CHARACTERIZATION AND PROPERTIES OF ECTO-5'NUCLEOTIDASE IN WHITE ADIPOSE TISSUE

by

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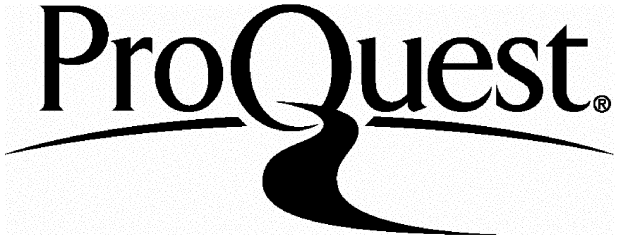

ABSTRACT

Membrane-bound 5'-nucleotidase (5'Nase, EC 3.1.3.5) activity was measured in rat white adipose tissue by radiochemical assay in several metabolic states, including normal fed, streptozotocin-diabetes, hypothyroidism, hyperthyroidism, and fasting. A doubling in 5'Nase activity was measured in the diabetic rats as compared to the normal fed control animals, whilst no statistical differences were noted in the other metabolic states tested. Two protein bands were detected at 76kDa and 74kDa in control and diabetic rat adipose tissue membrane samples by exposing a Western blot to a polyclonal anti-rat-ecto-5'Nase. The combined intensities of these two bands increased to the same extent in diabetics as did the membrane-bound 5'Nase activities when comparing with control values. *In vivo* insulin administration to the diabetic rats resulted in a reduction of membrane-bound 5'Nase activity to marginally below control activities over a period greater than 24 hours, but not exceeding 48 hours.

Partial purification of rat adipose tissue membrane-bound 5'Nase was achieved by a sulphobetaine detergent solubilization of the membranes, followed by concanavalin A-Sepharose, AMP-Sepharose and Superose-12 column chromatography. Only two protein bands were visualised by silver staining when this partially purified sample was subjected to denaturing gel electrophoresis. Characterization of the

partially purified 5'Nase demonstrated that its properties were similar to ecto-5'Nases purified from other tissues and species. These properties included: $K_{m(AMP)}$ = 11.1μM, pH optimum at pH7.5, no magnesium inhibition, competitive inhibition with K_i values for α,β-methylene-ADP in the nM range and for α,β-methylene-ATP in the μM range, IC_{50} = 2.3mM for dithiothreitol inhibition, and almost complete inhibition at 10μM concanavalin A. Although the rat adipose tissue membrane-bound 5'Nase was susceptible to cleavage by the glycosyl-phosphatidylinositol (GPI)-anchor cleavage enzyme, GPI-phospholipase C, a significant loss of 5'Nase activity meant that the procedure was not a viable option for use in the purification. Additionally, a factor derived from rat brain which was found to co-purify with "soluble" low-K_m 5'Nase, enhanced ATP inhibition of the partially purified adipose tissue membrane-bound 5'Nase by 40-fold.

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And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

T.S.Eliot

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ABBREVIATIONS

AMP	:	adenosine-5'-monophosphate
ADP	:	adenosine-5'-diphosphate
ATP	:	adenosine-5'-triphosphate
cAMP	:	cyclic-3',5'-adenosine monophosphate
Con A	:	concanavalin A
DTT	:	dithiothreitol
EDTA, EGTA	:	ethylenediaminetetra-acetic acid, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FPLC	:	fast protein liquid chromatography
GPI, GPI-PLC	:	glycosyl-phosphatidylinositol, GPI-phospholipase C
GTP, GTP-γ-S	:	guanosine-5'-triphosphate, guanosine-5'-O-thiotriphosphate
IMP	:	inosine-5'-monophosphate
IRS-1	:	insulin receptor substrate-1
K_i, K_m	:	inhibition constant, affinity constant
5'Nase, e5'Nase	:	5'-nucleotidase, ecto-5'-nucleotidase
NEFA	:	non-esterified fatty acids
Pi, PPi	:	orthophosphate, pyrophosphate
PI-PLC or D	:	phosphatidylinositol phospholipase C or D
PI-G, PI-G-PLC or D	:	phosphatidylinositol-glycan, PI-G-phospholipase C or D
PTU	:	propylthiouracil
SDS-PAGE	:	sodium dodecylsulphate-polyacrylamide gel electrophoresis
Tris.Cl^-	:	tris[hydroxymethyl]aminomethane, adjusted to stated pH with hydrochloric acid
V_{max}	:	maximum velocity
WAT	:	white adipose tissue

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 General Background

5'-Nucleotidase (5'Nase: EC 3.1.3.5) is an enzyme which catalyses the dephosphorylation of 5'-mononucleotides to their corresponding nucleosides, but has no effect on the 2'- and 3'-monophosphates (Baer *et al*, 1966; Burger & Lowenstein, 1970). The reaction, using 5'-adenosine monophosphate (AMP) as substrate for example, can be depicted as shown in Figure 1.1 below.

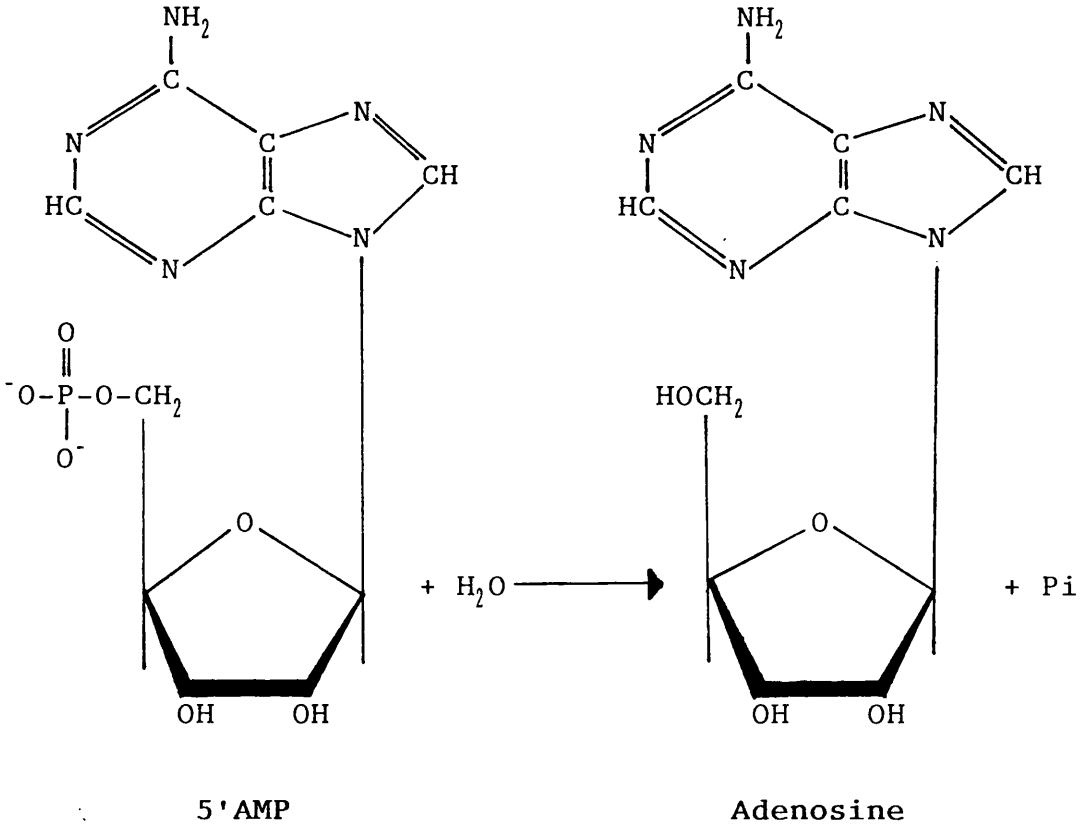


FIGURE 1.1: Reaction Catalysed by 5'Nase, Using AMP as Substrate

This enzyme is ubiquitous throughout vertebrate tissues and exists in a plasma membrane-bound and several soluble forms. In addition to possible compartmentation, each form varies in its size, preference for nucleotide substrate, kinetics, and sensitivity or tolerance to physiological factors. The plasma membrane-bound form of 5'Nase is an ecto-enzyme (Gurd & Evans, 1974; Newby *et al*, 1975), having its active site directed extracellularly and will therefore be referred to throughout this document as ecto-5'-Nucleotidase (e5'Nase). It has been used as a plasma membrane marker for many years without a clear understanding of its physiological role. Of all the nucleotide-5'-monophosphates, e5'Nase has the greatest affinity for AMP (Pearson & Coade, 1987; De Pierre & Karnovsky, 1974; Cusack *et al*, 1983). It therefore follows that e5'Nase may be important for the formation of extracellular adenosine; although facilitated transport of adenosine across the plasma membrane in both directions complicates this concept.

In recent years a novel membrane anchoring system has been elucidated for a wide variety of plasma membrane-bound proteins which are involved in adhesion and transmembrane signalling (Low, 1987 - review). Amongst the proteins identified as being thus anchored is e5'Nase (Low & Finean, 1978; Shukla *et al*, 1980). No structural or functional similarities have been determined for these proteins. The anchoring mechanism involves a phosphatidylinositol molecule which is associated with a glycan moiety and at least one

ethanolamine residue. In the literature this anchor is known interchangeably as glycosyl-phosphatidylinositol (GPI) or phosphatidylinositol-glycan (PI-G). Some authors prefer to retain the use of "PI-G" for the mammalian anchor exclusively which differs in small detail only from the non-mammalian anchors.

The proteins which are PI-G anchored are essentially hydrophilic, but their covalent linkage classifies them as membrane proteins. The anchor can be cleaved with a phosphatidylinositol-specific phospholipase C or D (PI-PLC or PI-PLD) (Shukla *et al*, 1980; Low & Prasad, 1988), though the sensitivity to these phospholipases varies from one anchor to the next. E5'Nase is one of the proteins which is partially resistant to cleavage and the extent of its resistance seems to be both species- and tissue-dependent (Zekri *et al*, 1989).

There is now strong evidence to suggest that insulin acts on the PI-G anchors by activating the endogenous membrane-bound PI-PLC (Saltiel & Cuatrecasas, 1986; Saltiel *et al*, 1986; Mato *et al*, 1987; Chan *et al*, 1988; Romero *et al*, 1988). Several reports have also demonstrated that exogenously administered PI-PLC can act as an insulin-mimetic, suggesting that the cleaved anchors may be responsible for some of insulin's actions in the cell (Saltiel & Cuatrecasas, 1986; Saltiel *et al*, 1986; Fox *et al*, 1987; Alemany *et al*, 1987; Saltiel and Sorbara-Cazan, 1987;

Alverez *et al*, 1988; Romero *et al*, 1988). The implications of this for the cellular metabolism in a variety of physiological and pathological states where insulin-receptor binding and/or insulin-receptor signal transduction is impaired may be highly significant.

1.2 Physiological Role of Adenosine

The role of adenosine has been the subject of extensive investigations since early this century. The first recorded report of adenosine administration to humans was made in 1914 by Thannhauser and Bommes. This was followed with a major contribution by Drury and Szent-Györgyi in 1929 when they described the effects of adenosine and adenosine 5'-monophosphate (AMP) on the cardiovascular system in particular, but also on cell function in general. However, it was left to Lindner and Rigler in 1931 to demonstrate the vasodilatory properties of adenosine and to postulate that adenosine is a physiological regulator of coronary blood flow. Unfortunately little more was published in this area until three decades later when Berne demonstrated the release of adenosine catabolites from hypoxic heart muscle (Berne, 1963) and in anoxic heart and skeletal muscles (Berne *et al*, 1963). Their work seemed to revive interest in the role of adenosine as a local metabolic regulator and in its vasoactive properties.

The establishment of "purinergic" neurones - i.e.

neurones releasing purines as transmitter substances instead of the conventional acetylcholine or noradrenaline (Burnstock, 1972), provided new insights in cellular modulation. However, it was his work on the purinergic-receptors (purinoceptors), specifically showing that some purinoceptors interacted with adenosine 5'-tri- or di-phosphate (ATP or ADP) preferentially, whilst others were specific for adenosine and to a limited extent AMP (Burnstock, 1978), which really opened new vistas.

1.3 Adenosine in White Adipose Tissue

Adenosine promotes a variety of cellular responses in white adipose tissue normally attributed to insulin such as increases in glucose transport (Green, 1983), lipoprotein lipase activity (Ohisalo *et al*, 1981), cyclic nucleotide phosphodiesterase activity (Wong *et al*, 1985), pyruvate dehydrogenase activity (Honeyman *et al*, 1983) and leucine oxidation (Honeyman *et al*, 1983).

The first evidence for the existence of adenosine receptors was the observation that adenosine stimulates cyclic-3',5'-adenosine monophosphate (cAMP) accumulation in brain slices, whilst theophylline had the reverse effect (Sattin & Rall, 1970). Subsequent studies on the catalytic enzyme adenylate cyclase in several mammalian cell types confirmed the existence of two distinct receptors and their respective antagonistic actions on adenylate cyclase (Londos

& Wolff, 1977; Van Calker *et al*, 1979; Londos *et al*, 1980). The route by which adenosine could exert its antilipolytic actions had been unclear until it was shown that adipocytes possess a subclass of these adenosine receptors, termed A_1 (Van Calker *et al*, 1979). More recently it has been shown that these A_1 receptors are coupled to adenylate cyclase via the inhibitory regulatory protein G_i (Londos *et al*, 1981; Moreno *et al*, 1983; Olansky *et al*, 1983) which provides an explanation for adenosine's ability to inhibit cAMP accumulation. However, this notion depends on the formation and presence of extracellular adenosine which is a subject still under general debate.

Of particular interest to this study was the discovery that adenosine, as well as some other paracrine (i.e. short-lived, locally acting) agents such as some prostaglandins, could exert potent antilipolytic effects in adipose tissue of various species including man (Schwabe *et al*, 1973; Fain & Wieser, 1975; Fredholm, 1976; Fernandez & Saggerson, 1978; Ohisalo *et al*, 1981; Kather *et al*, 1985 and 1985a; Saggerson, 1986), thus mimicking the well established role of insulin as antilipolytic hormone in white adipose tissue. Adenosine's antilipolytic action is highly significant in light of the fact that the responsiveness of adipocytes to adenosine is modified in a variety of physiological and pathological states where changes in receptor coupling are implicated, including streptozotocin-diabetes (Saggerson *et al*, 1991), fasting (Chohan *et al*, 1984), adrenalectomy

(Saggerson, 1980), obesity (Ohisalo *et al*, 1986), lactation (Vernon & Finley, 1985), ageing (Hoffman *et al*, 1984) and hypothyroidism (Saggerson, 1986; Ohisalo & Stouffer, 1979; Malbon *et al*, 1985; Milligan *et al*, 1987; Milligan & Saggerson, 1990).

At least of equal interest was the demonstration that adenosine is vasodilatory in white adipose tissue (Hedqvist & Fredholm, 1976). Coupled to the finding that adipose tissue blood flow generally varies with the intensity of lipolysis (Nielsen *et al*, 1968; Lewis & Matthews, 1970; Hoffbrand & Forsyth, 1973), adenosine appeared to be implicated in assisting the ordered removal of non-esterified fatty acids (NEFA). This was significant as increased circulating NEFA levels, which result from the mobilization of adipose tissue triacylglycerol stores in diabetic and other physiological and pathological states, cause increased hepatic handling of the NEFA and thus result in the formation of ketone bodies and hepatic triacylglycerol synthesis. Furthermore a rise in the utilization of NEFA as oxidative fuel by extrahepatic tissues may result in decreased glucose tolerance.

1.4.1 Adenosine Metabolism

The relative significance of adenine nucleotide synthesis and degradative pathways to each other, as well as the role of the adenosine molecule both intra- and extra-

cellularly are complicated. It appears that the primary source of adenosine formation is from the hydrolysis of AMP, catalysed by 5'Nase. Other sources such as S-adenosyl-1-homocysteine (Schrader *et al*, 1981; Ueland, 1982; Achterberg *et al*, 1985) have been largely dismissed with respect to adenosine synthesis due to the dynamics of the corresponding pathways (Ueland, 1982; Reddington & Pusch, 1983; Meghji *et al*, 1988). In the above example, the relevant catalysing enzyme, S-adenosyl-1 homocysteine hydrolase (EC 3.3.1.1), tends to push the equilibrium towards the synthesis of S-adenosyl-1-homocysteine (Ueland, 1982). Alternatively, acid and alkaline phosphatases are also capable of catalysing the dephosphorylation of AMP to adenosine, but they are non-specific for 5'-nucleotides and at physiological pHs their activities are very low. Conversely, although the kinetics vary for the different 5'Nases, generally they have pH optima close to physiological pH and are specific for 5'-nucleotides (Bodansky & Schwartz, 1968) with K_m values ranging roughly from 2μM to 2mM.

