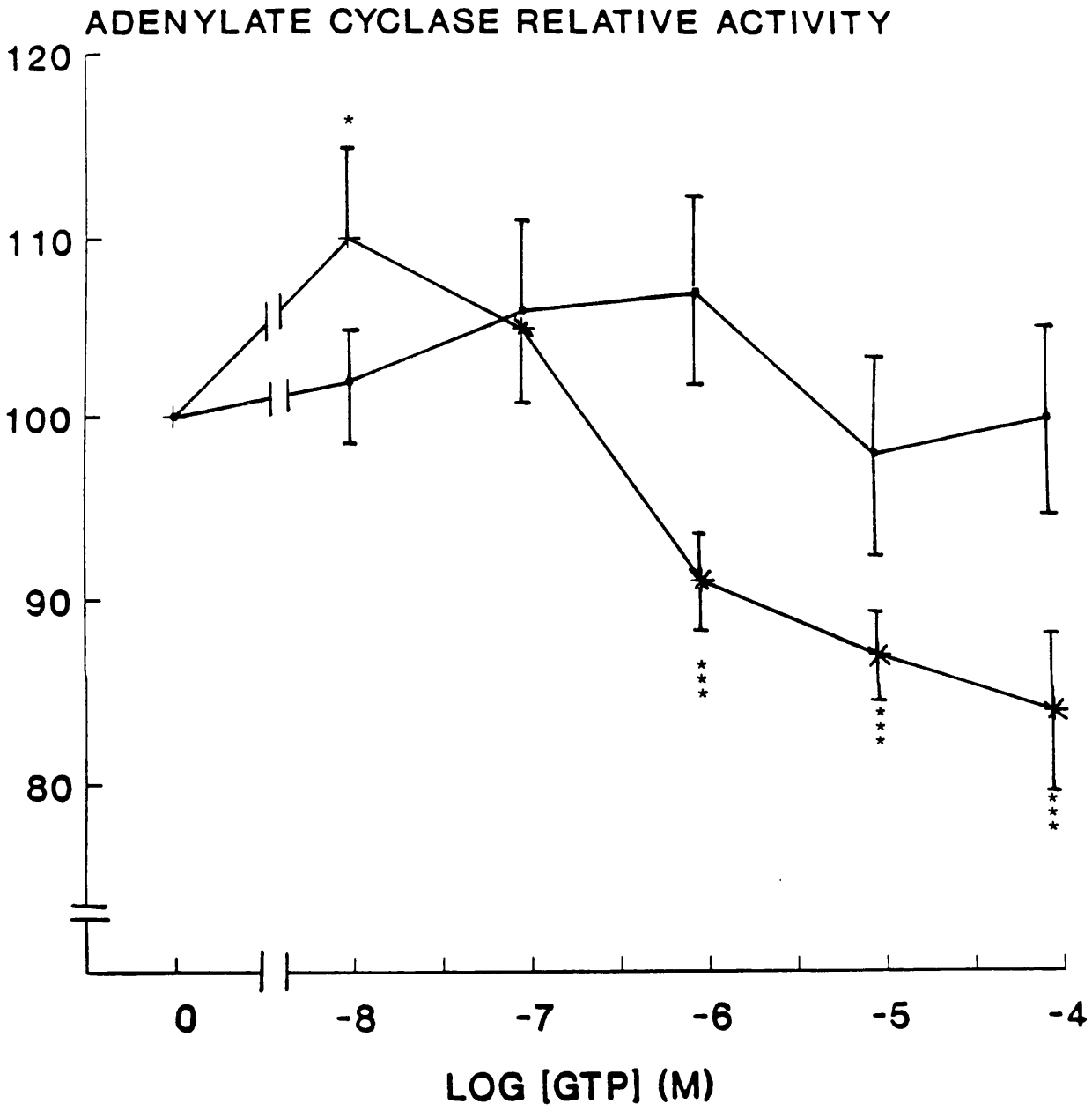

ADENYLATE CYCLASE RELATIVE ACTIVITY
120
110
100
90
80
0
-8
-7
-6
-5
-4
LOG [GTP] (M)

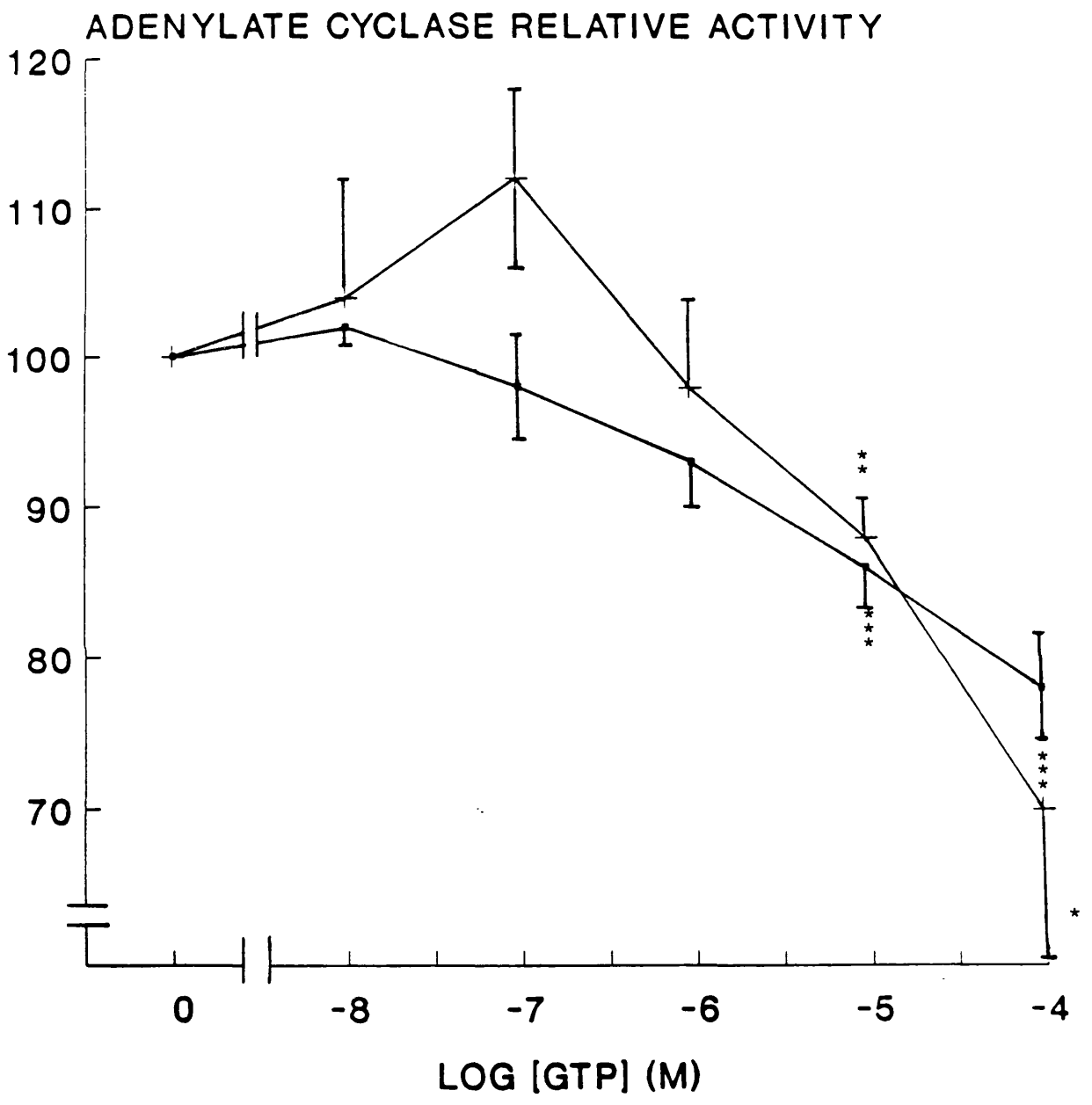
Fig. 3.12 Effects of GTP upon forskolin activated synaptosomal membrane adenylate cyclase from hypothyroid rats in the presence and absence of Na^+.

Synaptosomal membranes from hypothyroid rats were incubated with 10μM forskolin in the presence and absence of 100mM NaCl and the indicated concentrations of GTP, as described in the materials and methods section. The values are means ± S.E.M. and are expressed relative to those in the absence of GTP (100%). Significant effects of GTP are indicated by: *P<0.05, **P<0.025, **P<0.005 (paired differences).

■, hypothyroid without Na^+ (n = 5)

+, hypothyroid with Na^+ (n = 4).

(Note: the data presented in this diagram are the same as for Fig. 3.10)


ADENYLATE CYCLASE RELATIVE ACTIVITY
120
110
100
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80
70
0
-8
-7
-6
-5
-4
LOG [GTP] (M)

is not the case however in the hypothyroid state where GTP significantly inhibited the enzyme in the presence of Na^+ at 10 and 100μM ($p<0.025$, $p<0.05$).

Sodium ions are thought to act by decreasing basal and hormone-stimulated high affinity GTPases, both at Gs and Gi (Koski et al., 1982; Aktories et al., 1981) and also, as measured with the stable GTP analogue, GTP S, Na^+ ions inhibit the activation process of both Gs and Gi (Jakobs et al., 1984).

Na^+ by reducing the activity of Gi, reduces its ability to inhibit adenylate cyclase, even in the presence of high concentrations of GTP. The exact locus at which Na^+ acts within the adenylate cyclase system is presently unclear. It has been suggested that since sodium ions exert very similar effects at both Gs and Gi i.e., inhibiting the formation of the active, GTP-bound species of these guanine nucleotide proteins, then the site of action may be the β-proteins which are common to both proteins (Jakobs et al., 1984; 1985).

Taking the above into consideration, it seems possible that hypothyroidism induces a lesion at Gi such that Na^+ is not able to overcome the inhibitory action of GTP at concentrations $\geq 1\mu M$, the site of such a lesion may be at the β-subunit.

Since the concentration of GTP required to evaluate the agonist induced inhibition of adenylate cyclase is inhibitory in its own right, Na^+ is required in an assay to partially or totally counteract the GTP effect,

so that the degree of inhibition by agonist can be ascertained (Blume et al., 1979, 1980; Lichtshtein et al., 1979a,b; Jakobs et al., 1981; Londos et al., 1981). Furthermore, in the present study Na^+ attenuated the inhibitory effect of GTP in the euthyroid state and at 100μM GTP, 10μM forskolin and 100mM NaCl there was no significant difference in adenylate cyclase activity in control compared to test case. Therefore the effect of PIA on adenylate cyclase activity in synaptosomal membranes from euthyroid and hypothyroid rats was assayed in the presence of 10μM forskolin, a submaximal inhibitory concentration of GTP, which in this case was 10μM and 100mM NaCl.