Although the primary source of adenosine is from AMP catalysed by 5'Nase, other enzymes involved in the formation and degradation pathways are extremely important. The removal or absence of substrate for 5'Nase, as well as the phosphorylation and degradation of adenosine itself will have far reaching ramifications with respect to the physiological role of adenosine. Three enzymes, AMP deaminase, adenosine kinase and adenosine deaminase, which utilize AMP or

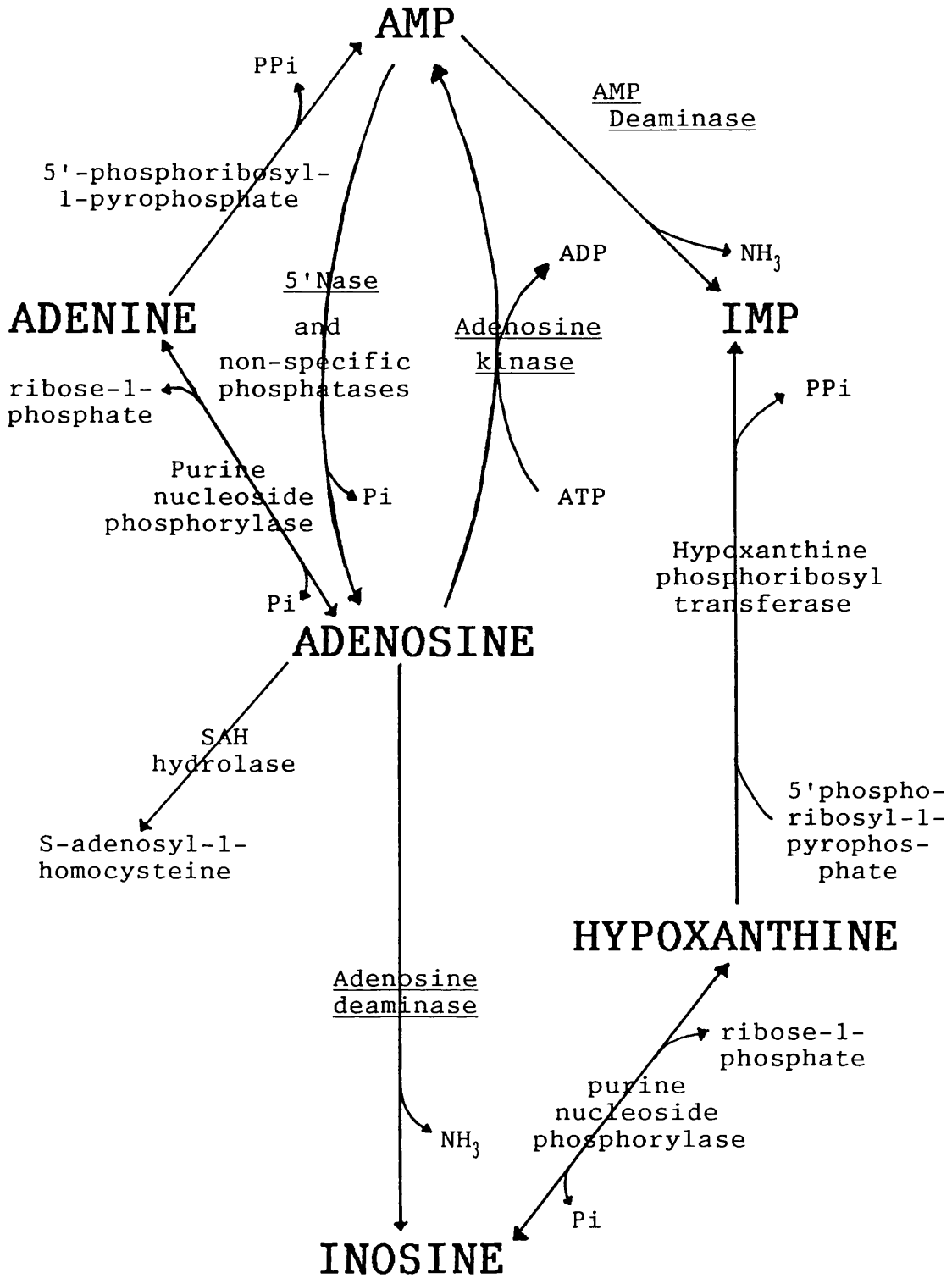


FIGURE 1.2: Pathways Important to Adenosine Metabolism

AMP = adenosine 5'-monophosphate
ADP = adenosine 5'-diphosphate
ATP = adenosine 5'-triphosphate
IMP = inosine 5'monophosphate
SAH = S-adenosyl-1-homocysteine
5'Nase = 5'-nucleotidase
Pi = orthophosphate
PPi = pyrophosphate

adenosine as substrate must be considered. Figure 1.2 shows the metabolic pathways of importance to adenosine formation and degradation.

1.4.2 AMP Deaminase (EC 3.5.4.6)

As can be seen in Figure 1.2, AMP deaminase catalyses the deamination of AMP to inosine-5'-monophosphate (IMP). Both these 5'-nucleotides are readily metabolised by 5'Nases though obviously the product derived from the dephosphorylation of IMP would be inosine. Furthermore, as will be seen in Section 1.5.1, certain 5'Nases prefer IMP as substrate, whilst others have a far greater affinity for AMP. AMP deaminase may therefore prove to be a strong regulator of cellular metabolism.

AMP deaminase has reported K_ms ranging from 60μM in rabbit muscle (Nikiforuk & Colowick, 1956) through to 2.7mM in calf brain (Setlow & Lowenstein, 1967). However, its apparent affinity for AMP is increased by the presence of ATP and lithium ions (and to a lesser extent sodium and potassium ions) without altering the maximum velocity (Setlow & Lowenstein, 1967). Additionally, both orthophosphate and pyrophosphate are competitive inhibitors (Lee, 1960). Under normal physiological conditions it therefore remains questionable whether this enzyme would be particularly active. Obviously, a reduction in the adenylate charge could easily alter this delicate balance. It has been suggested

that the route of AMP degradation is dependent on cell type. Those cells capable of generating ATP through anaerobic glycolysis (kidney medulla, erythrocytes, blood leukocytes, white skeletal muscle) tend to utilise the AMP deamination pathway to IMP; whilst the direct dephosphorylation of AMP to adenosine predominates in organs that must rely on oxidative phosphorylation (e.g.brain, heart) (Olsson and Pearson, 1990). There have been insufficient citations to confirm or repudiate this notion. Nevertheless, increasing findings of a low K_m, AMP-preferring, soluble 5'Nase (see section 1.5.2) would tend to favour the adenosine pathway.

1.4.3 Adenosine Kinase (EC 2.7.1.20)

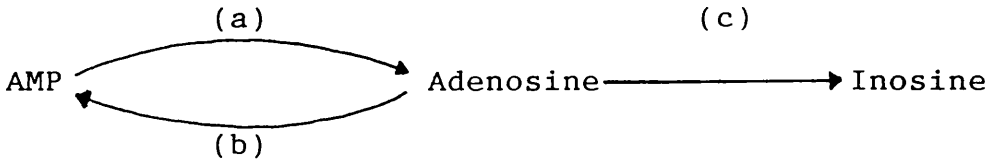
Adenosine kinase, also referred to in some documents as adenosine 5'phosphotransferase, catalyses the phosphorylation of adenosine back to AMP. This reaction can be shown as:

ATP + adenosine = ADP + AMP

The high energy requirement for ATP regeneration (Caputto, 1962), together with the enzyme's strong affinity for adenosine ($K_m \leq 2\mu M$) (Lindberg *et al*, 1967; Olsson *et al*, 1972; Miller *et al*, 1979; Fisher & Newsholme, 1984) makes the reaction essentially irreversible.

The kinetics of adenosine kinase suggest that this enzyme is the primary catalyst for adenosine removal, by recycling the nucleoside. This is perhaps necessary under

normal conditions when the many potent actions of adenosine, if allowed to interact with its receptors, are undesirable. However, if the steady-state is unbalanced, resulting in a rise in adenosine production, then experiments have shown an increase of inosine, and adenosine to a lesser degree, in the extracellular medium (Pull & McIlwain, 1972; Rubio *et al*, 1973; Berne & Rubio, 1974; Mentzer *et al*, 1975). This may be due, as suggested by Arch & Newsholme (1978), to the substrate cycle between adenosine kinase and 5'Nase with adenosine deaminase (depicted below) in which the kinase (b) is approaching or at its substrate saturation level under basal conditions, whilst the deaminase (c) is functioning well below its K_m. Thus a small rise in 5'Nase (a) activity will push the balance in favour of adenosine deaminase (c) catalysed removal of adenosine from the cell.



1.4.4 Adenosine Deaminase (EC 3.5.4.4)

Adenosine deaminase, mentioned in section 1.4.3 above, is responsible for the deamination of adenosine to inosine. The latter compound, though equally transportable through the plasma membrane (Plagemann & Wohlheuter, 1983), is not active at the adenosine receptors. Under neutral pH conditions, K_ms have been measured for vertebrate cytoplasmic

adenosine deaminase ranging from 46μM in rat liver (Arch & Newsholme, 1978) and 35μM in calf gut (Coddington, 1965) down to 6.6μM in domestic fowl heart (Arch & Newsholme, 1978).

There is also an ecto-adenosine deaminase which is reversibly bound to the extracellular surfaces of cells via a glycoprotein called adenosine deaminase-binding protein (Schrader & Stacy, 1977; Daddona & Kelley, 1978; Andy & Kornfeld, 1982; Schrader & Bryer, 1982; Trotta, 1982). The extracellular degradation of adenosine has a two-fold benefit: rapid removal of adenosine after interaction with its receptors, and prevention of raised blood adenosine levels circulating beyond the adenosine-generating tissue. The K_m of adenosine deaminase is as low and sometimes even lower than the K_m of the adenosine transporter in some tissues (Olsson & Pearson, 1990) which suggests that the ecto-enzyme may be yet another metabolic control on the "local hormonal" activity of adenosine.

1.5 5'-Nucleotidase (EC 3.1.3.5)

The physiological role of adenosine and the significance of the above mentioned enzymes involved in the adenosine metabolic pathways (sections 1.4.1-1.4.4) relates directly to the role of 5'Nase. Since it appears the 5'Nase is the primary enzyme responsible for adenosine formation, it remains to be determined what is the significance of the various different forms of 5'Nase in different tissues and

within any given cell type. The following sections describe the multiple forms of 5'Nase which have been reported to date, with especial emphasis on the ecto-enzyme.

1.5.1 Cytosolic 5'Nases

Early work investigating cytosolic 5'Nase in the late 1960s and throughout most of the next two decades was based on the assumption that there was one cytosolic form, one membrane-bound form, and later, one lysosomal form of the enzyme. However conflicting data, especially with respect to the kinetics of the cytosolic variety of 5'Nase, has in recent years led to the realisation that there are two different high K_m forms of the cytosolic 5'Nase and another low K_m soluble 5'Nase - possibly originating from the e5'Nase.

The cytosolic 5'Nase has been purified to varying degrees through to homogeneity and characterized from a variety of tissues and species including pigeon heart (Gibson & Drummond, 1972; Skladanowski & Newby, 1990); chicken liver (Naito & Tsushima, 1976); rat liver (Van den Berghe *et al*, 1977; Itoh, 1981); human placenta (Madrid-Marina & Fox, 1986; Spychala *et al*, 1988); rat heart (Itoh *et al*, 1986; Truong, 1988); rabbit heart (Yamazaki *et al*, 1991); bovine liver (Zekri *et al*, 1988); bovine brain cortex (Montero & Fes, 1982) and rat forebrain (Lai & Wong, 1991).

The conflicting reports with respect to cytosolic 5'Nase properties provoked a variety of explanations, not excluding in more recent years, the co-existence of more than one cytosolic 5'Nase. These explanations included species and tissue differences (Itoh & Tsushima, 1972; Van den Berghe *et al*, 1977; Newby, 1988), the combined regulation by a heterogeneous metabolic pool (Madrid-Marina & Fox, 1986) and adenylate charge fluctuations resulting from changes in the energy status of the cell (Itoh, 1981; Itoh *et al*, 1986).

The first purifications and characterizations demonstrated that cytosolic 5'Nase had a relatively high K_m (in the millimolar range) for IMP, GMP and AMP, though a distinct preference for IMP and GMP was usually observed. One notable exception to this where AMP was found to be the preferred substrate was in cytosolic 5'Nase from pigeon heart (Gibson & Drummond, 1972; Newby, 1988; Skladanowski & Newby, 1990). Generally it was found that the substrate-activity plots when using IMP as substrate were hyperbolic, but this changed to sigmoidal when AMP was used. A range of K_m values from different publications is presented in Table 1.1. Activities and values for maximum velocity (V_{max}) were much the same for all 5'-monophosphate nucleotides tested under the same conditions. These first cytosolic 5'Nases were also found to be absolutely dependent on divalent cations with Mg^{2+} being the most effective; inhibited by orthophosphate (Pi) which had the effect of changing the hyperbolic kinetics with IMP to sigmoidal and reducing the V_{max}; and activated by

ATP and ADP, except in a low K_m human placental cytoplasmic 5'Nase (Madrid-Marina & Fox, 1986); in rat kidney soluble-5'Nase (Le Hir & Dubach, 1988); and in rat brain soluble-5'Nase (Lai & Wong, 1991; Orford *et al*, 1991); with a pH optimum usually at pH6.5. Exceptions to this last point (i.e. an acidic pH optimum) include the soluble 5'Nase from rat liver which showed a pH optimum at 7.4 (Van den Berghe *et al*, 1977), from bovine brain with pH optimum between 7.25 and 7.5 (Montero & Fes, 1982) and from bovine liver with two pH optima at 7.5 and 9.5 (Zekri *et al*, 1988). The characterisations indicated above are listed in Table 1.1 along with the relevant references.

Most investigators began to realise that there may be more than one cytosolic 5'Nase in any given species, or even tissue, towards the mid- to late 1980's. However, the co-existence of two cytosolic 5'Nases was finally established within the rat heart by Truong *et al*, in 1988. The two soluble 5'Nases isolated by this group showed high K_ms (in the mM range) as with previously identified cytosolic 5'Nases, but by the criterion of V_{max}/K_{app} they demonstrated that one preferred IMP as substrate whilst the other had a preference for AMP. The co-existence of these two soluble 5'Nases within the rat heart led Truong and colleagues to propose that the AMP-preferring enzyme was likely to be active during times of hypoxia, increased workload and with metabolic stresses. This concept had previously been proposed for a single 5'Nase altering its role under the

varying conditions. It must be emphasized that the two proposals are not incompatible. Subsequent investigations also identified at least two soluble 5'Nases co-existing in other tissues (Spychala *et al*, 1989; Orford *et al*, 1991).

Several groups have since discovered a possible third form of soluble 5'Nase. Its co-existence with the earlier identified cytosolic 5'Nases has not yet been established, but it seems likely to be found in the same cells as its properties vary considerably and as such would not be redundant. This third form of soluble 5'Nase shares many properties with the ecto-enzyme, but does vary in some of its characteristics as described below. The suggestion by investigators such as Piec & Le Hir (1991) that it is artifactual and derived from the preparation of the original tissue homogenate therefore, does not seem to be the case. It is possible that the differences arise from post-translational modifications of the same gene product.

1.5.2 "Soluble" Low K_m 5'Nase

Unlike the previously discussed cytosolic 5'Nases which demonstrate high K_ms (in the mM range) for both IMP and AMP; have a slightly acidic pH optimum (~pH6.5) and are activated by ATP and ADP, the more recently discovered "soluble" 5'Nase prefers AMP as substrate with a low K_m ranging from 9.5μM (Le Hir & Dubach, 1988), 7-15μM (Spychala *et al*, 1989), and 28μM (Orford *et al*, 1991), to 270μM (Lai

& Wong, 1991). It has an alkaline pH optimum at ~7.5 and sometimes a second optimum at ~pH9.0; and is strongly inhibited by ATP and ADP.

This low K_m "soluble" 5'Nase has also been shown to contain 1 mole *myo*-inositol per mole protein (Klemens *et al*, 1990) and its hydrophobicity as determined by Triton X-114 phase separation can be altered by glycosylphosphatidyl-inositol-specific phospholipase C (PI-PLC) (Piec & Le Hir, 1991; Dr.M.Orford - private communication, 1993). This would suggest that, like the ecto-enzyme, it has had a glycosyl-phosphatidylinositol anchor at some stage in its processing. Furthermore this enzyme can be inhibited by antibodies raised against the ecto-enzyme (Piec & Le Hir, 1991) and appears to be glycosylated (Fritzon *et al*, 1986; Lai & Wong, 1991) which is highly unusual for a cytosolic protein. In fact, part of its purification by most groups involves the use of lectin affinity columns.

Despite the strong similarities outlined in the above paragraph between the low-K_m "soluble"-5'Nase and the ecto-enzyme, there are also some unavoidable differences. These include the work carried out on rat forebrain soluble-5'Nase versus ecto-5'Nase by Lai & Wong (1991) in which by using a variety of different glycosidases they demonstrated that the glycoprotein nature of the two enzymes differed. Furthermore, the molecular weights of the "soluble"- versus ecto-enzyme differ quite dramatically. The "soluble" 5'Nase

TABLE 1.1

Summary of Soluble 5'Nases:

Activities, Activators and Inhibitors, and pH Optima

Property	High K_m Cytosolic 5'Nase		Low K_m "Soluble" 5'Nase
	IMP-specific	AMP-specific	
Preferred substrate	IMP	AMP	AMP
K_m for preferred substrate	0.5-1.4mM	1-5mM	7-20µM
Subunit size	51-58kDa	40kDa	60-80kDa
pH optimum	6.5	6.5-7.0	7.0-8.0
Magnesium ions	Required	Required	Not Required
Phosphate ions	Inhibition	Inhibition	No inhibition
ATP	Activates in the mM range with ATP>ADP	No effect	K_i 60-100µM Inhibition is reversed by $MgCl_2$
ADP		Activates in the mM range	K_i 2-15µM
Anti-e5'Nase antibody	No inhibition	No inhibition	Potent inhibition
See reference numbers below:	1-8	7, 9-15	5, 15-25

Reference Numbers:

1. Itoh & Yamada, 1991
2. Newby *et al*, 1987
3. Bontemps *et al*, 1989
4. Naito & Tsushima, 1976
5. Spychala *et al*, 1988 & 1989
6. Tsushima, 1986
7. Itoh *et al*, 1986
8. Worku & Newby, 1983
9. Gibson & Drummond, 1972
10. Newby, 1988
11. Skladanowski & Newby, 1990
12. Collinson *et al*, 1987
13. Yamazaki *et al*, 1991
14. Truong *et al*, 1988
15. Zekri *et al*, 1988
16. Mallol & Bozal, 1983
17. Piec & Le Hir, 1991
18. Madrid-Marina & Fox, 1986
19. Klemens *et al*, 1990
20. Fritzson *et al*, 1986
21. Orford *et al*, 1991
22. Lai & Wong, 1991
23. Montero & Fes, 1982
24. Le Hir & Dubach,1988
25. Fini *et al*, 1990

has been reported at 131 kDa (Lai & Wong, 1991) and 53 kDa subunit weight (Dr.M.Orford, personal communication) by polyacrylamide gel electrophoresis, whilst the e5'Nase has a well established molecular weight of 72-76 kDa. Finally, there are also conflicting reports for the requirement of Mg^{2+} ions. Some reports suggest an absolute requirement of these divalent ions for the "soluble"-5'Nase (Fritzon *et al*, 1986; Le Hir & Dubach, 1988), whilst others found that the "soluble" enzyme was active in the absence of $MgCl_2$, but that activity increased up to four-fold on the addition of 1mM $MgCl_2$ (Fritzson *et al*, 1986). Still others found no Mg^{2+}-sensitivity at all when tested directly, but that inhibition caused by ATP could be partially or wholly reversed by the addition of these divalent cations (Mallol & Bozal, 1983; Dr.M.Orford, private communication). It therefore appears that there is a third "soluble" 5'Nase, but it has not yet been established whether it is cytosolic, secreted extracellularly or cleaved from the membrane by phosphatidylinositol-specific phospholipase C.

1.5.3 Lysosomal 5'Nase

Several reports have been made supporting the concept of the existence of a distinct lysosomal 5'Nase (Maguire & Luzio, 1985; Wada *et al*, 1987; Draye *et al*, 1987). Lysosomes from rat liver were shown to contain both membrane-bound and soluble forms of 5'Nase in a ratio of 3:1 respectively. However, characterization of the membrane-bound form appears

to correspond to the ecto-enzyme in most respects (Tanaka *et al*, 1989) except for the calculated K_m which may be due to differing assay conditions. It was suggested by Zimmermann in his 1992 review that the soluble-5'Nase present in lysosomes may result from an endogenous GPI-specific phospholipase C cleavage of the membrane-bound form.

1.5.4 Ecto-5'Nase (e5'Nase) - General

The e5'Nase is the most thoroughly characterised of all the 5'Nases, but still there remain many unanswered questions. E5'Nase is ubiquitous throughout vertebrate tissues (Drummond & Yamamoto, 1971; Riemer & Widnell, 1975; Arch & Newsholme, 1978; for review see Zimmermann, 1992) and as such has been used for many years as a plasma membrane enzyme marker. In humans, the gene encoding e5'Nase is located on chromosome 6 (Boyle *et al*, 1988). E5'Nase catalyses the hydrolysis of nucleoside 5'-monophosphates exclusively, with no activity for 2'- and 3'-monophosphates (Baer *et al*, 1966; Burger & Lowenstein, 1970). Furthermore, this enzyme is stereo-selective of its substrates, having no activity with the L-enantiomer (Cusack *et al*, 1983). Of all the nucleoside 5'-monophosphates, AMP is the preferred substrate for e5'Nase (Pearson & Coade, 1987; De Pierre & Karnovsky, 1974; Cusack *et al*, 1983) with K_m values being reported in the low micromolar range as shown in Table 1.2. However, it should be noted that this activity would be dependent on the appropriate substrate being extracellular

as the enzyme is an ectoenzyme, i.e. is located on the external surface of the plasma membrane (Gurd & Evans, 1974; Newby *et al*, 1975). Generally, a single broad pH optimum has been identified at approximately pH7.5 for most mammalian e5'Nases studied, but removal of bovine liver e5'Nase from its membrane environment appeared to significantly reduce its tolerance to changes in pH (Harb *et al*, 1983). There has also been a report of two pH optima for bull seminal plasma 5'Nase (Bodansky & Schwartz, 1963).

E5'Nase has a subunit molecular weight of ~72kDa, although the reports vary ±8kDa depending on the species and tissue of origin (see Table 1.2). Biosynthetic labelling with [^{35}S]-methionine and using pulse chase experiments in rat liver (Wado *et al*, 1986) and a rat hepatoma cell line (Van den Bosch *et al*, 1986) has demonstrated that e5'Nase is initially synthesized as a 61kDa precursor, including a cleavable 2kDa signal sequence. Later production of rat liver and human placental e5'Nase cDNA enabled Misumi *et al* (1990; 1990a) to calculate a molecular weight of almost 64kDa from the open reading frames, however the cDNA was shorter by 0.7kb and 0.55kb respectively, than the major fragments identified with the mRNAs, indicating incomplete cDNAs in both instances. This discrepancy may benefit from further investigation as the authors also made predictions regarding a signal sequence at the N-terminal end and a sequence at the C-terminal suitable for phosphatidylinositol-glycan (GPI)-anchorage (see section 1.5.5).

TABLE 1.2

Examples of E5'Nase Properties Reported in Different Tissues and Species

Property	Value/Effect Reported for E5'Nase	Reference Number Listed Below: (Noted only when variation exists between tissues and/or reports)
Preferred substrate	AMP	-
K_m for AMP in mammals	15.3µM 4.7µM 3.2(±1.6)µM 83µM	1 2 3 14
Molecular weight of subunits (homodimeric)	70kDa 73kDa 72kDa 80kDa	1 4,13 5,6,14 7
Glycosylation sites	4 in placenta 5 in liver	8 5,9,10
pH Optimum	7.5 (7.5-8.3) 8.5	1 11
EDTA and EGTA	Both inhibitory, reversible by divalent cations	1,7
Metalloprotein	Suggested Mn^{2+}/Mg^{2+} Suggested Ca^{2+} Identified Zn^{2+}	1 7 12
ATP	K_i 1.5µM K_i 410µM	2 14
ADP	K_i 0.082µM K_i 3.4µM	2 14
α,β-methylene-ADP	K_i 0.006µM K_i 0.23µM	2 14

References: (SEE ALSO ZIMMERMANN, 1992 - FOR REVIEW)

1. Harb *et al*, 1983
2. Collinson *et al*, 1987
3. Dieckhoff *et al*, 1987
4. Thompson *et al*, 1987
5. Wado *et al*, 1986
6. Baron & Luzio, 1987
7. Fini *et al*, 1985
8. Misumi *et al*, 1990a
9. Van den Bosch *et al*, 1986
10. Misumi *et al*, 1990
11. Riemer & Widnell, 1975
12. Fini *et al*, 1990
13. Klemens *et al*, 1990
14. Lai & Wong, 1991

The difference between precursor (~61kDa) and mature protein (~72kDa) sizes can be accounted for mostly by the asparagine-linked glycosylation which takes place in the endoplasmic reticulum, bringing the molecular weight up to 68kDa. Four sites in human placenta (Misumi *et al*, 1990a) and five sites in rat liver (Van den Bosch *et al*, 1986; Wado *et al*, 1986; Misumi *et al*, 1990) have been predicted for N-linked glycosylation. Evidence supporting the suggestion of extensive glycosylation is provided by the binding of a variety of lectins, including strong non-competitive inhibition by concanavalin A (Harb *et al*, 1983). Final post-translational modifications, including sialylation and the development of glycosylation complexity occur in the golgi body to form the mature 72kDa protein.

The mature e5'Nase appears to exist *in vivo* as a homodimer (Harb *et al*, 1983; Fini *et al*, 1985; Buschette-Brambrink & Gutensohn, 1989; Fini *et al*, 1990). The addition of dithiothreitol was shown to inhibit e5'Nase by 80% to 100% indicating the importance of disulphide bridges for catalytic activity (Harb *et al*, 1983; Fini *et al*, 1985), but it was not determined whether these bridges were necessary to maintain a functional dimeric form or whether the monomer could be active on its own with the importance of the disulphide bridge being in the active site. Inhibition studies carried out by Worku *et al* (1984) indicated that the active site of e5'Nase contains an important histidyl residue and possibly also a susceptible cysteinyl residue. Inhibition of the

enzyme with diethylpyrocarbonate (DEPC) followed by rapid reactivation with hydroxylamine (NH_2OH) implicated either histidine or serine. However phenylmethylsulphonylfluoride (PMSF) did not affect the catalysis which negated the possibility of a serine esterase. Activity was not completely restored with NH_2OH which also indicated the presence of a cysteinyl residue. Chelating agents such as EDTA and EGTA were also shown to be inhibitory (Fini *et al*, 1985). Early studies suggested that e5'Nase is a metalloprotein - incorporating magnesium or manganese (Naito & Lowenstein, 1981; Harb *et al*, 1983), but more recent evidence has shown that e5'Nase is a zinc metalloprotein with one zinc ion per subunit (Fini *et al*, 1990). They showed that removal of the zinc ions, followed by their replacement resulted in a linear reactivation indicating that both zinc ions are equally involved in the catalytic process.

Metabolic inhibitors of e5'Nase include adenosine tri- and di-phosphates (ATP and ADP respectively), as well as some of their analogues such as α,β-methylene-ATP and -ADP. Unlike the cytosolic 5'Nases, neither ortho- or pyro-phosphates have any effect on e5'Nase activity. ATP, ADP and their analogues are potent competitive inhibitors of e5'Nase activity with inhibition constant (Ki) values generally in the low micromolar, or even nanomolar, range (see Table 1.2) and it has been suggested that ATP and ADP may act as endogenous regulators of e5'Nase in addition to the more obvious modulation of cytosolic 5'Nase activity. Recent work

in this laboratory by Dr.M.Orford has identified a high molecular weight (i.e.≥30kDa) factor which partially co-purifies with the "soluble" low-K_m 5'Nase previously discussed in section 1.5.2. Replacement of this factor confers significantly greater ATP-sensitivity both to the "soluble" 5'Nase and, as will be shown in Chapter 4, to the e5'Nase. It is interesting to note the work of Buschette-Brambrink & Gutensohn (1989) in which it was shown that chemical crosslinking of isolated isoforms of human placental e5'Nase produced dimers of ~140kDa (confirming the α,α-homodimeric protein previously discussed), but the same treatment of e5'Nase whilst still bound to the membrane produced a protein of ~97kDa (i.e. the partner to e5'Nase being a protein ~30kDa). The potential for the "soluble" low-K_m 5'Nase being an endogenously released form of e5'Nase, combined with the above studies regarding the ATP-sensitivity conferring factor and the crosslinked 30kDa protein, require further investigations to determine whether there is any connection.

In addition to the putative role of e5'Nase for extracellular adenosine formation (depending on the availability of extracellular substrate), a further two roles have been suggested as a result of e5'Nase interaction with components of the extracellular matrix. Dieckhoff *et al* (1986) first showed that fibronectin inhibited and laminin stimulated the hydrolase activity of e5'Nase, whilst collagen had no discernable effect. They proposed that e5'Nase could be the mediator for fibronectin and laminin to organise the

intracellular cytoskeleton. A more direct role was later proposed by the same laboratory in which the cellular adhesiveness to components of the extracellular matrix could be regulated by cleavage of the e5'Nase GPI-anchor (Stochaj *et al*, 1989a). Based on the ability of e5'Nase to bind lectins and the assumption of the availability of extracellular AMP to produce adenosine locally, another group much earlier suggested that concanavalin A may regulate cAMP accumulation via adenosine-receptor interaction (Dornand *et al*, 1980). It appears that an area of investigation would be to determine whether fibronectin and laminin could similarly regulate cAMP accumulation via the activity of e5'Nase.

1.5.5 E5'Nase - Phosphatidylinositol-glycan (PI-G) Anchor

In the last fifteen years many ecto-enzymes have been identified as being covalently anchored to the external surface of the plasma membrane via a novel mechanism involving a glycosylated phosphatidylinositol molecule. This is referred to as glycosyl-phosphatidylinositol (GPI) or phosphatidylinositol-glycan (PI-G) anchorage. This form of anchoring is widely distributed in eukaryotes from protozoa through to mammals, but has not yet been found in higher plants or in prokaryotic cells. All these proteins are hydrophilic and without the anchor it appears that they would be soluble proteins. However, that is where their similarities cease (see examples in review by Low, 1987). There

appears to be no functional or structural resemblance between these proteins, unless it is a requirement for regulation by selective cleavage, or for dispersal, mobility or packing-density within the membrane which could be satisfied by the anchor itself. In polarized epithelial cells it has been shown that the anchor may be involved in the mechanism for protein sorting with GPI-anchored proteins always being transported to the apical membrane (Lisanti *et al*, 1988; Lisanti & Rodriguez-Boulan, 1990).

A schematic diagram of the GPI-anchor is shown in Figure 1.3. As can be seen in the illustration, the anchor consists of a *myo*-inositol-containing phospholipid molecule which is associated with the membrane via the diacylglycerol portion, and extracellularly is glycosidically linked via glucosamine to a glycan moiety containing several mannose residues and possibly also galactose in some cases. The glycan has a mannose-6-phosphate at its non-reducing end which is phosphodiesterically linked to the hydroxyl residue of ethanolamine (usually two such linkages in mammals). The ethanolamine is then covalently bound to the C-terminal end of the protein via a peptide bond. The membrane fatty acids vary, but generally consist of any combination of stearic acid, myristic acid and palmitic acid. Several good reviews exist on the structure and potential functions of GPI-anchors (Cross, 1987; Low, 1987; Low & Saltiel, 1988; Ferguson & Williams, 1988).

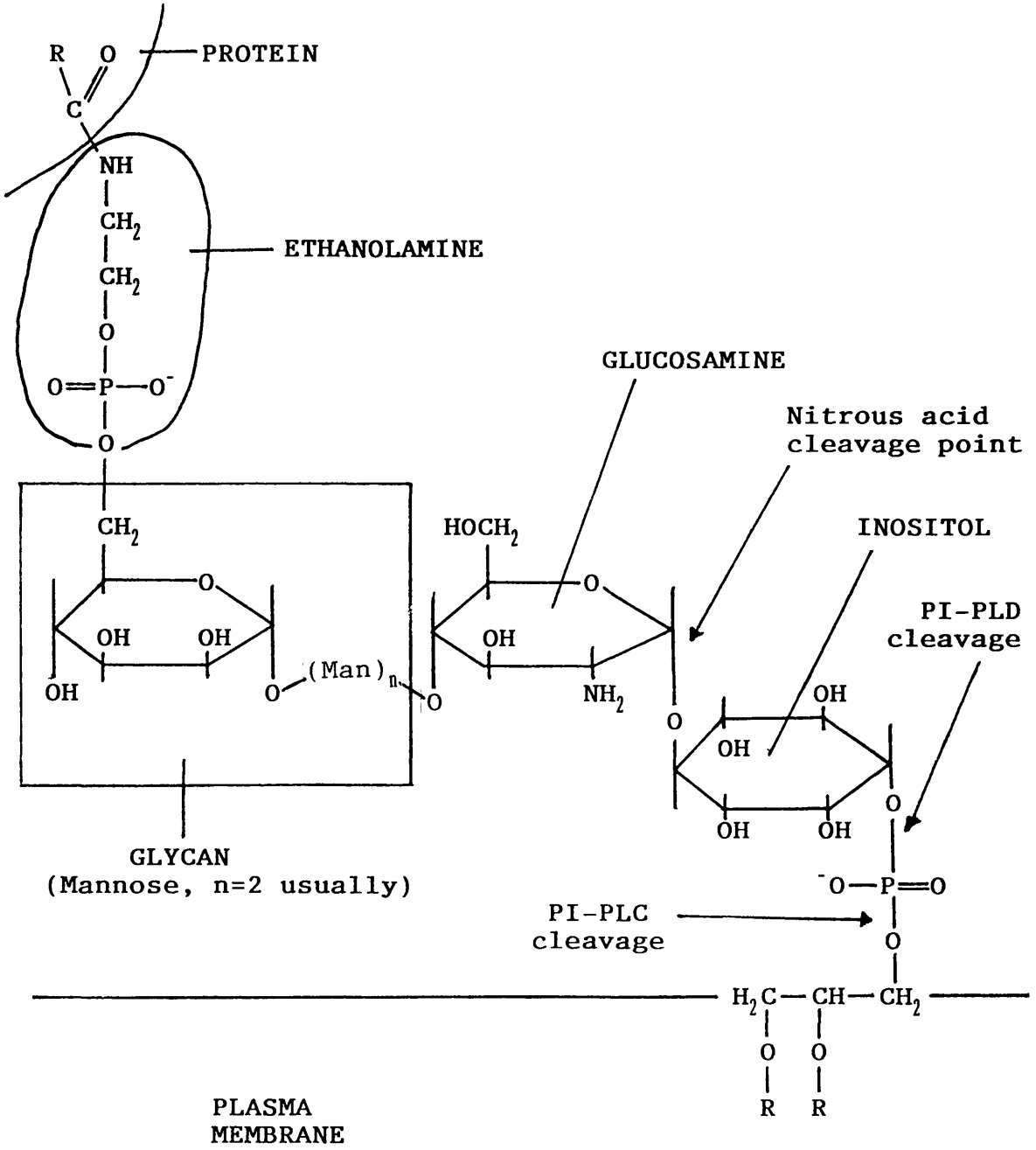


FIGURE 1.3: Schematic Diagram of a Phosphatidylinositol-Glycan Anchor (PI-G anchor)

Biosynthetic labelling and site-directed mutagenesis studies have shown that the attachment of the PI-G anchor occurs after both the anchor and the protein are fully formed (Ferguson *et al*, 1985; Berger *et al*, 1988; Doering *et al*, 1989; Caras & Weddell, 1989). Despite there being no sequence homology in all the proteins studied to date, several groups have determined some of the requirements for GPI-anchoring. These include a short N-terminal signal sequence of 2-3kDa for translocation into the endoplasmic reticulum and also a cleavable C-terminal stretch of between 15 to 31 variable amino acids (conferring hydrophobicity) with a strong requirement for a serine residue, followed by either an aspartate, an alanine, a glycine, or a cysteine at the actual cleavage/substitution site (Micanovic *et al*, 1990; Kodukula *et al*, 1991; Moran *et al*, 1991). Micanovic *et al* (1990) using a rabbit reticulocyte lysate processing system for their *in vitro* translations with their site-directed mutations, postulated that the enzyme responsible for both the C-terminal cleavage and condensation reaction with the anchor is a putative transamidase, but this would require further investigation.