3.6 EFFECTS OF PIA ON FORSKOLIN STIMULATED ADENYLATE CYCLASE ACTIVITY IN EUTHYROID AND HYPOTHYROID STATES

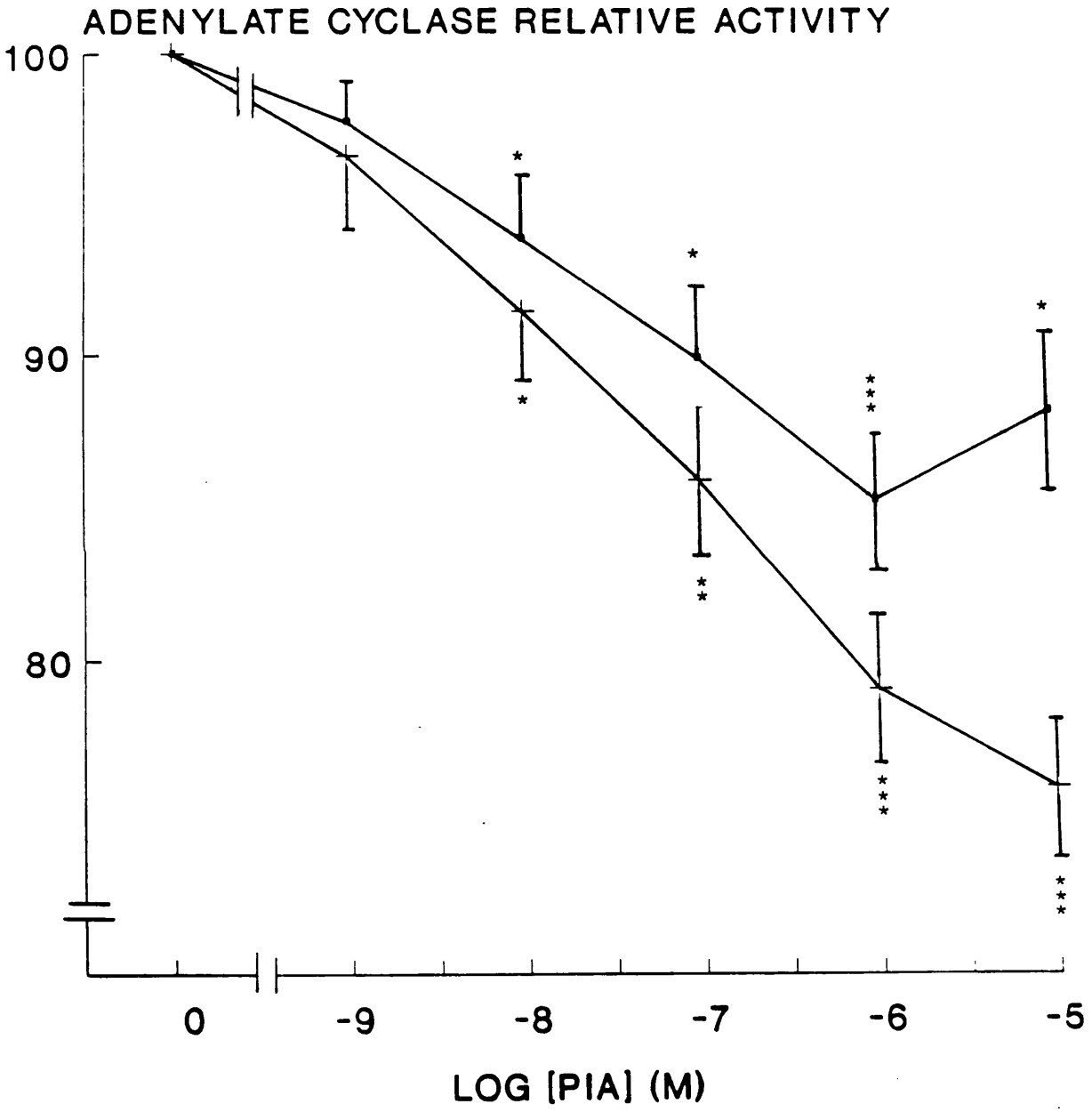
Under the conditions outlined, Fig. 3.13 shows that PIA significantly decreased adenylate cyclase activity at concentrations of 0.01μM and greater in both euthyroid and hypothyroid states, maximal inhibition was observed at 1μM and 10μM PIA in euthyroid and hypothyroid states respectively. Similar results were obtained by Cooper et al., (1980), though this group achieved a greater inhibition of cyclase at 1μM PIA (34% as compared with 15%, in the present study). Ebersolt et al., (1983) studied the effect of PIA on three fractions of rat brain namely: adult rat striatum, striatal neurones and cerebral cortex. The maximal inhibitory concentration of PIA and the resulting

Fig. 3.13 Effect of PIA on adenylate cyclase activity in synaptosomal membranes from euthyroid and hypothyroid rats.

Synaptosomal membranes were incubated with 10μM forskolin, 10μM GTP and 100mM NaCl and were assayed for adenylate cyclase activity as described in the materials and methods section. The values are means ± S.E.M. and are expressed relative to those in the absence of PIA (100%). Significant effects of PIA are indicated by: *P<0.05, **P<0.01, ***P<0.05 (paired differences). In addition, the effect of 10μM-PIA was significantly greater in hypothyroidism than in the euthyroid state (P<0.05 by an unpaired test).

■, euthyroid (n = 5)

*, hypothyroid (n = 6)


ADENYLATE CYCLASE RELATIVE ACTIVITY
100
90
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-9
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-7
-6
-5
LOG [PIA] (M)

degree of inhibition differed, depending on the fraction in question. Maximal inhibition in striatum and cerebral cortex occurred at 10μM PIA with 32 and 25% inhibition respectively. Maximal inhibition of striatal neurone adenylate cyclase was observed at approximately 1μM PIA giving an approximate 16% inhibition. It is noteworthy that different fractions yield different responses so when assaying adenylate cyclase in whole brain synaptosomal membranes the overall response will tend to approximate to that region of either greatest mass or of greatest change.