Evidence in favour of PI-G anchorage for e5'Nase is very strong (Low & Finean, 1978; Shukla *et al*, 1980; Panagia *et al*, 1981; Baron *et al*, 1986; Bailyes, *et al*, 1990; Ogata *et al*, 1990; Misumi *et al*, 1990; Misumi *et al*, 1990a). PI-G anchored proteins tend to be identified by the ability to release them with phosphatidylinositol-specific phospholipase C

(PI-PLC) cleavage and concomitantly to convert them into a hydrophilic and soluble form. Although e5'Nase certainly falls into this category by virtue of PI-PLC cleavage, it is only partially susceptible. Partial cleavage of e5'Nase has been reported from a variety of species and tissues including bovine synaptosomes - 10.5%, lymphocytes - 12.5% and liver - 18% (Zekri *et al*, 1989); rat hepatocytes - 30% (Shukla *et al*, 1980); chicken gizzard and human pancreatic adenocarcinoma cells - 40 to 50% (Stochaj *et al*, 1989); and human placenta - ~70% (Thompson *et al*, 1987). Zekri *et al* (1989) noted a strong influence of species on susceptibility of e5'Nase to PI-PLC cleavage, as opposed to PI-PLC cleavage of alkaline phosphatase which varied little if at all. Complimentary DNA has been made for rat liver and human placental e5'Nase which supports the claim for PI-G anchorage (Misumi *et al*, 1990; Ogata *et al*, 1990; Misumi *et al*, 1990a; Suzuki *et al*, 1993). There have however, been several reports supporting the idea of two species of e5'Nase - the first being susceptible to PI-PLC cleavage and therefore GPI-anchored, whilst the second is PI-PLC resistant and may be using a transmembrane segment for anchorage (Zachowski *et al*, 1981; Dieckhoff *et al*, 1987; Grondal & Zimmermann, 1987; Thompson *et al*, 1987; Stochaj *et al*, 1989; Klemens *et al*, 1990). There is a precedent for this duality in membrane anchoring previously established for the LFA-3 and N-CAM cell adhesion glycoproteins (Dustin *et al*, 1987; Gower *et al*, 1988). Alternatively, resistance to PI-PLC cleavage in some GPI-anchored proteins such as acetylcholinesterase (AChE) has

been shown to result from palmitoylation of the inositol ring (Roberts *et al*, 1988). This group also demonstrated that an endogenous plasma GPI-specific phospholipase D (GPI-PLD) was able to cleave the intact acylated anchor. In direct contrast, Toutant *et al* (1989) found that they had to remove the acylation from the inositol ring of the AChE anchor by treatment with hydroxylamine prior to either PI-PLC or GPI-PLD cleavage and that PI-PLC was more effective.

Several research groups have identified and purified endogenous PI-G specific phospholipases including *Trypanosoma brucei* membrane-bound PI-PLC (Bulow & Overath, 1986; Hereld *et al*, 1986; Fox *et al*, 1986), rat liver plasma membrane PI-PLC (Fox *et al*, 1987), rat and rabbit plasma PI-PLD (Low & Prasad, 1988), bovine plasma PI-PLD (Huang *et al*, 1990), human plasma PI-PLD (Davitz *et al*, 1987; Davitz *et al*, 1989; Balsinde & Mollinedo, 1990), and a human serum acid-lipase which, based on its cleavage products, appeared to be neither a PLC or a PLD (Cardoso de Almeida *et al*, 1988). The isolation of these endogenous phospholipases demonstrated the existence of an *in vivo* mechanism for anchor cleavage, but most of the groups who undertook the purifications also found that the phospholipases required activation in one form or another. Thus, the identification of an endogenous activator/regulator was sought and appears to have been quickly found. It was suggested by several groups that PI-PLC/D may be targeted by insulin. Concomitant research had identified an insulin-sensitive phosphatidylinositol-glycan at the outer

surface of insulin-sensitive cell membranes (Mato *et al*, 1987; Mato *et al*, 1987a; Alvarez *et al*, 1988; Macaulay & Larkins, 1990; Varela *et al*, 1990). It is interesting to note that Varela *et al* (1990) found a distinct lack of glycosyl-phosphatidylinositol on the outer surface of erythrocytes which are insulin-insensitive cells. The phospholipase-released inositol glycan headgroup was shown to mimic many of insulin's actions on intermediary metabolism, but not on glucose transport, in a variety of cell types such as adipocytes (Alemany *et al*, 1987; Saltiel & Sorbara-Cazan, 1987; Macaulay & Larkins, 1990), BC_3H cells (Saltiel *et al*, 1986; Romero *et al*, 1988), and in rat liver (Larner *et al*, 1988). Further evidence to support the idea of insulin activation of endogenous phospholipases and the subsequent release of an inositol glycan headgroup as mediator comes from studies on disease states such as diabetes mellitus in which insulin action is impaired. This will be discussed in detail in Section 1.8.1, but essentially it has been found that incorporation of radiolabelled components into glycosylated-phosphatidylinositol is impaired in the absence of insulin (Bergh *et al*, 1988; Thakkar *et al*, 1990) and insulin re-exposure appears to rectify this situation, even in the presence of the protein *de novo* synthesis inhibitor cycloheximide, after an initial period of PI-G anchored protein release (Lisanti *et al*, 1989). Furthermore, it has been found that control of protein phosphorylation by insulin can be inhibited by exogenous addition of individual components of the inositol glycan headgroup (Strålfors & Alemany, 1990)

and by the addition of anti-inositol glycan antibodies (Romero *et al*, 1990). Nevertheless, it still remains unclear whether the inositol glycan headgroup released by insulin is derived from the PI-G anchor or as some authors suggest (see for example Larner *et al*, 1988) from a molecule which resembles the anchor closely, but is not identical. Certainly PI-G anchored proteins have been shown to be released by the action of insulin (Ishihara *et al*, 1987; Romero *et al*, 1988; Chan *et al*, 1988; Lisanti *et al*, 1989) and whether the anchors act directly as insulin mediators, or whether their cleavage is incidental, the implications for the regulation of proteins which are PI-G anchored, such as e5'Nase, during insulin fluctuations cannot be ignored.

1.6 White Adipose Tissue (WAT)

White adipose tissue consists predominantly of lipid-laden cells (adipocytes), connective tissue (collagenous matrix), nerves and blood vessels. The adipocyte has been described as having a "signet-ring" shape resulting from the large droplet of lipid at its centre which flattens the nucleus against the plasma membrane, thus causing a protrusion. Other components of the cell found in the thin cytoplasmic layer surrounding the lipid droplet include typical subcellular organelles such as smooth endoplasmic reticulum, scattered ribosomes, a small Golgi zone and various mitochondria with random distribution. Very rarely, rough endoplasmic reticulum, microtubules and microfilaments,

and glycogen particles can also be observed in mature white adipocytes (Slavin, 1985).

Primary innervation of WAT appears to be adrenergic, regulating the lipolytic activity and vascular responses of the capillary bed (Ballard & Rosell, 1971; Ballard *et al*, 1974; Slavin & Ballard, 1978). Although only a few adipocytes are directly innervated, cellular junctions between the adipocytes appear to transfer information between the innervated and non-innervated cells via electrical coupling (Sheridan, 1971). Other nerve fibres identified are peptidergic, using peptide release for both vasoregulation and as sensory neurones (Lundberg *et al*, 1982; Rökeus *et al*, 1983).

Stimulation of the adrenergic neurones in WAT results not only in increased lipolysis and blood flow (Fredholm, 1974; Rosell & Belfrage, 1979), but also in increased adenosine production (Hjemdahl *et al*, 1979). The adrenergic receptors mediating lipolysis and vasodilation (β-adrenoceptors) are believed to be localized at some distance from the nerve terminals, possibly even responding to circulating catecholamines, whilst α-adrenoceptors which regulate vasoconstriction are close to the nerve terminals (Rosell & Belfrage, 1979).

Adenosine's actions on WAT are mediated via adenosine receptors present on both the vasculature (Sollevi &

Fredholm, 1981) and the adipocytes (Trost & Stock, 1979). The adenosine receptors on the vasculature responsible for vasodilation may possibly be A_2 receptors due to the higher potency of 2-chloro-adenosine and N^6-phenylisopropyladenosine over adenosine (Sollevi & Fredholm, 1981). However, the identification of adenosine receptors on vasculature in all tissues is still unresolved (for review see Olsson & Pearson, 1990). Adenosine's action on vasculature appears to be directly vasoactive rather than involving the endothelium, the latter being non-essential for vasodilation (Furchgott, 1984). Conversely, the receptors located on the adipocytes are clearly of the A_1 type (Van Calker *et al*, 1979). The signal is transduced by a G_i protein (Londos *et al*, 1981; Moreno *et al*, 1983; Olansky *et al*, 1983) which inhibits adenylate cyclase activity and thus the accumulation of cAMP.

The increased adenosine production observed from adrenergic stimulation is likely to be due to the fatty acid turnover which in WAT results from the fatty acyl-CoA ligase reaction (Saggerson & Greenbaum, 1970; Halperin & Denton, 1969). Adipose tissue AMP concentrations rise in the presence of non-esterified fatty acids (NEFA) and may well leave the adipocytes via an as yet unknown route, to be converted to adenosine by e5'Nase. The adenosine, being a potent antilipolytic and vasodilatory agent, could then act as a metabolic signal to the vasculature regarding the extent of fatty acid turnover.

1.7 Insulin Action and its Role in WAT

In response to increased blood levels of glucose and other blood nutrients such as amino acids and digestion products, insulin is released into the circulation. Its interaction with specific insulin receptors on the surface of various cell types induces numerous cellular responses.

The insulin receptor is a tetramer consisting of $\alpha_2\beta_2$ subunits. The wholly extracellular α-subunits bind the insulin, whilst the β-subunits, which traverse the plasma membrane and protrude into the cytoplasm, contain tyrosine kinase activity (Ullrich *et al*, 1985). Insulin's interaction with its receptor results both in the autophosphorylation of tyrosine residues on several locations of the β-subunits (White *et al*, 1988; White & Kahn, 1989; Myers *et al*, 1991) and in the tyrosyl phosphorylation of multiple sites on a protein of 185kDa as determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels (White *et al*, 1985; White *et al*, 1987). The 185kDa protein is known as IRS-1 which is the acronym for Insulin Receptor Substrate-1.

IRS-1 is uniformly distributed throughout the cell with the exception of the nucleus where it is absent (Sun *et al*, 1992). Prior to insulin stimulation it is phosphorylated on serine and threonine residues, but requires the insulin stimulation for tyrosine phosphorylations and has been

identified as an important component of insulin signal transmission (Sun *et al*, 1992). The phosphorylated tyrosine residues of IRS-1 are located amongst specific amino acid motifs (either Tyr-Met-Xaa-Met or Tyr-Xaa-Xaa-Met) which, when phosphorylated, bind certain proteins containing src homology-2 and -3 (SH2 and SH3) domains (Sun *et al*, 1991; Auger *et al*, 1992). SH2 and SH3 domain-containing proteins that bind to IRS-1 which have been identified to date include: pp85-α - the regulatory subunit of phosphatidylinositol 3'-kinase (Sung & Goldfine, 1992; Hayashi *et al*, 1992; Shoelson *et al*, 1993); pp85-β - an isomer of pp85-α with unknown associations (see Myers & White, 1993 for review; Augustine *et al*, 1990); SHPTP2 - a tyrosine phosphatase (Freeman *et al*, 1992); and GRB-2/sem-5 - believed to be an adapter molecule for $p21^{ras}$-GAP ($p21^{ras}$-GTPase activating protein), thereby stimulating MAP-kinase (mitogen-activated-protein kinase) activities via $p21^{ras}$ (Lowenstein *et al*, 1992; Baltensperger *et al*, 1993; Skolnik *et al*, 1993). Some of these SH2 and SH3 containing proteins are regulated by binding directly to certain tyrosine kinase receptors when the latter are phosphorylated (e.g.epidermal growth factor [EGF] receptor) without the intermediate of IRS-1 phosphorylation (Coughlin *et al*, 1989; Cantley *et al*, 1991). More SH2 domain-containing proteins are being investigated and it is likely that some of these will be found to bind with phosphorylated IRS-1. Each IRS-1-associated protein may potentially be the beginning of an independent signalling pathway. This could explain both the pleiotrophy of insulin

action and quite conceivably how insulin-mimetics can act on individual pathways independently of the others.

Adipocytes are highly responsive to variations in circulating insulin concentrations. For many years it has been recognised that insulin plays a major counter-regulatory role in lipolysis at the level of the adipocyte. In the normal fed state *in vivo*, only noradrenaline from the sympathetic innervation can stimulate the hormone sensitive lipase at the beginning of the lipolytic pathway. Other lipolytic hormones such as glucagon and adrenaline do not circulate in high enough concentrations to stimulate the lipase, though they have been shown to do so *in vitro*. Insulin is not able to inhibit the hormone-sensitive lipase directly, although the absence of insulin may be sufficient to allow the circulating hormones mentioned above (i.e. glucagon and adrenaline) to accentuate the effect. All the lipolytic hormones act via increased levels of cAMP, thus activating the cAMP-dependent protein kinase which in turn phosphorylates a specific serine residue on the hormone sensitive lipase. The degree of phosphorylation is directly proportional to the level of activation of the lipase. Insulin, on interacting with its receptors, lowers the cAMP levels in WAT - partially by stimulating phosphodiesterase activity (Smith *et al*, 1991; Manganiello *et al*, 1992). However, it has been shown that the resulting reduction in cAMP levels is not sufficient alone to prevent lipolysis in highly stimulated cells (Londos *et al*, 1985) and it must therefore

be concluded that insulin is also acting via other mechanisms which are presently being elucidated, such as the activation of protein dephosphorylation (Strålfors & Honnor, 1989). One suggestion that has been made is that there may be two different insulin receptors - one cAMP-modulating and the other cAMP-independent, but no direct evidence for this has been provided (Ciaraldi, 1988).

In addition to adenosine's antilipolytic role (discussed in sections 1.3 and 1.6), adenosine has been shown to modulate insulin's actions for a wide variety of responses including stimulation of glucose transport (Green & Newsholme, 1979; Joost & Steinfelder, 1982; Smith *et al*, 1984; Kuroda *et al*, 1987; Lonnroth *et al*, 1987), glucose oxidation (Schwabe *et al*, 1974; Fain & Weiser, 1975), pyruvate dehydrogenase (Wong *et al*, 1984), cAMP phosphodiesterase (Wong *et al* 1985), acetyl-CoA carboxylase (Brownsey *et al*, 1984; Borthwick *et al*, 1990) and lipogenesis (Joost & Steinfelder, 1982). The interaction of adenosine with the A_1 receptors increases adipocyte's responsiveness and sensitivity to insulin in two ways: by maintaining a high efficiency of insulin action early in the signalling process and also by maintaining optimal activity once insulin's actions have been stimulated after the insulin-receptor dissociation (Schwabe *et al*, 1975; Ciaraldi, 1988). The effect of adenosine on insulin action has been shown to be distal to insulin's interaction with its receptor by the administration of adenosine deaminase. In the presence of

this adenosine utilizing enzyme, basal levels of lipolysis are increased and the anti-lipolytic action of insulin is reduced (Ebert & Schwabe, 1973; Schwabe *et al*, 1973), whilst the insulin-receptor binding is left unaffected (Hashimoto *et al*, 1987; Ciaraldi, 1988). Along the same lines, down-regulation of adenosine receptors has also been shown to alter insulin action with no concomitant change in insulin binding (Green, 1987).

One action of insulin in white adipose tissue which could possibly be attributed to the presence of extracellular adenosine is an apparent regulation of blood flow. Two groups have reported a decrease in blood flow in white adipose tissue resulting from the administration of insulin to rats which were fasted (Madsen & Malchow-Mõller, 1983) and to streptozotocin-diabetic rats (Jamal & Saggerson, 1988). Madsen & Malchow-Mϕller hypothesized that the decreased blood flow may be due to the release of adenosine from the cells, as adenosine is a potent vasodilator in WAT. The activity of WAT e5'Nase has been shown to increase in streptozotocin-diabetic rats and subsequently decrease after insulin administration *in vivo* (Jamal & Saggerson, 1987). Assuming that there is a suitable extracellular source of AMP, the e5'Nase activity changes could provide the link between insulin administration and adenosine levels.