Nevertheless, hypothyroidism significantly enhanced the inhibitory effect of PIA upon brain adenylate cyclase activity (at 10μM PIA, $p<0.05$, fig. 3.13). However, this difference was slight compared to that achieved in fat cells (Malbon et al., 1985). In the fat cell system 1μM GTP inhibited forskolin-stimulated adenylate cyclase activity by about 25% in euthyroid and by nearly 70% in hypothyroid rat fat cell membranes. In comparison, rat brain synaptosomal membranes yielded a 15 and 21% inhibition at the same concentration of GTP, although the maximal inhibitory response in the hypothyroid state occurred at 10μM PIA, giving a 44% inhibition. As with the GTP effect, the modest inhibition observed in the present study must be viewed with consideration of the complexity of the tissue and the likelihood of regional differences in sensitivity of adenylate cyclase to inhibition by PIA (Ebersolt et al., 1983).

3.7 THE EFFECT OF HYPOTHYROIDISM ON ECTO 5'-NUCLEOTIDASE ACTIVITY IN SIX REGIONS OF THE RAT BRAIN

The multi-factorial role of adenosine within the CNS has been extensively documented. Physiological effects such as, sedation; decreased motor activity, anti-convulsant actions and depression (Williams, 1984; Williams, 1987; Eldridge & Milhorn, 1987) have been attributed primarily to the inhibition of excitatory neurotransmitter release, but also to other effects such as hyperpolarisation of the post synaptic membrane (Phillis & Wu, 1981; Williams, 1984; Trussel & Jackson, 1985; Trussel & Jackson, 1986). Pharmacological evidence has shown that the receptor responsible for one or more of these actions is the A_1 receptor (Reddington et al., 1982). Via the A_1 receptor, linked to a G protein-coupled potassium channel, adenosine is able to increase K^+ conductance at the postsynaptic membrane.

Presynaptic inhibition of neurotransmitter release has been attributed to the inhibition of action potential induced influx of Ca^{2+} ions into the axon terminal (Fredholm & Dunwiddie (1988). However, the data reported so far on this particular effect of adenosine is far from conclusive. Changes in the intracellular level of calcium or its coupling to the secretory process has also been implicated as a possible mechanism, (Schubert & Heinemann, 1988).

The complete functional significance of 5'-nucleotidase in the nervous system is as yet unclear.

Nonetheless, the predominant site of 5'-nucleotidase localisation (i.e., on the external aspect of membranes) suggests a role in the hydrolysis of extracellular nucleotides. ATP has been shown to be packaged and released as a co-transmitter with classical neuro-transmitters such as acetylcholine (Nagy et al., 1976), noradrenaline (Geffen & Livett, 1971; Lagercrantz, 1971) or 5-hydroxytryptamine (Da Prada & Pletscher, 1968). AMP, derived ultimately by hydrolysis of ATP released (Zimmerman et al., 1986; Richardson & Brown, 1987a,b) can be acted upon by 5'-nucleotidase to yield adenosine. It is likely therefore that the activity of ecto 5'-nucleotidase exerts an influence on the modulatory actions of adenosine.

Pathophysiological alterations as caused by diabetes, or hypothyroidism, have been shown to cause alterations in the activity of membrane bound 5'-nucleotidase in adipose tissue and skeletal muscle (Jamal & Saggerson, 1987; Karnielli et al., 1987; Klip et al., 1988). Hypothyroidism has for many years been associated with: somnolence and depression, and to a far lesser extent with paranoia and schizophrenia. Since adenosine itself has been shown to have sedative and depressive effects, any change in the availability of adenosine caused by hypothyroidism may be a factor in the expression of these neurological symptoms of hypothyroidism. Hence 5'-nucleotidase activity was measured in six regions of rat brain after chemical induction of hypothyroidism.

Moreover, membrane bound 5'-nucleotidase has been observed in purified myelin and synaptosomal membrane fractions from all six tested brain regions. Association of a significant production of brain 5'-nucleotidase with myelin has been noted by Cammer et al., (1980), Cammer et al., (1984) and Casado et al., (1980). Subcellular fractionation studies have repeatedly shown the enzyme to be associated with synaptosomal fractions (see e.g. Phillips & Newsholme, 1979). However, cytochemical evidence suggests that most of this 'synaptosomal' activity is not derived from neurones but from glial cell membranes (Kreutzberg & Barron, 1978; Kreutzberg et al., 1978, 1986; Heymann et al., 1984). Hence 5'-nucleotidase was assayed both in myelin and synaptosomal fractions. However, Richardson et al., (1987 a,b) have provided some evidence that 5'-nucleotidase is associated with post-synaptic membranes of cholinergic nerve terminals. Table 3.3 shows that 5'-nucleotidase activities in the myelin fraction were relatively uniform between regions, except for a slightly lower activity in the medulla oblongata, in the euthyroid state. Interestingly, in the synaptic fractions, cortical 5'-nucleotidase was notably lower than all other fractions (3.31 + 0.26 nmoles/min/mg protein) with the exception of the medulla oblongata, which had an activity of approximately 8.7 ± 2.2 nmoles/min/mg/protein). These synaptosomal membrane activities compare well with those found by Sun et al., (1976) in tissue dispersions from seven regions of rat brain. They found the lowest

activities associated with the cortical region and the highest with cerebellum and medulla oblongata. Phillips & Newsholme (1979) measured 5'-nucleotidase in three broad regions of the rat brain; namely in the synaptic fractions of cortex, cerebellum and hindbrain. There was very little difference in activity between the three though activity in the cortex was the lowest 6.3, 8.6 and 11.2 nmol/min/mg protein for the cortex, hind brain and cerebellum respectively.

Of interest is that Richardson et al., (1987a) in studies using immunoaffinity purified cholinergic nerve terminals from the cortex and striatum, suggest that rat brain cholinergic neurons have two distinct feedback control mechanisms. One determined by presynaptic adenosine receptors and the activity of ectophosphophohydrolases on adjacent cells (in the striatum), and one determined by presynaptic inhibitor acetylcholine receptors (in the cortex). In fact these cortical terminals can produce only low amounts of nucleosides owing to low activities of both ecto-5'-nucleotidase and ecto-(ADP)ase. As in the study of Richardson et al., (1987a) the present study found synaptosomal membrane cortical 5'-nucleotidase to be markedly lower than other fractions, whereas the myelin enzyme is in the higher range of 14.2 ± 2.8 nmol/min/mg protein. However, since 5'-nucleotidase activity wss observed in the cortex it is likely that adenosine does have a neuromodulatory role in the region, though not

necessarily associated with cholinergic neurons. The high activity of ecto-5'-nucleotides associated with myelin in the cortex may also signify an important role for adenosine, other than neuromodulation, in this region.

Table 3.3 shows that hypothyroidism caused significant increases in 5'-nucleotidase activity associated with synaptosomal membranes from the cerebellum (5.5-fold), cortex (4.8-fold), striatum (3.9-fold) and hippocampus (2.3-fold). There were no significant changes in the medulla oblongata or the hypothalamus. Previous studies using homogenates or membrane preparations from rat or mouse brain have shown a decrease in brain 5'-nucleotidase activity in hypothyroidism (Smith et al., 1980; King et al., 1983; Shanker et al., 1984). However their studies were conducted in developing animals where thyroid deficiency would influence the biochemical specialisation of cells. The aforementioned authors were not however able to identify the structures or cell types responsible for the decrease in 5'-nucleotidase activity. By contrast, the 5'-nucleotidase activity in the myelin fraction was unchanged, except for a 1.9-fold increase in the medulla oblongata. Furthermore, it can be seen that hypothyroidism brings about a region-specific increase in membrane bound 5'-nucleotidase activity that is almost entirely confined to structures at or near terminals; an interesting point since the activity of 5'-nucleotidase close to the synaptic cleft is thought to be rate limiting in the production of adenosine (Richardson et al., 1987b).

TABLE 3.3

Effects of hypothyroidism on 5'-nucleotidase specific activity in six regions of rat brain.

5'-nucleotidase activity was determined as described in the materials and methods section. The values are expres as nmol/min per mg of protein and are means ± S.E.M. for the number of measurements shown in parenthes Statistical significance of changes relative to the control is indicated by a, b, c, which represent P<0.05, <0. <0.001 respectively.

		Brain region					
Enzyme	Condition	Cerebellum	Cortex	Hippocampus	Hypothalamus	Striatum	Medulla oblongata
5'-nucleotidase (myelin)	Control (4-5)	9.03±2.41	14.2±2.8	13.9±3.2	14.4±2.9	10.6±2.2	6.78±1.89
	Hypothyroid (4-5)	7.25±0.36	14.0±1.6	16.8±2.7	15.0±2.2	15.8±2.7	12.9±0.7[a]
5'-nucleotidase (synaptic)	Control (4-5)	8.99±2.27	3.31±0.26	8.28±2.81	8.27±1.59	9.36±2.08	16.4±3.7
	Hypothyroid (4-5)	49.3±4.4[c]	16.0±1.2[c]	18.8±1.4[c]	9.68±1.3	36.2±4.8[b]	17.6±1.4

Therefore the increase in 5'-nucleotidase effected by hypothyroidism in the cerebellum, cortex, striatum and hippocampus in the synaptosomal membrane fraction (in which 5'-nucleotidase is likely to be associated with glial membranes or perhaps post-synaptic membranes) may result in an increase of adenosine near nerve terminals and consequently increase the bioavailability of, and hence the degree of neuromodulation exerted by the nucleoside on pre- and post-synaptic membranes via the A_1 receptor.

It is noteworthy that the initial attempts to show a correlation between A_1 receptor distribution and ecto 5'-nucleotidase activity by Goodman & Snyder (1982) were dispelled by Schubert et al., (1986). The aforementioned authors found a relative consistency in the distribution of hippocampal A_1 receptors, suggesting that the function of these sites is conserved across the broad range of species examined. This, however, is in sharp contrast to the diversity of 5'-nucleotidase activity found in the species examined. It remains possible, however, that 5'-nucleotidase might be associated with a different type of purine receptor, e.g., the A_2 receptor whose exact distribution has yet to be elucidated. A correlation may therefore exist between A_1 and A_2 receptors with 5'-nucleotidase, although this has yet to be established. Moreover, previous studies have shown a correlation between the distribution of A_1 adenosine sites and the depressive action of adenosine (Lee et al., 1983 a,b).

Such a correlation would add further substance to hypothyroidism enhancing the neuromodulatory action of adenosine through an increase in extracellular adenosine.

3.8 THE EFFECT OF HYPOTHYROIDISM ON ADENOSINE DEAMINASE AND ADENOSINE KINASE ACTIVITY IN SIX REGIONS OF THE RAT BRAIN

Even though the physiological actions of adenosine such as neuromodulation or changes in local cerebral blood flow are mediated extracellularly via its receptors, metabolism of adenosine occurs intracellularly. Intracellular adenosine, derived either by reuptake via a carrier mediated process or by intracellular formation from AMP, is converted to either inosine or AMP by the cytosolic enzymes adenosine deaminase (ADA) or adenosine kinase (AK) respectively (Pull & McIlwain 1974; Arch & Newsholme 1978 a,b; Phillips & Newsholme 1979, Zimmerman et al., 1986). In general, non-specific phosphatases and purine nucleotide phosphorylase are considered not to be involved in adenosine metabolism (Arch & Newsholme 1978b). Overall, the concentration of adenosine is controlled by the relative rates of formation and degradation. A means therefore of altering adenosine concentrations could be by modifying the activities of ADA and/or AK. These two soluble enzyme activities were therefore measured in the present study.