In recent years one important mechanism for insulin's second messenger system has been coming to light. This

mechanism involves a phosphatidylinositol-glycan (PI-G) cleavage by insulin-stimulated phosphatidylinositol-specific phospholipase C (PI-PLC). The glycosylated-phosphatidyl-inositol may either be part of a protein-anchor entity or a molecule which shares great structural similarities as discussed in Section 1.5.5.

Early studies in this area demonstrated that insulin stimulated the hydrolysis of a novel inositol-glycolipid releasing two inositol-containing phospholipids which modulated the action of cAMP phosphodiesterase in hepatic plasma membranes (Saltiel & Cuatrecasas, 1986) and cultured myocytes (Saltiel *et al*, 1986). Work quickly followed showing that both cAMP-dependent and cAMP-independent events were mimicked by these inositol-containing phospholipid headgroups including activation of pyruvate dehydrogenase (Saltiel, 1987; Suzuki *et al*, 1987), inhibition of adenylate cyclase (Saltiel, 1987), inhibition of phospholipid methyltransferase (Kelly *et al*, 1986), glucose oxidation and lipogenesis in intact adipocytes (Saltiel & Sorbara-Cazan, 1987), and inhibition of catecholamine-stimulated lipolysis (Kelly *et al*, 1987). Isolation of these glycosylated phospholipids and reintroduction to the incubation medium of intact cells exhibited similar time-courses of protein phosphorylation as did insulin (Alemany *et al*, 1987). However, in adipocytes the inositol glycan headgroups, despite being shown to stimulate the same signalling pathways as insulin via phosphorylations and dephosphorylations, had

no effect on the insulin-sensitive glucose transporter (Alemany *et al*, 1987). This would suggest that the regulation occurs at some point distal to a bifurcation in the pathways between glucose transport and intermediary metabolism. Furthermore, their work is consistent with the reports that insulin controls the mobilization of glucose transporters without affecting their phosphorylation state.

The structures of the two inositol-containing phospholipids which acted as insulin mediators needed to be elucidated to help determine their origin. Studies carried out by Mato and colleagues suggested that these insulin-mediators were generated by the action of an insulin-sensitive phospholipase C (Mato *et al*, 1987). Purification of a phosphatidylinositol-glycan-specific phospholipase C from rat liver plasma membranes (Fox *et al*, 1987) also pointed to this mechanism as a target of insulin action. However, despite the identification of a non-N-acetylated glucosamine being glycosidically linked to the lipid moiety as with the GPI-anchor, ethanolamine and *myo*-inositol were not incorporated into the purified glycolipid from H35 hepatoma cells (Mato *et al*, 1987). Furthermore, in the H35 cells, the cleaved phosphatidylinositol was determined to contain *chiro*-inositol rather than the *myo*-inositol of the GPI-anchor (Mato *et al*, 1987a; Larner *et al*, 1988). Nevertheless, much earlier work on insulin mediators purified from rat liver had been identified as containing glucosamine, *myo*-inositol and galactose (Larner *et al*, 1974). This led

to the suggestion that there are multiple insulin mediators, possibly varying with tissue and species.

Evidence that the insulin-sensitive phospholipase C cleaved the GPI-anchor came from studies demonstrating that insulin stimulated the release of lipoprotein lipase (Chan *et al*, 1988) and alkaline phosphatase (Romero *et al*, 1988) and that insulin induced a decrease in ecto-5'Nase in skeletal muscle membranes (Klip *et al*, 1988), each of these proteins being PI-G anchored. Furthermore, the insulin-sensitive phosphatidylinositol-glycan was localized at the outer surface of the cell membrane (Alverez *et al*, 1988) and the distribution of the PI-G anchored proteins was shown to be regulated by insulin in a variety of cell types (Lisanti *et al*, 1989; Varela *et al*, 1990). This suggested that both the inositol glycan headgroup and the PI-G anchored proteins which are released by insulin's activation of GPI-phospholipase C, are in fact the same rather than two distinct molecules. Finally, by using anti-inositolglycan antibodies raised specifically against the protein anchor, Romero *et al* (1990) demonstrated that metabolic actions of insulin, but not the glucose transporter regulation, could be selectively blocked in intact BC_3H1 cells. These findings also implied that the action of the insulin mediator had to occur either by interaction with specific PI-G receptors on the cell surface or by facilitated uptake of the mediator. The release of proteoheparan sulphate metabolizing enzyme with its inositol-glycan followed by cellular uptake in a receptor

mediated way was shown to be blocked by inositol phosphates and glucose-6-phosphate (Ishihara *et al*, 1987). The recognition site was therefore concluded to be the *myo*-inositolphosphate moiety. Support for the idea of a receptor mediated mechanism was provided in work carried out by Strålfors and Alemany (1990) in which it was shown that the effects on protein phosphorylation due to insulin in intact adipocytes could be blocked selectively with *myo*-inositol-hexakisphosphate, glucose-6-phosphate and non-acetylated-glucosamine, but not with glucose, glucose-6-sulphate, N-acetyl-glucosamine, *myo*-inositol, or *myo*-inositol-hexakis-sulphate. Similarly, insulin-stimulated lipogenesis was also blocked by inositol-phosphate and glucosamine (Machicao *et al*, 1990). All the blockers being previously identified components of the insulin GPI-mediators.

1.8 Changes in Metabolic Status in WAT

The responsiveness of adipocytes to insulin and adenosine alters in a variety of physiological and pathological states including fasting/starvation, streptozotocin-diabetes (a type I diabetes model), and hypo- and hyper-thyroidism. Many studies have been carried out on animal models of the above states investigating the changes which occur in enzyme activities (Saggerson & Carpenter, 1987; Jamal & Saggerson, 1987; Suhail & Rizvi, 1989), sensitivity of lipolysis to stimulatory and inhibitory agonists (Saggerson, 1986; Saggerson *et al*, 1991), the levels and

activities of G-proteins (Gawler *et al*, 1987; Houslay *et al*, 1989; Strassheim *et al*, 1990; Saggerson *et al*, 1991), and phosphatidylinositol-glycan turnover (Bergh *et al*, 1988; Thakkar *et al*, 1990; Macaulay & Larkins, 1990).

1.8.1 Diabetes Mellitus

Diabetes mellitus is characterized by an inability to control blood glucose. There are two forms of this disease: type I or insulin-dependent (IDDM) results from a lack of insulin production by the pancreas and has an early age onset; type II or non-insulin-dependent (NIDDM) sometimes has a partial insulin deficiency, but is better known by an insensitivity to insulin and has a later-life onset. The animal models of alloxan- or streptozotocin-diabetes mimic the insulin-dependent form of diabetes mellitus by destruction of the β-cells in the pancreas which produce the insulin. It is predominantly this type I form and the associated animal models which are of interest to this study.

Rapid mobilization of fatty acids from adipose tissue is a key characteristic of insulin dependent diabetes. The combination of insulin-resistance and lack of insulin have been shown to be responsible for excessive lipolysis during this state of insulin deficiency (Bell *et al*, 1986; Singh *et al*, 1987). A mechanism distal to insulin-receptor binding also appears to be implicated in type I diabetes. The first evidence that a pathological state could induce a G_i-protein

dysfunction was found in the liver of streptozotocin-diabetic rats, with insulin treatment being capable of reversing the lesion (Gawler *et al*, 1987). This abolition of G_i-protein activity was also demonstrated in adipocytes, whilst the levels of expression were not reduced (Houslay *et al*, 1989; Strassheim *et al*, 1990) and in some studies were found to be raised (Saggerson *et al*, 1991). The effect of this G_i-protein dysfunction to raise the basal level of adenylate cyclase activity, and hence the intracellular levels of cAMP (Gawler *et al*, 1987), may help to explain the findings of decreased diabetic activities in a variety of adipose-tissue triacylglycerol synthesis enzymes (Saggerson & Carpenter, 1987) by the removal of G_i-inhibition rather than by direct G_s activation.

Streptozotocin-diabetic rats were shown to have reduced phosphatidylinositol turnover in cardiac tissue which increased with the duration of the diabetic state (Bergh *et al*, 1988). Treatment with insulin appeared to restore normal turnover rates. However, it was not made clear whether the phosphatidylinositol of the study was involved with the second messenger cascade pathway or whether it was of the PI-G anchor or insulin-mediator variety. Streptozotocin-diabetes was also shown to impair insulin-stimulated PI-G cleavage in rat adipocytes (Macaulay & Larkins, 1990). This could support the findings of raised levels of some PI-G anchored proteins in the diabetic state, such as e5'Nase (Jamal & Saggerson, 1987) and acetylcholinesterase (Suhail

& Rizvi, 1989).

1.8.2 Hypo- and Hyperthyroidism

Thyroid disorders arise frequently in areas of iodine deficiency, and are also known to result from autoimmune processes. Animal models of thyroid disorders can be made by thyroidectomy or by providing animals with propyl-thiouracil (PTU)-containing drinking water coupled with an iodine-poor diet for hypothyroidism; or to simulate hyper-thyroidism, daily injections for 3-7 days of triiodothyronine (T_3) or thyroxine (T_4).

In hypothyroid adipose tissue the adrenergic stimulation of lipolysis is severely impaired, but binding studies on the abundance and affinity of adipocyte β-adrenergic receptors mostly showed no change from the euthyroid controls (Goswami & Rosenberg, 1978; Malbon *et al*, 1978; Ros *et al*, 1988). Similarly, no significant changes in function or abundancy were found when investigating the stimulatory G_s-protein (Malbon *et al*, 1984; Rapiejko *et al*, 1989; Milligan & Saggerson, 1990). However, a series of reconstitution experiments between G_s-containing membranes and β-adrenergic receptors, obtained from both euthyroid and hypothyroid, indicated a lesion in the coupling mechanism between receptor and G-protein (Malbon *et al*, 1984). These findings were consistent with previous evidence showing that activation of adenylate cyclase by cholera toxin was

unimpaired in hypothyroidism (Malbon & Gill, 1979) and that maximal levels of cAMP accumulation in hypothyroid adipocytes in response to forskolin were unaffected (Malbon & Graziano, 1983).

It is important to note that the demonstration of unimpaired forskolin-stimulated cAMP accumulation in hypothyroid rats discussed above, was carried out in the presence of adenosine deaminase. Malbon & Graziano (1983) found this to be necessary because adipocytes from hypothyroid rats were shown to have increased sensitivity to antilipolytic regulation by adenosine as compared to those from euthyroid rats. This was confirmed by Malbon *et al* (1985) who also demonstrated that the increased sensitivity appeared to occur at the level of adenylate cyclase activity inhibition. Later studies implicated the adenosine receptor-coupled inhibitory G_i-protein in this altered sensitivity (Malbon *et al*, 1985; Saggerson, 1986). Adipose tissue adenosine contents were found to be very low in hypothyroid rats, but were found to increase significantly if the rats were subsequently injected with T_3 or T_4 (Ohisalo *et al*, 1987). The adipocyte e5'Nase activity which could convert extracellular AMP to adenosine was also shown to be reduced by 50% in hypothyroidism (Jamal & Saggerson, 1987). These combined findings prompted the conjecture that the reduced adenosine levels lessened the effect of G_i-protein up-regulation, as discussed by Saggerson (1992).

In contrast to hypothyroidism, hyperthyroidism is characterized by increased lipolysis and increased concentrations of plasma non-esterified fatty acids. Incubated fat pieces from rats treated with T_3 or T_4 injections to induce hyperthyroidism showed an exaggerated lipolytic response to adrenaline or noradrenaline (Debons & Schwartz, 1961; Deykin & Vaughan, 1963). However, attempts to locate the mechanism responsible for the increased response to catecholamine-induced lipolysis are still being elucidated. β-adrenergic receptor studies showed no changes in abundance in either adipocytes (Goswami & Rosenberg, 1978) or adipocyte membranes (Malbon *et al*, 1978). Similarly, the G_s-protein coupled to the β-adrenergic receptor was also studied, with no changes measured either in abundance (Rapiejko & Malbon, 1987; Rapiejko *et al*, 1989) or in function (Rapiejko & Malbon, 1987).

Observations made when investigating catecholamine-induced adipocyte cAMP accumulation in hyperthyroidism showed the same increased response as did lipolysis (Caldwell & Fain, 1971; Malbon *et al*, 1978; Mills *et al*, 1986). However, when adenosine deaminase was added to the system, despite higher levels of cAMP accumulation in all studies, the hyperthyroid animals did not have as great a level of cAMP accumulation as the euthyroid controls (Rapiejko & Malbon, 1987). This suggested a reduced sensitivity to adenosine regulation in the hyperthyroid state. It therefore appears that although no upregulation of the stimulatory pathway for

lipolysis has been identified, at least the small amount of downregulation in the adenosine-associated inhibition may contribute to increased lipolysis in the hyperthyroid state.

1.9 The Purpose of Further Studies of E5'Nase in WAT

The changes in e5'Nase in the streptozotocin-diabetic rat white adipose tissue and in the subsequent readministration of insulin (Jamal & Saggerson, 1987) were to be investigated further both *in vivo* and *in vitro* to determine whether these activity changes were quantitative or qualitative. The objective of this study was to determine the role of insulin in regulating e5'Nase in WAT in varying metabolic states, and to investigate the possibility of insulin-stimulated cleavage of the e5'Nase phosphatidyl-inositol-glycan anchor. The near immediate changes noted in white adipose tissue blood flow in response to insulin status led to the idea that insulin-regulation of e5'Nase activity could, in turn, control the formation of adenosine locally, thereby being important during lipolysis for the removal of the released non-esterified fatty acids. Furthermore, the potential for insulin-stimulated cleavage of e5'Nase PI-G-anchor could explain the reduction in e5'Nase activity noted on insulin administration to streptozotocin-diabetic rats.

Polyclonal antibodies raised against e5'Nase detected two proteins on a Western blot from a sodium-dodecylsulphate polyacrylamide gel. It therefore became necessary to purify

WAT e5'Nase and raise monoclonal antibodies in order to investigate the possibility that the aforementioned proteins may be two species of e5'Nase anchored in the membrane by different mechanisms.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Materials

Unless otherwise listed below, reagents were always used at the highest possible grade and were obtained from Sigma Chemical Co. Ltd., Poole, Dorset; FSA Laboratory Supplies Ltd., Loughborough, Leics.; May & Baker Ltd., Dagenham, Essex and BDH Ltd., Poole, Dorset.

In particular, albumin, bovine - initial fractionation by heat shock, essentially fatty acid free (BSA); streptozotocin mixed anomers; concanavalin A - sepharose 4B; adenosine 5'-monophosphate - sepharose 4B; inosine 5'-monophosphate, disodium salt (IMP); adenosine 5'-triphosphate, disodium salt (ATP); Freund's adjuvant, complete and incomplete; Nonidet P-40 (NP-40); N,N,N',N'-tetramethylethylenediamine (TEMED); polyoxyethylene sorbitan monolaurate (Tween 20); donkey anti-sheep IgG (whole molecule) peroxidase conjugated; concanavalin A, type IV; deoxyribose nucleic acid, calf thymus type V (DNA); α,β-methyleneadenosine 5'-triphosphate (AOPCPOP) and α,β-methyleneadenosine 5'-diphosphate (AOPCP); 4-chloro-1-naphthol; protein A immobilized on 250μm acrylic beads; methyl α-D-mannopyranoside (α-methyl-D-mannoside); antibiotic-antimycotic 100x solution (penicillin/streptomycin/amphotericin-B); polyethylene glycol 50% solution (PEG) "Hybri-Max", average molecular weight 1450; dimethyl-

sulphoxide (DMSO) "Hybri-Max" and glutamine were obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

[2-^{3}H]Adenosine 5'-monophosphate, ammonium salt and [8-^{14}C]Inosine 5'-monophosphate, ammonium salt were obtained from Amersham International plc, Aylesbury, Bucks.

Goat anti-rabbit IgG (H+L) peroxidase conjugated; rabbit anti-mouse IgG (H+L) peroxidase conjugated; silver nitrate ($AgNO_3$); RPMI-1640 cell culture medium; Sp2/0.Ag-14 myeloma cells were obtained from ICN Immunobiologicals (FLOW), High Wycombe, Bucks.

Hypoxanthine/aminopterine/thymidine & hypoxanthine/ thymidine 50x supplements; gentamycin; foetal calf serum "Myoclone Plus" were obtained from Gibco BRL, Life Technologies Ltd., Uxbridge, Middex.

Human insulin (prb) Insulin zinc suspension, crystalline was obtained from Eli Lilly and Co. Ltd., Basingstoke.

Collagenase A from *Clostridium histolyticum* (EC 3.4.24.3); Mouse-Hybridoma-Screening-Kit; adenosine 5'-monophosphate (AMP), disodium salt, crystallised; N-Glycosidase F (PNGase F) from *Flavobacterium meningosepticum*; Insulin, sterile, lyophilised from bovine pancreas; glucose-6-phosphate dehydrogenase (G6P-DH, EC 1.1.1.49) from yeast

Grade II and hexokinase (HK, EC 2.7.1.1) from yeast were obtained from Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Lewes, East Sussex.

Zwittergent 3-14 (N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate) and MEGA 9 (nonanoyl-N-methyl-glucamide), ultrol grade detergents were obtained from Calbiochem Corp., Nottingham.

Phosphatidylinositol-specific phospholipase C (PI-PLC, EC 3.1.4.10) from *Bacillus thuringiensis* was obtained from Peninsula Laboratories Europe Ltd., St.Helens, Merseyside.