ADA activity was relatively uniform through the cortex, cerebellum, striatum and hippocampus (see table 3.4); although two and three-fold differences in ADA activity were seen between the hippocampus and medulla

oblongata and hypothalamus respectively. Overall the distribution of enzyme activity in the six brain regions examined compares well with that obtained by Geiger & Nagy, (1986). The results obtained, from highest to lowest activity, are hypothalamus (3.62) > medulla oblongata (2.37) > cortex (1.87) > cerebellum (1.78) > striatum (1.34) > hippocampus (1.27) (numbers in parentheses represent activities in terms of nmol min^{-1} mg^{-1} protein). However a much larger difference in ADA activity was reported by Geiger & Nagy (1986) between rat hypothalamus and hippocampus (6.6 fold as compared with 3 fold). On the whole little agreement has been reached in the literature with respect to relative activities of ADA in brain regions. Sun et al., (1976) and Davies & Taylor (1979) examined brain regions in the rat and found ADA activity to be about two-fold higher in the hypothalamus than in the striatum or neocortex. These disparate findings may be due to species variations in ADA activity or differences in dissection procedures or assay methodologies. Table 3.4 shows that hypothyroidism had no significant effect on ADA activity.

As can be seen from table 3.4 there were no marked differences in AK specific activities between the striatum, hippocampus, medulla oblongata, hypothalamus and cortex. However, a 2.6-fold greater activity was observed in cerebellum compared to the hypothalamus. Phillips & Newsholme, 1979) also found AK activities to be relatively uniform in the nine regions of human brain assayed. The

TABLE 3.4

Effects of hypothyroidism on adenosine deaminase and adenosine kinase specific activities in six regions of brain.

Adenosine deaminase and adenosine kinase specific activities were determined as described in the materials methods section. The values are expressed as nmol/min per mg of protein and are means ± S.E.M. for the numb of measurement shown in parentheses.

Statistical significance of changes relative to the control is indicated by a, b, c, which represent P<0. <0.02, <0.01 respectively.

		Brain region					
Enzyme	Condition	Cerebellum	Cortex	Hippocampus	Hypothalamus	Striatum	Medulla oblongata
Adenosine kinase	Control (5)	0.69±0.8	0.41±0.05	0.37±0.05	0.27±0.03	0.38±0.06	0.34±0.05
	Hypothyroid (4)	0.39±0.04[b]	0.25±0.05	0.23±0.02[a]	0.15±0.02[c]	0.22±0.02[a]	0.25±0.03
Adenosine deaminase	Control (4-5)	1.78±0.13	1.87±0.15	1.27±0.40	3.62±0.95	1.34±0.34	2.37±0.46
	Hypothyroid (5-6)	1.80±0.25	1.98±0.30	1.42±0.20	2.66±0.16	2.03±0.33	3.12±0.54

main difference is that in the human brain the hypothalamus is the region of greatest AK activity. Table 3.4 shows that hypothyroidism decreased AK activity by approximately 40% in the cerebellum, hippocampus, hypothalamus and striatum.

Interpretation of the possible functional significance of the distribution pattern of ADA and AK within the rat CNS and also the decrease in AK specific activity effected by hypothyroidism requires a consideration of the role of ADA and AK in the intracellular metabolism of adenosine.

Unfortunately it is not possible from the present study to determine the actual cell types responsible for the changes precipitated by hypothyroidism. However previous studies have shown ADA to be present in neurons and, in some species, namely rats and to a greater degree in mice and guinea pigs, in glia (Yamamoto et al., 1987). Furthermore, Pull & McIlwain (1974) concluded that ADA is contained within the cytoplasm of neurons, including nerve endings. Moreover, a close correspondence between brain areas containing structures immunoreactive for ADA and those containing high levels of nucleoside transporter sites, (as determined by analysis of [^{3}H] NBI binding), has been found (Geiger & Nagy 1986); a result that would imply a role for ADA and by inference, for AK in the disposal of adenosine after its reuptake into neuronal endings. It has been postulated therefore that ADA and AK in conjunction

with the nucleoside transporter may participate in regulating the bioavailability of adenosine at its physiologically relevant sites of action (Geiger & Nagy, 1986).

Intracellularly adenosine can be incorporated into S-adenosylhomocysteine (SAH) by the enzyme S-adenosylhomocysteine hydrolase. However the formation of SAH has been shown to be only a minor product in brain slices even after they are exposed to relatively high concentrations of adenosine (Reddington & Pusch, 1983). In fact, even though the equilibrium of the reaction catalysed by SAH hydrolase favours synthesis the reaction proceeds in the direction of hydrolysis because both adenosine and homocysteine are further metabolised (Lloyd et al., 1988). Alternatively, adenosine may either be deaminated by ADA or phosphorylated by AK. The precedence of one pathway over the other would seem to depend on the intracellular concentration of adenosine, since the Km and Vmax values of AK are much lower than those of ADA. AK, Km and Vmax values in rat brain homogenates were 2μM and 25.0 nmol min^{-1} g wet wt^{-1} (Phillips & Newsholme, 1979). Partially purified AK from rat brain had a Km of 20μM (Shimzu et al., 1972), whilst that of the purified rat brain enzyme was 0.2μM (Yamada et al., 1980). Interestingly, this last study found that maximum activity was observed at 0.5μm adenosine; higher concentrations strongly inhibited the enzyme. Km values for ADA activity in homogenate of whole rat brain have been reported at 17, 34, 100, 54-57 and 47μM