Dehydrated milk powder ("Make 5") was obtained from Tesco Stores Ltd., Cheshunt, Herts.

Ecoscint A scintillation fluid was obtained from National Diagnostics, Aylesbury, Bucks.

Pressurised gases (95%O_2:5%CO_2 and oxygen-free N_2) were obtained from BOC Ltd., London.

"Clinistix" glucose reagent strips were obtained from Miles UK Ltd., Slough.

2.2 Animals

2.2.1 Rats

These were either University College London home bred or Charles River bred Sprague-Dawley males, unless specified to the contrary. All animals had access to water *ad libitum* and were fed on Rat and Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.) which contained (w/w) 21% digestible crude protein, 4% digestible crude oil and 39% starches and sugars. The light/dark cycle was 13 hours/ 11 hours with light from 06:00 to 19:00. Rats used for control versus physiological and pathological status investigations were approximately six weeks old, weighing 160-180g. Those used for enzyme purification were mixed male and female, ranging between 8-10 weeks and weighing 200-250g.

2.2.1a Streptozotocin-Diabetes and Insulin Treatment

Rats were injected subcutaneously with 80mg Streptozotocin per kilogram body weight, made up in 0.9%(w/v) NaCl, 50mM sodium citrate buffer, pH4.0. The induction of streptozotocin-diabetes was monitored by the use of Clinistix™, reagent strips for the analysis of glucose in urine. After 7/8 days the rats were killed and glucose levels were measured in blood plasma taken from ventricular heart punctures.

For insulin treatment the rats were injected subcutaneously with 20 IU each of human insulin in a zinc crystalline suspension. Non-diabetic control and streptozotocin-diabetic control animals received equivolume 0.9%(w/v) saline injections. These injections were administered at 10:00a.m. on Day 7 after the streptozotocin injections for the short-term treatments, Days 6 and 7 for the "24 hour" treatments and on Days 6, 7 and 8 for the "48 hour" treatments. Unless specified otherwise, the rats were killed two hours after the last insulin/saline injection.

Body weights before treatment and during treatment were closely monitored. These are shown in Table 2.1 overleaf.

2.2.1b Hypothyroids

Hypothyroidism was induced in rats from the age of 4 weeks (80-90g) by the procedure outlined in Chohan *et al* (1984) and in Saggerson and Carpenter (1986). They were fed on an iodine-deficient version of the No.3 Breeding diet and drank water containing 0.01%(w/v) 6-n-propylthiouracil (PTU) for four weeks at which point they reached a body weight of 140-170g. Euthyroid age-matched controls fed on the normal diet then weighed 260-280g.

TABLE 2.1

Body Weights of Normal Control Rats and Streptozotocin-Diabetic Animals [A]

Day Number	Controls		Diabetics	
	Relative [B] Mean Weights (g)	Mean Change (g)	Relative [B] Mean Weights (g)	Mean Change (g)
1	180.0 (n=42)	-	180.0 (n=60)	-
2	186.3 (n=42)	6.3	172.7 (n=60)	-7.3
3	193.1 (n=30)	6.8	171.8 (n=36)	-0.9
4	195.8 (n=6)	2.7	171.2 (n=6)	-0.6
5	204.8 (n=6)	9.0	171.3 (n=12)	0.1
6	218.6 (n=24)	13.8	182.7 (n=41)	11.4
7	224.7 (n=42)	6.1	182.5 (n=59)	-0.2
8	230.7 (n=40)	6.0	194.6 (n=36)	12.1

Notes:

[A] Weights taken from Control and Diabetic, saline treated animals in the "*In vivo* insulin administration" experiments.

[B] Relative denotes that the starting weight (and therefore subsequent weights) were recalculated to equal 180g for ease of comparison. Generally, starting weights ranged from 160g to 220g.

2.2.1c Hyperthyroids

The procedure for inducing hyperthyroidism as described by Sugden *et al* (1983) was by subcutaneous injection of 1mg/kg body weight triiodothyronine (T_3) dissolved in 10mM NaOH, 0.03%(w/v) bovine serum albumin at 7-8 weeks old. The T_3 injection was repeated at 24 and 48 hours after the initial injection. Body weights dropped by 4-6g. Euthyroid controls were injected with the NaOH/BSA diluent and showed a 20-25g increase in body weight over the same period.

2.2.1d Fasting

Rats were selected at approximately 260g body weight. The fasting period began at 11:30a.m. and was continued for a 48 hour period.

2.2.2 Mice

These were University College London home bred Balb/c females, selected at 10 weeks of age. All mice had access to water *ad libitum* and were fed on Rat and Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.) which contained (w/w) 21% digestible crude protein, 4% digestible crude oil and 39% starches and sugars. The light/dark cycle was 13 hours/11 hours with light from 06:00 to 19:00.

2.3 Isolation of Adipocytes and "Non-adipocytes"

The method used to isolate viable white adipocytes for whole cell studies was that described by Rodbell (1964). Krebs-Henseleit buffer (100 parts 0.9%(w/v) NaCl, 1 part 2.11%(w/v) KH_2PO_4, 1 part 3.82%(w/v) $MgSO_4.7H_2O$, 4 parts 1.15%(w/v) KCl, 1.5 parts 1.6%(w/v) $CaCl_2.2H_2O$, 21 parts 1.3%(w/v) $NaHCO_3$ (fresh)) was made up, pre-warmed to 37°C and aerated with a 95% oxygen/5% carbon dioxide mixture. Rats were killed by cervical dislocation and the epididymal fat pads were dissected out, the main blood vessels were extracted and the pads were washed in pre-warmed Kreb's buffer. Fifty millilitre conical flasks which had previously been coated with dimethyldichlorosilane solution to reduce friction were used as digest flasks. These contained 15mg Collagenase (0.25 IU/mg) and 1%(w/v) bovine serum albumin (BSA -fatty acid free) in 5mls aerated Kreb's buffer. Between 5 and 8 epididymal pads were chopped up into these digest flasks which were then stoppered with rubber caps. To ensure gaseous exchange, the oxygen/carbon dioxide mixture was introduced directly through the caps and provision was also made for gaseous escape. The digest flasks were then vigorously shaken at 37°C for 45 minutes. After this time the digests were forced through a nylon mesh (250µm pore size) to remove connective tissue and any undigested tissue. The resulting cells were transferred to 15ml plastic centrifuge tubes, made up to 10mls with 1%(w/v) BSA, Krebs-Henseleit buffer and spun for 20 seconds at approximately

400xg to remove broken cell debris and separate any released lipids. The infranatant below the adipocytes and the pellet were removed, but retained for alternative "non-adipocyte" membrane preparations. The wash procedure was repeated twice more as above, but the infranatant was discarded on these subsequent washes.

These adipocytes remained viable in Krebs-Henseleit buffer at 37°C with aeration for several hours and were used for incubation experiments to investigate the potential for release of ecto-5'Nase by insulin in the presence and absence of adenosine triphosphate (ATP) and guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S). Similarly, membranes derived from the adipocytes and other fractions obtained during adipocyte isolation (described below) were also used in these types of study.

For membrane preparations from the adipocytes, wash-infranatant and wash-pellet, the following procedures were used. Both the adipocytes and the pellet were resuspended in a volume of 50mM Tris.Cl^-, pH7.4 equal to that of the infranatant. All three fractions were subjected to homogenisation in a Potter-Elvejhem homogeniser with a 0.2mm clearance, for ten strokes which had the effect of disrupting any whole cells. The fractions were centrifuged at 18,000rpm (30,000xg_{av}) in a Sorvall SM34 rotor for 30 minutes as described for crude membrane preparations in Section 2.4 below. The supernatants were discarded, whilst the pellets

were resuspended in a minimal volume of 50mM Tris.Cl^-, pH7.4. In some instances, the infrantant and pellet were combined and used as one fraction.

2.4 Crude Membrane Preparation

Rats were killed by cervical dislocation. In females the white fat surrounding the fallopian tubes and ovaries was removed, whilst in males the epididymal fat pads were dissected out and the main blood vessel extracted. Approximately five fat pads, or their equivalent, were transferred to 25mls ice cold Tris.Cl^-, pH7.4 (tris[hydroxy-methyl]amino-methane, adjusted with hydrochloric acid). From this stage onwards the preparation was always maintained on ice or at 4°C during centrifugation.

The tissue was completely disrupted with ten rapid passes of an ultraturrax homogenizer. Addition of protease inhibitors such as antipain, leupeptin and pepstatin A had no discernable effect on the resulting activity of 5'Nase and were not therefore used despite the vigour of this preparation.

The homogenate was transferred to 50ml centrifuge tubes and spun at 4,500rpm (1,500xg_{av}) in a Sorvall SS34 rotor for one minute. This formed a solid fat upper layer, a nuclear pellet and an infranatant primarily consisting of cytosol and membranes. The infranatant was transferred to

clean 50ml open-end centrifuge tubes by piercing the fat layer and pouring the infranatant through the hole thus created. The infranatant was spun at 18,000rpm (30,000xg_{av}) in a Sorvall SS34 rotor for 30 minutes. The supernatants were discarded. The pellets were resuspended in a minimal volume of 50mM Tris.Cl^-, pH7.4 using a Potter-Elvejhem homogeniser which had 0.2mm clearance. This resuspension was used in experiments as a source of crude membranes and latterly for the further purification of 5'Nase.

2.5 Isolation of the ATP-Sensitivity-Conferring Factor from Rat Brain Cytosol

Rats were killed by cervical dislocation. Whole brains were dissected out and transferred to ice cold 0.32M sucrose, 10mM Tris.Cl^-, pH7.4. The brains were finely diced, washed free of blood and homogenised in fresh buffer as above [~15% (w/v) tissue/buffer]. The homogenate was subjected to a slow speed centrifugation at 1,500xg for three minutes; the pellet was discarded and the supernatant was then centrifuged at 105,000xg for one hour to obtain a cytosolic fraction.

Ammonium sulphate ($(NH_4)_2SO_4$) was added dropwise to the supernatant/cytosol, whilst stirring on ice, to 65% saturation and then stirring was continued for a further hour on ice. The remaining supernatant was discarded, whilst the $(NH_4)_2SO_4$-precipitated pellet was resuspended in 0.1%(v/v) Tween-20, 20mM Tris.Cl^-, pH8.5 and dialysed overnight at 4°C

against the same detergent-buffer.

The dialysed fraction was divided into ~10mg aliquots which were individually applied to a pre-equilibrated (three column volumes of 0.1% (v/v) Tween-20, 20mM Tris.Cl^-, pH8.5) 1ml Mono Q ion exchange column (Pharmacia Biotech Ltd) at a flow rate of 1ml/min. Two peaks of 5'Nase activity were obtained; one which did not bind to the column (and which was excluded from being "overload") and the other which was eluted with a sodium chloride (NaCl) gradient at ~150-200mM NaCl.

The "Unbound" fraction contained 5'Nase activity which was found to have an ATP-sensitivity-conferring factor associated with it. It was possible to isolate this factor from the 5'Nase activity by passing the entire fraction through a Concanavalin A affinity column by the procedure described in section 2.9.2. The 5'Nase activity bound to the column whilst the factor was contained in the wash-through proteins. To ensure that no residual 5'Nase activity remained associated with the factor, the wash-through proteins were passed through the concanavalin A column a second time. The resulting "factor fraction" was dialysed overnight at 4°C against 0.1% (v/v) Tween-20, 50mM Tris.Cl^-, pH7.4 in a ratio of 1(fraction):100(dialysis buffer). After protein determination by the method described in section 2.6, the factor was used immediately to assess its effects on ATP inhibition of e5'Nase activity as described in section 2.11.1, part (g).

2.6 Protein Determination

Protein quantitation in samples was carried out by the method of Lowry *et al* (1951) modified as follows. Buffer solution was freshly prepared consisting of 100 volumes of 2%(w/v) Na_2CO_3 in 0.1M NaOH: 1 volume 1%(w/v) $CuSO_4$: 1 volume 5%(w/v) K.Na.tartrate. To 2mls of this buffer either 0-100µg bovine serum albumin (BSA) for calibration, or sample (maximum 50µl), was added in a total volume of 200µl (made up with 0.1M NaOH). Sample buffer of 50mM Tris.Cl^-, pH7.4, ± relevant detergents, was used as reaction background for the samples. Fifty microlitres of Folin & Ciocalteau's reagent were added to each tube and immediately whirlimixed thoroughly. The tubes were allowed to stand at room temperature for 30 minutes to enable full colour development, after which time the absorbance was read at 660nm. Protein was determined in the samples by comparison with the bovine serum albumin standard calibration.

2.7 DNA Estimation

DNA was determined by the method of Switzer & Summer (1971) with slight modifications as necessary for tissue homogenates. Fifty microlitres of homogenate were added to 50µl of 50%(w/v) trichloroacetic acid (TCA) pre-cooled on ice in 1.5ml microfuge tubes. Homogenate buffer was also added to the TCA as a blank. The tubes were left on ice for 30 minutes to precipitate DNA. Precipitates were collected by

microcentrifugation at 2,000xg for two minutes. The supernatants were removed and tubes were inverted on paper towelling to allow pellets to drain. Lipids and the TCA were removed with one alcoholic extraction by adding 400µl of 0.01M potassium acetate in absolute ethanol to the pellets and vortexing for 30 seconds. These resuspensions were sonicated for 15 seconds and microfuged at 2,000xg for two minutes. Supernatants were removed, inverted on paper towelling and allowed to air dry overnight. Meanwhile, a series of DNA standards were prepared by dissolving calf thymus DNA to 100µg/ml in deionized water and pipetting out 0-4.0µg DNA in 0.5µg increments. These were also allowed to air dry overnight.

Fresh 2M diaminobenzoic acid (DABA) was made up the next day and 20µl DABA was added to each standard sediment, reagent blank and sample. These were vortexed for 30 seconds and sonicated for 15 seconds. All tubes were incubated at 60°C for 30 minutes, then cooled. To each tube 0.58mls of 0.6M perchloric acid was added and whirlimixed. Fluorescence was measured with excitation at 420nm and emission at 520nm in a Perkin-Elmer fluorescence spectrometer, model 3000.

2.8 Glucose Determinations in Blood Plasma

The method used to determine the blood glucose levels in control and diabetic rats *post mortem* was as follows. Assay buffer was made up as 0.013%(w/v) $NADP^+$ (nicotinamide

adenine dinucleotide phosphate), 0.022%(w/v) albumin, 0.030%(w/v) ATP (adenosine triphosphate), 0.203%(w/v) magnesium chloride all in 50mM Tris.Cl^-, pH8.0. The enzymes glucose-6-phosphate dehydrogenase and hexokinase required for the assay were obtained from Boehringer Mannheim both at 140 IU/mg. All plasmas were diluted ten-fold with deionised water. The assay consisted of 2mls assay buffer, 0.02ml (2.8 IU) glucose-6-phosphate dehydrogenase, 0.02ml diabetic/ 0.05ml control plasmas and 0.95ml/0.92ml deionised water accordingly. It was started with the addition of 0.01ml (2.8 IU) hexokinase and allowed to go to completion (~10 minutes). Absorbance was monitored at λ=340nm, and 50µl 500mM glucose were added at the end of the assay to confirm that the system was still functioning. Plasma concentrations were calculated using the $m\epsilon_{340nm}=6.22mM^{-1}.cm^{-1}$. Generally, control rats had glucose plasma concentrations of 7.1±0.8mM (n=8), whilst rats were confirmed as being diabetic when their glucose plasma concentrations were in the region of 27.5±1.8mM (n=16). Sample values which provided the afore-mentioned mean values are shown in Table 2.2.

2.9 5'Nucleotidase Purification

Crude membrane preparations of white adipose tissue as described in section 2.4 were used as the source of ecto-5'Nase for purification. Various attempts (as will be described in section 2.13) were made to cleave the putative phosphatidylinositol-glycan anchor thus releasing a

TABLE 2.2

Glucose Plasma Concentrations Measured in Control and Streptozotocin-Diabetic Rats

Status	Batch 1 Plasma Glucose (mM)	Batch 2 Plasma Glucose (mM)	Mean Plasma Glucose ± S.E.[A] (mM)
Control	8.1	5.4	
	9.5	5.5	7.1±0.8
	9.0	5.0	(n=8)
	10.1	4.3	
Diabetic	32.6	20.0	
	35.2	23.2	
	28.2	22.9	
	35.5	16.4	27.5±1.8
	36.2	19.3	(n=16)
	36.9	28.2	
	35.0	21.0	
	29.2	20.7	

[A] indicates that S.E. = Standard Error

hydrophillic form of the ecto-5'Nase, but these were unsuccessful. The following procedures were therefore used, modifying the procedures used by Harb *et al* (1983) who purified the enzyme from bovine liver.

2.9.1 Solubilization of Ecto-5'Nucleotidase

Zwittergent-14 (a sulphobetaine detergent) was added to the crude membrane preparation to give a final concentration of 0.4%(w/v). This was stirred at 4°C for four hours, followed by centrifugation at 40,000rpm (105,000xg_{av}) in a Beckman 75Ti rotor for 90 minutes. The supernatant was retained for purification, whilst the pellet was resuspended in a volume of 0.4%(w/v) Zwittergent-14, 50mM Tris.Cl^-, pH7.4 equivalent to that of the supernatant to ensure there was no residual 5'Nase activity.

2.9.2 Concanavalin A-Sepharose 4B: Affinity Chromatography

A 5.5ml Concanavalin A (Con A)-Sepharose 4B column (7cm x 1cm) was equilibrated with three column volumes of 0.4%(w/v) Zwittergent-14 in 50mM Tris.Cl^-, pH7.4 containing 1mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$ (Buffer A). The salts were required for activation of the lectin. The supernatant containing solubilized 5'Nase, from section 2.9.1 above, was loaded onto the column at a rate of 3.6ml/hr and washed with Buffer A until no further protein was detected coming off the

column at 280nm. Elution then commenced with a second buffer which was the same as Buffer A, but in addition containing 0.3M methyl α-D-mannopyranoside (α-methyl-D-mannoside) (Buffer B). Two millilitre fractions were collected for the next eight column volumes. The chart recording obtained from monitoring the run at 280nm is depicted in Figure 2.1. Fractions collected from the column were assayed for 5'Nase activity as outlined in Section 2.11.1 and those with the highest activity were pooled together.

The α-methyl-D-mannoside, salts and Zwittergent-14 had to be removed from the pooled fractions without precipitating out the protein. The former two components interfered with the next stage (AMP-Sepharose 4B affinity chromatography) and the latter interfered with protein determination at higher 5'Nase concentrations and with PAGE (polyacrylamide gel electrophoresis) used later (see Section 2.10). This removal was achieved by concentrating the pooled fractions down to ~2mls using an Amicon stirred ultrafiltration cell through a YM30 membrane. The concentrate was rediluted to 50mls with 0.1%(v/v) Tween-20 in 50mM Tris.Cl^-, pH7.4 (Buffer C) and reconcentrated to ~2mls. The redilution and reconcentration were repeated twice more, thus producing a final concentrated eluate in Buffer C with negligible traces of Buffer A or B, far more rapidly than by conventional dialysis.

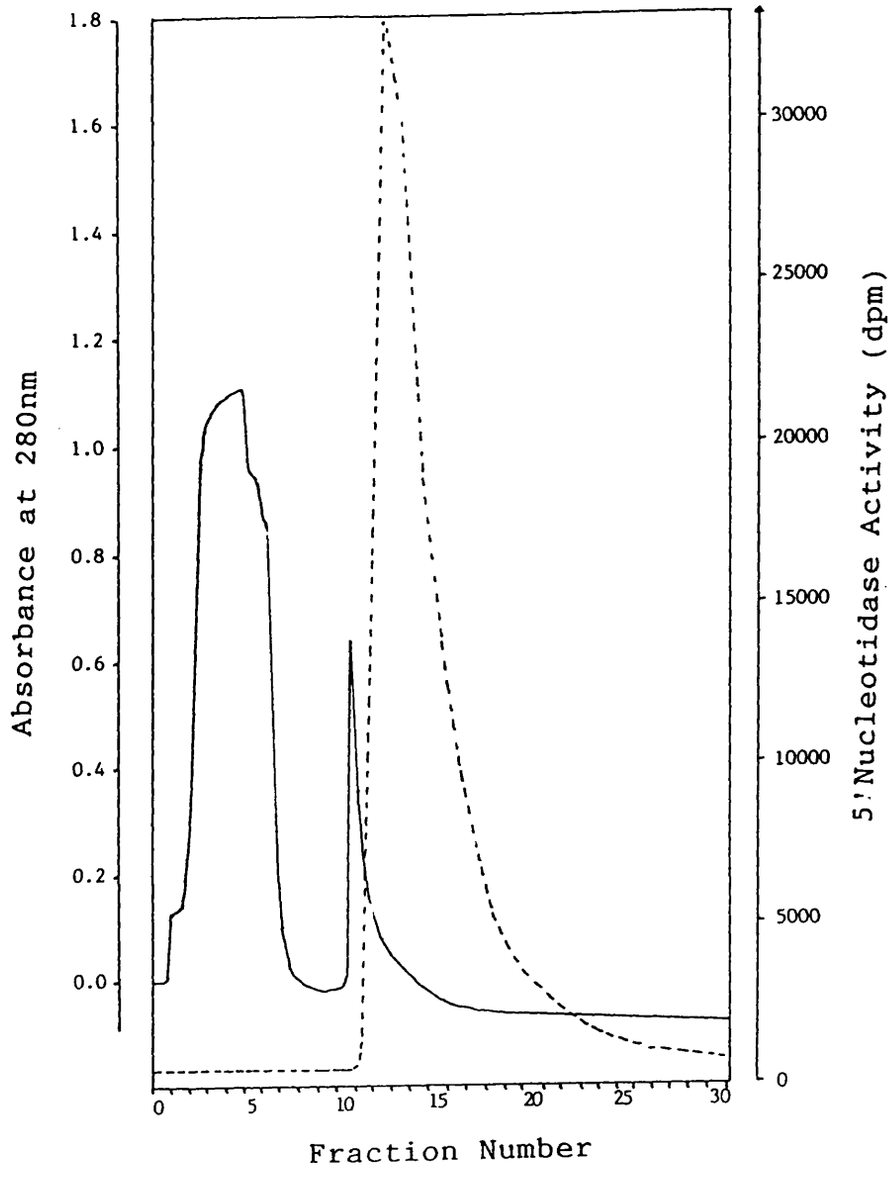


FIGURE 2.1: Concanavalin A-Sepharose 4B Affinity Column Trace with Superimposed 5'Nase Activity

———— = Absorbance at 280nm
---------- = 5'Nucleotidase Activity

2.9.3 AMP-Sepharose 4B: Affinity Chromatography

This chromatography medium had to be prepared fresh before use by swelling 1g medium at room temperature in 0.1M phosphate buffer and, after pouring the 4ml column, washing with a further twenty column volumes. The column was then equilibrated with three column volumes of Buffer C or until a steady base line was achieved. The final concentrated eluate from section 2.9.2 above was loaded on the column at a rate of 3.6ml/hr and then continued to be washed with Buffer C for four column volumes after the apparent return of protein levels to the base line, as monitored at 280nm. This was followed by elution with 0.2M NaCl in Buffer C for 2.5 column volumes and finally 1M NaCl in Buffer C for one column volume to completely strip it. Fractions were collected throughout the run as a large proportion of 5'Nase passed through the column more slowly than other non-binding proteins, but did not actually require elution. This can be seen in Figure 2.2 which depicts the monitor's chart recording read at 280nm, overlaid with 5'Nase activity measured by radiochemical assay. Fractions were 2mls.

The pre-elution fractions containing 5'Nase activity, which also showed negligible traces of the main bulk of "wash-through" proteins according to the chart recording, were pooled together. Similarly, the fractions containing 5'Nase activity which bound to the column were also pooled together. These were referred to as "AMP-Bound" and

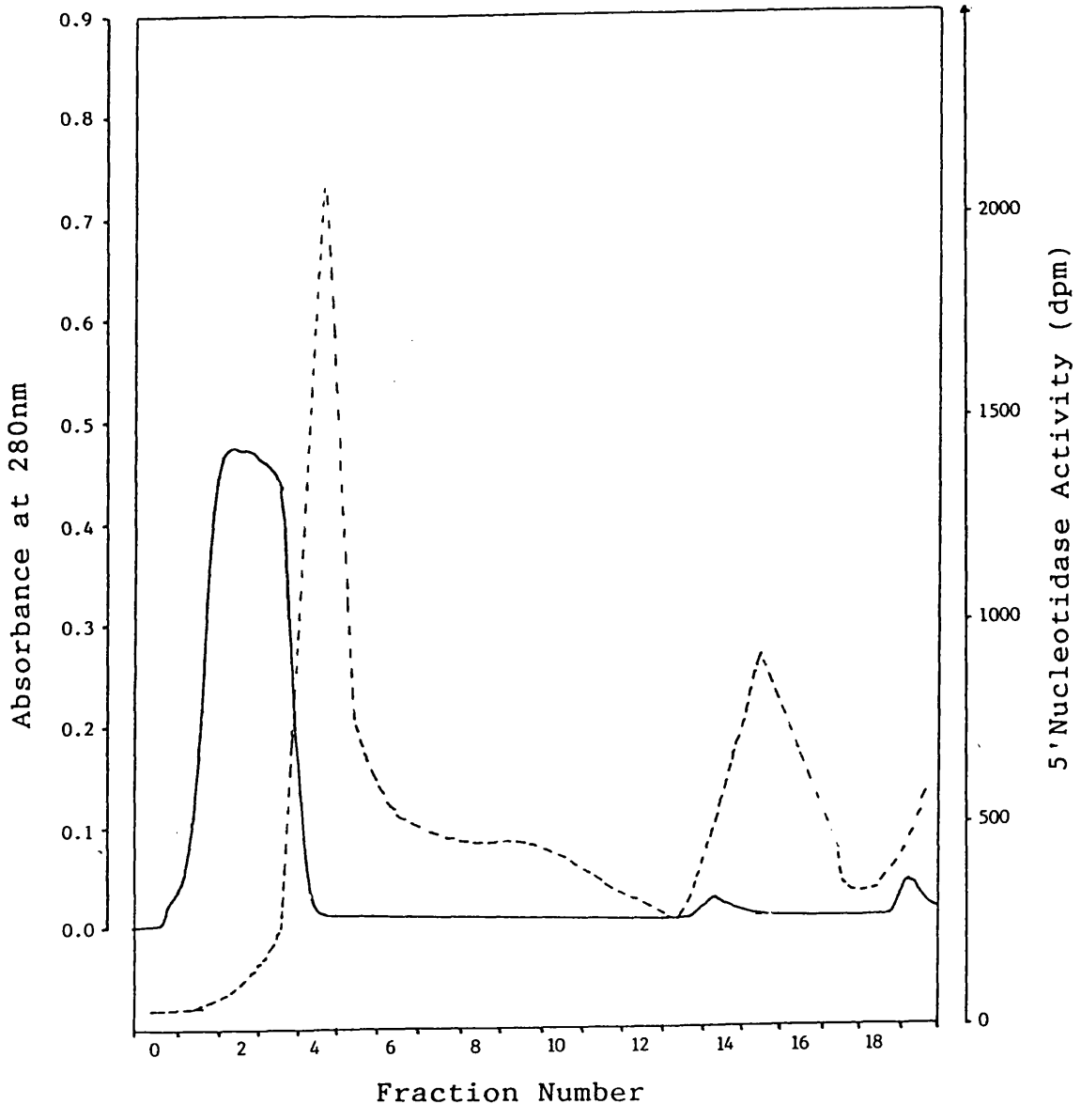


FIGURE 2.2: AMP-Sepharose 4B Affinity Column Trace with Superimposed 5'Nase Activity

———— = Absorbance at 280nm
- - - - - - - - = 5'Nucleotidase Activity

"AMP-Unbound" in the characterization studies. For purification both pools of 5'Nase activity were combined and concentrated down first in an Amicon stirred ultrafiltration cell through a YM30 membrane to ~4mls and then in an Amicon Centricon-30 microconcentrator unit down to 1-1.2mls.

2.9.4 FPLC Superose 12: Gel Filtration Chromatography

The FPLC Superose 12 column is a Pharmacia prepacked gel filtration column containing 25mls of medium, has a separation range of 1,000 to 300,000 kDa and has a maximum loading volume of 0.2mls of sample. The column was equilibrated with two column volumes of Buffer C or until there was a steady base line. The concentrated eluate from Section 2.9.3 was loaded in five to six looped runs, each run using 0.2mls successively, at a rate of 0.75 ml/min. Each run was 25mls long, by which time all protein had travelled the length of the column and had been collected in 1ml fractions. Two successive runs are depicted in Figure 2.3 with the overlay of 5'Nase activity clearly shown. The fractions containing highest 5'Nase activity were pooled and concentrated down to 1ml in an Amicon Centricon-30 microconcentrator unit.

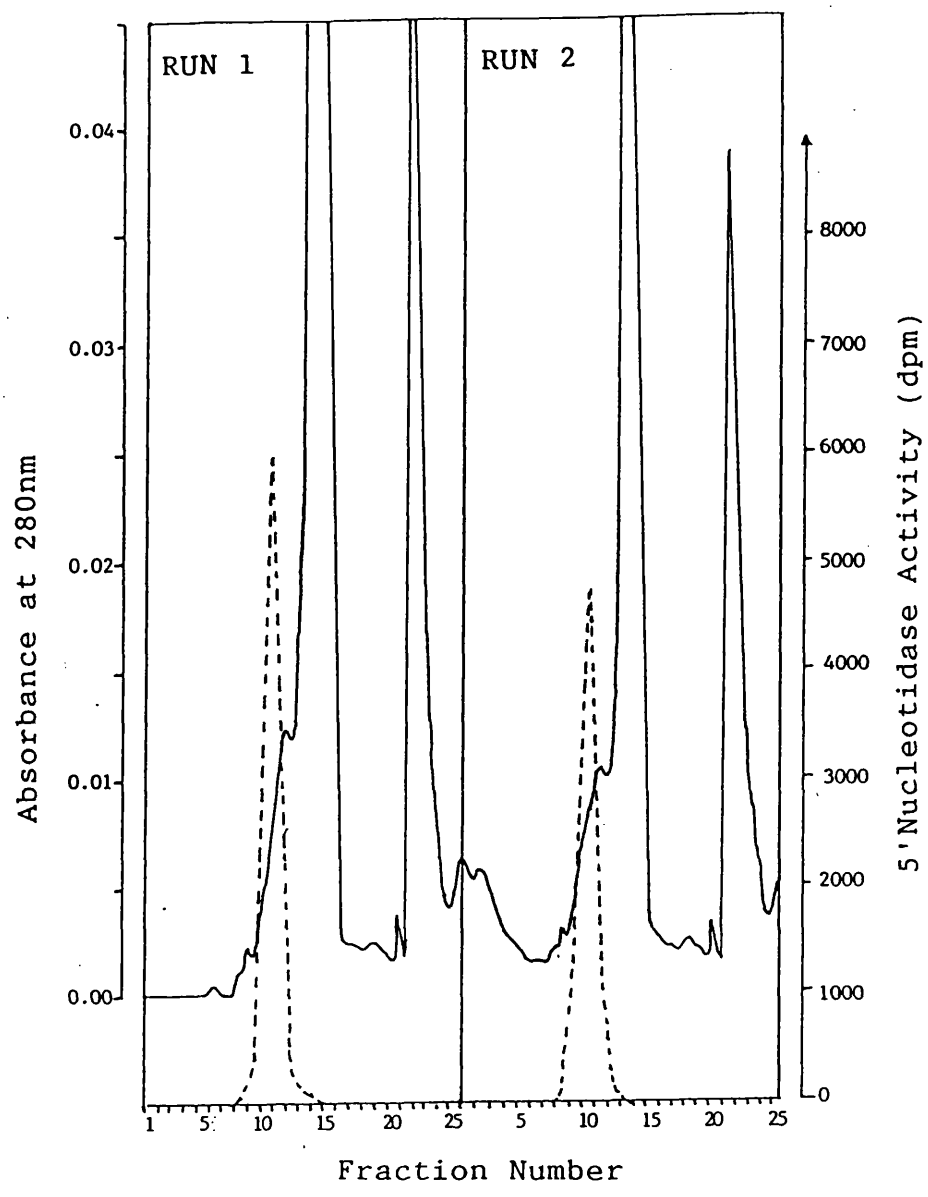


FIGURE 2.3: Superose 12 Gel Filtration Trace with Superimposed 5'Nase Activity - Two Consecutive Runs

_____ = Absorbance at 280nm
\- - - - - - - = 5'Nucleotidase activity

2.10 Electrophoresis

2.10.1 SDS PAGE - Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis

The method used was that of Laemmli (1970) using 0.1%(w/v) SDS throughout, except in the sample buffer which was at a final concentration of 2%(w/v) SDS. The separating gel was made to a final concentration of 10%(w/v) acrylamide, 0.27%(w/v) N,N'-methylenebisacrylamide, 0.1%(w/v) SDS, 0.375M Tris.Cl^-, pH8.8 and polymerization achieved with 5µl/ml 10%(w/v) ammonium persulphate, catalysed with 1µl TEMED (N,N,N',N'-tetramethylethylenediamine). Dimensions of the gel were 15cm vertical x 10cm horizontal x 1.25mm width. Stacking gel was layered on top at a final concentration of 5%(w/v) acrylamide, 0.13%(w/v) N,N'-methylenebisacrylamide, 0.1%(w/v) SDS, 0.125M Tris.Cl^-, pH6.8 and polymerized as above for the separating gel. Dimensions of this gel were 5cm vertical x 10cm horizontal x 1.25mm width, containing wells of 120µl capacity.

The samples were prepared by mixing sample and sample buffer to a final concentration of 10-100µg protein (depending on purity of 5'Nase in sample), 62.5mM Tris.Cl^-, pH6.8, 2%(w/v) SDS, 10%(v/v) glycerol, 0.001%(w/v) bromophenol blue and freshly added 5%(v/v) 2-mercaptoethanol. This mixture was boiled for 15 minutes and then loaded in the wells of the stacking gel with a Hamilton syringe.

The gel was run discontinuously with an electrode buffer containing 0.192M glycine, 25mM Tris.Cl^-, pH8.3, 0.1%(w/v) SDS at a limiting current of 40mA for the duration of the stacking gel and rising to a limiting current of 50mA for a minimum of 10cm of separating gel, noted by the progress of the dye front. The gel temperature was maintained by use of a cooling fan.

The gel was then stained directly by a neutral silver staining technique (see Section 2.10.2) or was subjected to Western blotting in order to transfer the proteins to a sheet of nitrocellulose for further analysis (see Sections 2.10.3 - 2.10.5).