(Arch & Newsholme, 1978b; Phillips & Newsholme, 1979; Pull & McIlwain, 1974; Skolnick et al, 1978; Geiger & Nagy, 1986). These same authors reported Vmax values of 174, 54, 115 and 105 nmol min^{-1} g wet wt^{-1}. Basal intracellular concentrations of adenosine have been estimated at 3.1, 0.6 and 0.52 nmol g wet wt^{-1} (Hagberg et al., 1987; Rehncrona et al., 1978; Winn et al., 1980). Furthermore, free exchangeable concentrations of adenosine in intact rat brain have been estimated at 1-2μM. Under such conditions the kinase may function at or slightly below its Km value. Conversely such concentrations of adenosine will be far below the km value for ADA. It is likely therefore that under basal conditions phosphorylation predominates. In fact studies evaluating the chemical nature of [^{14}C] adenosine derivatives prepared from guinea pig neocortex showed 78-88% of the derivatives to be present at the 5'-nucleotides (ATP, ADP & AMP). Some 1-18% of the ^{14}C was present as adenosine and there were small amounts of adenine, inosine, hypoxanthine and cAMP (Kuroda & McIlwain, 1974). Studies using nerve terminals from Torpedo electric organ gave similar results (Zimmerman et al., 1979).

However under conditions where intracellular concentrations of adenosine are raised such as hypoxia increased seizure activity or exposure of CNS tissues to depolarisation (Hagberg et al., 1987, Fredholm et al., 1984; Dragunow, 1986), deamination would predominate since under such conditions AK is likely to be inhibited. Levels of adenosine in hypoxia have been estimated at well above

100μM (Fredholm et al., 1984, Hagberg et al., 1987). Moreover, such conditions have caused marked increases in inosine and hypoxanthine levels with little change in AMP levels (Daval & Barberis, 1981, Lewin & Bleck, 1981; 1983), thus supporting the predominant role of ADA in metabolising elevated concentrations of adenosine. Therefore those regions in rat brain with the higher specific activities of ADA i.e. medulla oblongata and hypothalamus should be more effective at metabolising adenosine when the cell is put under metabolic stress. In support of this statement Fredholm et al., (1984) found that one of the main mechanisms involved in recovering the energy charge of the cell was adenylate dephosphorylation with the formation of adenosine and its further metabolites.

It is presently difficult to predict the effect that a decrease in adenosine kinase, as effected by hypothyroidism, in the cerebellum, hippocampus, hypothalamus and striatum would have on basal adenosine levels. The substrate cycle proposed by Arch & Newsholme (1978 a,b) states that under all conditions AK approaches saturation with substrate, whereas, ADA is not saturated and adenosine concentrations may be below the Km of this enzyme for the nucleoside. Therefore, an increase in the activity of the nucleotidase or a decrease in that of the kinase will markedly increase the conversion of AMP into inosine. However, the only reason why the deaminase activity should increase under these conditions is an increase in the concentration of adenosine. Using such

arguments a decrease in AK without an alteration in the rate of dephosphorylation by 5'-nucleotidase(s) would result in an increase in basal adenosine concentrations. Conversely should a new steady state be reached and the 5'-nucleotidase activity approximate to the phosphorylation rate of AK then a decrease in basal adenosine would be seen. This scheme is further complicated in nervous tissue as the main intracellular pool of adenosine is fed from synaptically and intracellularly derived adenosine (Zimmerman et al., 1986; Pons et al., 1980). Therefore both an intracellular 5'-nucleotidase(s) and the ecto 5'-nucleotidase would be involved in the substrate cycle. However, whether or not the substrate cycling theory can be applied to nervous tissue remains to be tested.

Nevertheless, the decreases in AK activity in the cerebellum, hippocampus and striatum are in fact accompanied by increases in ecto 5'-nucleotidase, whereas, the hypothalamus shows a decrease in AK specific activity with no alteration in ecto 5'-nucleotidase. According to the above argument a decrease in AK activity should result in a decreased re-utilisation via the phosphorylation pathway with a consequent possible increase in extracellular adenosine in all the regions affected. Moreover, such an action would accentuate the increase in extracellular adenosine effected by an increase in 5'-nucleotidase in the cerebellum, hippocampus and striatum. However, it would be useful to ascertain the contribution made by the intracellular 5'-nucleotidase(s) to the

intracellular pool, and consequently synaptic release of adenosine by nerve terminals, in the control and hypothyroid states.

CHAPTER 4

GENERAL DISCUSSION

4. GENERAL DISCUSSION

It is evident from these studies that thyroid hormones retain their ability to modulate biochemical parameters in the adult rat brain since hypothyroidism increases the effectiveness of an inhibitory agonist namely PIA at rat brain adenylate cyclase. The role of the A_1 receptor in this increase in sensitivity can be excluded as competition binding studies with [^{3}H] DPX as radioligand and PIA as competing ligand showed no significant changes in either K_D or Bmax in the absence of GTP in the hypothyroid state. [^{3}H] PIA saturation binding studies also revealed no alterations in K_D, although, somewhat paradoxically a small but significant decrease in A_1 receptor density was observed in the hypothyroid state. The increased effectiveness of GTP at changing the affinity of agonist binding and an increased ability of agonist to promote the formation and stabilise the high affinity complex HRG would imply that the hypothyroid adenylate cyclase system has an increased ability to inhibit adenylate cyclase at the level of R-Gi. This increased ability may reside either, in an increase in affinity of R for Gi or, as already established in the fat cell system, an increase in the α subunit of αGi.

Certainly the increase in sensitivity cannot be explained by an increase in the catalytic subunit of adenylate cyclase as basal and forskolin-stimulated activity was the same in euthyroid and hypothroid states. Unlike the fat cell system where an increase in αGi was reflected by an increased sensitivity to inhibition of

adenylate cyclase by GTP, the brain membrane system revealed no such increase in the hypothryoid state; although this lack of difference must be viewed with regard to the complexity of the tissue and the likelihood of regional differences in any potential alterations of αGi. In this context it would be interesting to directly evaluate any changes in αGi in the hypothyroid state by utilising anti peptide antisera to αGi both in the whole brain and brain regions. It is possible, however, that hypothyroidism results in an alteration of function of Gi by firstly, impairing the inhibitory action of sodium on forskolin stimulated adenylate cyclase in the presence or absence of GTP and secondly, by preventing sodium ions from overcoming the inhibitory action of GTP. The physiological relevance, however, of these two lesions is presently unknown. Nevertheless, since two of the mechanisms of Na^+ action have been shown to be an inhibition of GTPase activity and the activation process at both Gi and Gs then it may be that by impairing these inhibitory actions at Gi, hypothyroidism may increase the sensitivity of adenylate cyclase to inhibitory agonists. Further studies would be required to investigate the validity of this statement.

The more effective coupling of the A_1 receptor to adenylate cyclase along with the impairment of sodium action on adenylate cyclase would imply that Gi is the major locus of change in hypothyroidism. If this is in fact the case then it is to be expected that inhibition of brain adenylate cyclase through receptors other than the A_1

receptor [e.g., opiate (Hume et al., 1979, 1980; Cooper et al., 1982; Koski et al., 1982) or muscarinic cholinergic (Lichtshtein et al., 1979)] would also be enhanced in this state.

Unfortunately, although pharmacological evidence points to the A_1 receptor subtype being the mediator of adenosine's neuromodulatory actions, (Reddington et al., 1982) the role of adenosine-modulated decreases in intracellular cAMP via the A_1 receptor are less clear. Although a contributory role has not been totally excluded, direct tests have failed to demonstrate that inhibitors of adenylate cyclase including 2'-5'-dideoxyadenosine inhibit transmitter release; and in electrophysiological, as well as biochemical studies it has proved difficult to block the presynaptic inhibition by adenosine analogues by increasing cAMP levels (Dunwiddie & Fredholm, 1985). The neuromodulatory actions of adenosine as potentially effected by: presynaptic inhibition of calcium uptake, changing the intracellular level of calcium or its coupling to the secretory process (Fredholm & Dunwiddie, 1988; Schubert & Heinemann, 1988) or by opening K^+ channels on the post-synaptic membrane (Trussel & Jackson, 1985; 1986) may nevertheless, still be accentuated by increasing the bioavailability of adenosine by increasing the activity of ecto-5'-nucleotidase or in fact decreasing that of AK as found in certain regions of the rat brain in the hypothyroid state.

The brain regions showing the largest changes in synaptosomal-membrane 5'-nucleotidase activity were cerebellum, cortex, striatum and to a lesser extent hippocampus. A high content of A_1 receptors is found in the thalamus, cerebellum and hippocampus with lesser amounts in the cortex, medulla oblongata and pons (Williams & Risely, 1980; Goodman & Snyder, 1982). A moderate A_1 receptor density is found in the striatum, whereas this region has the highest density of A_2 receptors, though lower levels are found in all other regions. A direct correlation between the distribution of A_1 receptors and 5'-nucleotidase as suggested by Goodman & Snyder (1982) was ruled out by Lee at al., (1986) and Fastbom et al., (1987) who found no such correlation in mammalian brain. Nevertheless, studies by Lee et al., (1983 a,b) have demonstrated a correlation between the distribution of A_1 adenosine sites and the depressive action of adenosine. Richardson et al., (1987) have suggested that an increase in the synthesis or expression of glial and perhaps post-synaptic membrane 5'-nucleotidase would enhance modulation through an increase in adenosine, as found in rat brain in the hypothyroid state. Hence, a demonstration of a direct correlation between A_1 receptors and 5'-nucleotidase would add further credence to hypothyroidism enhancing the modulatory action of adenosine through an increase in extracellular adenosine in the cerebellum, cortex, striatum and hippocampus of rat brain.

The highest levels of ADA were found in the hypothalamus and medulla oblongata with lesser amounts in the cortex, cerebellum, striatum and hippocampus. Since deamination predominates in conditions when adenosine levels are markedly increased e.g. hypoxia (Hagberg et al., 1987; Fredholm et al., 1984) those regions in rat brain with relatively greater specific activities of ADA i.e. medulla oblongata and hypothalamus should be more effective at metabolising adenosine when the cell is put under metabolic stress. Significant decreases in AK activity as found in the cerebullum, hippocampus, hypothalamus and striatum, of approximately 40%, could result in an increase in the bioavailability of extracellular adenosine as a consequence of diminised re-utilisation after reuptake into nerve terminals in all the regions affected. Furthermore, since extracellular adenosine concentration is mainly determined by local formation, the increase in extracellular adenosine effected by increases in ecto 5'-nucleotidase in the cerebellum, hippocampus and striatum could be accentuated by the concomitant decrease in the intracellular phosphorylation pathway of adenosine in these regions. In this context it would be interesting to investigate the contribution made by the intracellular 5'-nucleotidase(s) in regulating the intracellular pool and, extracellular biovailability of adenosine in euthyroid and hypothyroid states, in both whole brain and specific brain regions.

Finally, it would be interesting to speculate that the increased biovailability of adenosine in certain brain regions along with the more effective coupling of the A_1 receptor to adenylate and impairment of sodium action on adenylate cyclase may be a causative factor in CNS dysfunction seen in the hypothyroid state.

CHAPTER 5

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REFERENCES

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