2.10.2 Silver Staining (neutral pH)

The method used was described in the laboratory manual edited by Harlow & Lane (1988). To fix the proteins and prevent gel shrinkage, the gel was placed in five gel volumes of 30%(v/v) ethanol, 10%(v/v) acetic acid overnight with gentle shaking. The next day, the ethanol/acid mixture was replaced with five gel volumes of 30%(v/v) ethanol and left for 30 minutes with gentle shaking. This was repeated with a fresh solution of 30%(v/v) ethanol. The gel was then washed three times for 10 minutes each, with deionised water. The water was removed and the gel incubated in a 0.1%(w/v) silver nitrate solution for 30 minutes in the dark with gentle shaking. The silver solution was then quickly removed

under a stream of deionised waster for 20 seconds and the colour was developed by adding five gel volumes of a solution of 2.5%(w/v) sodium carbonate, 0.02%(v/v) formaldehyde and gentle shaking. When the bands had developed to their maximum capacity and the background just began to show colour, the reaction was stopped by pouring off the sodium carbonate solution and washing the gel in 1%(v/v) acetic acid, followed by several washes of deionised water.

2.10.3 Western blotting

This procedure is a modified technique of that which was described by Towbin *et al* (1979). It was carried out by apposing the gel and a sheet of nitrocellulose which had been moistened in transfer buffer (25mM Tris base - unaltered pH, 0.192M glycine, 20%(v/v) methanol), sandwiched between first two sheets of Whatmann No.1 filter paper and then two sponges of the scouring pad variety. These were held together firmly between sheets of rigid plastic, punctured to allow flow-through. The "sandwich" was then submerged vertically in the aforementioned transfer buffer. Two sheet electrodes were lowered into the transfer buffer on either side of the "sandwich" making sure the cathode was on the gel side and the anode on the nitrocellulose side. A limiting voltage of 50v was passed through for one hour, by which time any protein remaining in the gel was negligible. The sheet of nitrocellulose, or blot, was then used for further analysis.

2.10.4 Amido Black Staining

Amido black stain was made up as 0.1%(w/v) amido black dye, 10%(v/v) methanol, 10%(v/v) acetic acid. This was added to the blot (prepared in Section 2.10.3) in a container and incubated at room temperature for 10 minutes with gentle shaking.

The stain was poured off and excess stain removed from the blot by washing with deionised water, followed by several washes with 25%(v/v) isopropanol, 10%(v/v) acetic acid. When the background was almost white the blot was washed again in deionised water and then removed to air dry.

2.10.5 Immunoblotting

Alternatively, the blot from Section 2.10.3 was exposed to specific antibodies by the following procedure. The blot was washed for 90 minutes in a non-specific blocking agent consisting of 3%(w/v) dehydrated milk powder in 0.5M NaCl, 20mM Tris.Cl^-, pH7.4 (TBS) at room temperature. The blot was then exposed to a polyclonal raised against rat liver ecto-5'Nase (kindly donated by Dr.J.P.Luzio, Department of Clinical Biochemistry, University of Cambridge, in the first instance, and later by Dr.A.C.Newby, Department of Cardiology, University of Wales - College of Medicine, in the second instance) in 1%(w/v) dehydrated milk powder in TBS overnight at room temperature with gentle shaking. The

technique was also tried with antibodies in a monoclonal hybrid supernatant without success.

The following day, the anti-5'Nase was removed and the blot washed successively: once with deionised water, twice with 0.05%(v/v) Tween-20 in TBS, and twice with TBS on its own. Each wash lasted ten minutes. The blot was then exposed to a second antibody raised against antibodies of the animal in which the first anti-5'Nase were raised (i.e. anti-rabbit for Dr.J.P.Luzio's antibody and anti-sheep for Dr.A.C.Newby's antibody). This second antibody was conjugated to horseradish peroxidase and was in a solution of 1%(w/v) dehydrated milk powder in TBS. The blot was incubated with this second antibody for two to three hours. The same succession of washes as before - deionised water, Tween-20 in TBS, and TBS alone - were repeated. The blot was then ready for the detection system.

As rapidly as possible, 30mg 4-chloro-1-naphthol was dissolved in 10mls ice-cold methanol. At the same time, 30µl of 100vol. hydrogen peroxide were added to 50mls TBS. The 4-chloro-1-naphthol mixture was then added to the hydrogen peroxide mixture and immediately poured onto the blot. When background began to colour slightly, the detection solution was poured off and the blot washed extensively with deionised water. The blot was then air-dried.

2.11.1 Radiochemical 5'Nucleotidase Assay

This assay measures the release of [^{3}H]-adenosine from [^{3}H]-adenosine 5'-monophosphate (AMP) as per the method of Avruch & Wallach (1971), modified by Newby *et al* (1975) and again as follows. The assay mixture consisting of 450µl of 20µM AMP, 0.1µCi [2-^{3}H]-AMP as tracer, 5mM β-glycerophosphate all in 50mM Tris.Cl$^-$, pH8.0, was pre-incubated at 37°C for five minutes in microfuge tubes. The assay was started by the addition of 0.05-2.5µg protein (depending on the purity of 5'Nase in the sample) in a volume of 50µl, diluted with 50mM Tris.Cl$^-$, pH7.4. The diluent was also used alone in the assay to provide a background value. The reaction was stopped by the addition of 200µl 0.15M $ZnSO_4$ and vortexing for five seconds, followed by the addition of 200µl 0.15M $Ba(OH)_2$ and vortexing for a further 15 seconds to form a precipitate of the remaining AMP. Microfuging the tubes subsequently for three minutes at 2,000xg enabled the easy removal of 100µl supernatant which was transferred to 5mls of Ecoscint A for scintillation counting.

Variations of this assay to determine the properties of ecto-5'Nase included the following modifications:

a) Addition of 0-2µM α,β-methylene ADP (AOPCP) to the assay buffer at three different substrate concentrations (i.e. 5, 15, and 25µM AMP).

b) Addition of 0-10µM α,β-methylene ATP (AOPCPOP) to the assay buffer at 5, 15, and 25µM AMP.

c) Addition of 0-10μM Concanavalin A to the assay buffer.

d) Addition of 0-2mM dithiothreitol (DTT) to the assay buffer.

e) Addition of 0-20mM magnesium chloride to the assay buffer.

f) Addition of 0-500μM adenosine triphosphate, disodium salt (ATP,Na_2) to the assay buffer.

g) Addition of 0-40μM ATP,Na_2 to the assay buffer in the presence and absence of a high molecular weight factor (45.5μg - partially purified) which is recognised to date as an ATP-sensitivity-conferring soluble factor for 5'Nase isolated from rat brain cytosol (Dr.M.Orford - personal communication, See Section 2.5 for preparation).

h) Varying the pH of the assay buffer between pH 5.0 to pH 10.5 at half pH unit increments. To avoid variations due to buffer changes required by this pH range, the assay buffer was made up as indicated in the main section (2.11.1), but additionally contained 50mM glycine and 50mM MES (2-[N-Morpholino]ethanesulfonic acid).

2.11.2 Determination of K_m and V_{max}

The assay buffer was made up without either the radiolabelled or the cold substrate as these were added separately in order to maintain constant ratio between the

varying substrate concentrations and the corresponding radiolabel. Thus, the substrate was made up as 2mM AMP; 2µCi/ml [2-^{3}H]-AMP in 5mM β-glycerophosphate; 50mM Tris.Cl^{-}, pH8.0 and diluted for the necessary substrate concentrations in the same diluent. For each final substrate concentration, ranging between 0-200µM, fifty microlitres of the appropriate substrate dilution was added to 400µl assay buffer and incubated for five minutes prior to assay. The remainder of the assay followed the same procedures as outlined in Section 2.11.1 above.

Where inosine 5'-monophosphate (IMP) was used as substrate, the above procedures were followed precisely whilst the radiolabelled IMP was [8-^{14}C]-IMP.

The K_m and V_{max} values were derived from plotting a double reciprocal Lineweaver-Burke graph. These values were estimated by linear regression analysis.

2.11.3 Liquid Scintillation Counting

The Packard CA1500 liquid scintillation counter was used which incorporated appropriate quench curves to determine disintegrations per minute. A counting time of 10 minutes was always used for both ^{3}H- and ^{14}C-samples, or until 40,000cpm were detected.

2.12 Deglycosylation of Purified 5'Nase

A commercial N-glycosidase F (PNGase F) from Boehringer Mannheim was used in these studies. Following the manufacturer's instructions, sodium dodecylsulphate (SDS) was added to 100µl purified 5'Nase in 50mM Tris.Cl^-, pH 7.4 to a final concentration of 1%(w/v) SDS and was boiled for 30 minutes. The denatured sample was made up with the following ingredients to give a ten-fold dilution and provide the following final concentrations: 40mM ethylenediamine-tetra-acetic acid (EDTA), 1%(v/v) Nonidet P-40, 0.1%(w/v) SDS, 1%(v/v) 2-mercaptoethanol, 50mM Tris.Cl^-, pH7.4. In a second experiment the detergent Nonidet P-40 was replaced with MEGA 9 (nonanoyl-N-methyl-glucamide). This incubation mixture was divided into two equal 1ml portions. To one portion 60 IU of PNGase F were added, whilst the same volume of deionised water was added to the second portion. These two tubes were incubated at 37°C for 18 hours. The incubations were then run side by side on a 10% SDS-PAGE gel as described in Section 2.10.1.

2.13 Phosphatidylinositol-glycan (PIG) Anchor Manipulation

2.13.1 Phosphatidylinositol-Specific Phospholipase C (PI-PLC) Treatment and Triton X-114 Separation

The Triton X-114 (TX-114) was precondensed prior to

use in the phase separation according to the method of Bordier (1981). The remaining procedure was essentially that described by Hooper and Bashir (1991) which had in turn been modified from the method of Pryde and Phillips (1986). To 1.75mls crude membranes (~2.5µg protein), 250µl (~3 IU) PI-PLC or water were added and incubated at 37°C for 30 minutes. These samples were then subjected to both 2-phase differential TX-114 separation and 3-phase temperature-induced TX-114 separation as follows.

Two-phase separation involved adding 5µl TX-114 to 495µl PI-PLC-treated sample, vortexing and leaving on ice for 5 minutes. These were then centrifuged at 2,000xg at 4°C for 10 minutes. The supernatant was retained for later assaying and the pellet was resuspended in 0.5ml 50mM Tris.Cl^-, pH7.4 by vortexing and spun again to wash. The supernatant was again retained and added to the first supernatant whilst the pellet was resuspended finally in 1ml 50mM Tris.Cl^-, pH7.4. Thus for each sample there was a solubilised and insoluble fraction to assay for 5'Nase activity by the radiochemical method described in Section 2.11.1.

For three-phase, temperature-induced separation 20µl TX-114 was added to 980µl of each sample, vortexed and left on ice for 5 minutes. These were centrifuged as outlined above. Similarly the pellet was washed and resuspended in a final 1ml volume of 50mM Tris.Cl^-, pH7.4 as before. However, the supernatant was carefully layered onto a 1.5ml

sucrose/TX-114 separating cushion [6%(w/v) sucrose, 10mM Tris.Cl$^-$, 150mM NaCl, 0.06%(v/v) TX-114] and incubated at 30°C for 3 minutes. The tubes were centrifuged at room temperature for 3 minutes at 3,000xg in a swing-out rotor. The top aqueous layer was carefully removed (~1ml) and mixed with 20µl TX-114, vortexed and re-layered onto the original sucrose/TX-114 separating cushion. The 30°C incubation, 3,000xg centrifugation and TX-114 additions were repeated twice more, each time removing the aqueous layer, adding 20µl TX-114, vortexing and relayering onto the original sucrose/TX-114 separating cushion. On the final repetition, the aqueous layer (1ml) was removed and retained; the sucrose cushion formed an infranatant and was removed and discarded, whilst the underlying layer (~50µl) was made up to 1ml with 50mM Tris.Cl$^-$, pH7.4 and retained. The lattermost underlying layer contained non-aqueous, TX-114 soluble components.

2.13.2 Phosphatidylinositol-Phospholipase C (PI-PLC) Cleavage After Modification of Proteins by Hydroxylamine

The method used was essentially that described by Toutant *et al* (1989). The pH of crude adipocyte membranes was altered to pH 10.8 with sodium hydroxide. Solid hydroxylamine chloride was added to 1M concentration and stirred at 4°C overnight (16.5 hours). Using an Amicon ultrafiltration unit fitted with a YM30 membrane, this mixture was diluted 3 x 10-fold (i.e. 1,000-fold) with

0.1%(v/v) Triton X-100 in 50mM Tris.Cl^-, pH7.4 and reconcentrated to 6mls. Half an international unit of PI-PLC was added to the concentrate which was then incubated at 37°C for 60 minutes and then centrifuged at 40,000rpm (105,000xg_{av}) in a Beckman 70.1Ti rotor for 90 minutes at 4°C. Only the supernatant which contained PI-PLC-cleaved proteins was retained for 5'Nase radiochemical assay.

2.14 Monoclonal Antibody Production

The techniques used for monoclonal antibody (mAb) production are fairly standard. However, the detailed procedures which were used here are those taught on the four-day course offered at Keele University, Department of Biological Sciences. With the exception of immunisation, all the following techniques took place under sterile conditions normally used for cell culture work.

2.14.1 Immunisation Schedule

Two balb/c mice were immunised in tandem during the initial stages of immunisation. Control mice were also selected from the same batch and isolated at the same time as the immunised animals. The immunisation schedule commenced with a 0.2ml priming intraperitoneal (i/p) injection of Pristane on Day 0, followed by 2 x 0.25ml i/p injections of 1:1 5'Nase:Freund's Complete Adjuvant. On Day 14 the mice received 2 x 0.25ml i/p injections of 1:1

5'Nase:Freund's Incomplete Adjuvant. This last injection was repeated again on Day 28. Test bleeds were taken from the tail vein of both immunised and control animals on Days 21 and 30 to check that the animals had responded to the immunisation. Finally on Days 35, 36 and 37 the mouse with the strongest immunogenic response received 0.2ml i/p boosts of 5'Nase in physiological buffered saline (PBS). This mouse was then used on Day 38.

2.14.2 Cell Culture Media

The following cell culture media were used in Sections 2.14.3 - 2.14.6. These are standard solutions, often commercially available "ready-made".

Plain medium - RPMI-1640 containing 0.2%(w/v) sodium carbonate and glutamine. Also further supplemented with 2mM glutamine, 2mM sodium pyruvate, 100 IU/ml each penicillin and streptomycin, 2.5µg/ml amphotericin-B, 100µg/ml gentamycin.

HT/HAT medium - Plain medium plus hypoxanthine/thymidine or hypoxanthine/thymidine/aminopterin supplements (50-fold dilutions).

Foetal calf serum - added to both plain and HT/HAT mediums, 20%(v/v) for hybridoma culturing medium or 10%(v/v) for myeloma cell culturing medium.

Sodium pyruvate - additive to myeloma cell culturing medium

at 2mM, absolute requirement.

2.14.3 Preparation of Macrophage "Feeder" Layer

The day before fusion a "feeder" layer of macrophage cells was laid down in the 96-well plates to provide growth factors and mop-up cell death debris. One control mouse was sacrificed. A small flap of skin was cut away on the side of the abdomen, thus exposing the peritoneal wall. Using a 25G needle, the peritoneal cavity was injected with 5mls ice-cold plain medium. The needle was withdrawn and the abdomen massaged to wash the peritoneal cavity with medium. Changing to a 19G needle and reinserting the point through the same area (bevel side uppermost), as much as possible of the fluid was then withdrawn. This was usually ~3-3.5mls. This fluid was transferred to a sterile 20ml universal tube and topped up with a further 10mls plain medium. The tube was centrifuged at 300xg for 5 minutes to spin down the cells. Supernatant was sucked off and 2mls (precisely) of HAT medium were added to resuspend the cells. The cells were counted using an improved Neubauer Haemocytometer slide under 100x microscope magnification. The cells were diluted with HAT medium to give $6x10^4$ cells/ml. Five to six 96-well plates were filled with these cells at a density of approximately 4000 cells/well. The plates were then transferred to an incubator set at 37°C with a controlled oxygen/carbon dioxide environment.

2.14.4 Preparation of Splenocytes & Fusion

A plasmacytoma cell line called Sp2/0-Ag14 was selected as a suitable immortalised line for fusion with the balb/c splenocytes. It is a commercially available cell line, derived originally from balb/c mice, which not only is lacking in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), but also does not secrete immunoglobulins. Furthermore, the Sp2 myeloma cells tend not to adhere to the surface of the flasks in which they are grown too strongly which makes manipulations easier. This was a problem encountered with another cell line called P3X63.Ag8-653 that was tried and abandoned.

Ten days to two weeks prior to fusion the Sp2 myelomas were grown up in small (25ml) flasks, such that on the day of fusion there were 4-6 flasks available of healthy, exponentially growing myeloma cells. Depending on the density of growth, a number of these flasks were selected on the day and their contents transferred to 20ml universal tubes.

To isolate the splenocytes, one mouse was selected which demonstrated a strong immunogenic response. The mouse was killed by cervical dislocation and the spleen dissected out directly into a glass petri dish containing two $1cm^2$ pieces of gauze and 5mls plain medium. The spleen was trapped between the gauze and gently squeezed with the flat

end of a syringe plunger until the tissue appeared to be totally disrupted. Using a pasteur pipette the remaining cells trapped between the gauze were washed loose with surrounding medium. The medium was then transferred from the petri dish to a 20ml universal tube and made up to 15ml.

The myeloma cells and the splenocytes were then handled simultaneously from this stage. The cells were centrifuged at 300xg for 5 minutes. The supernatant was aspirated from the cells which were then resuspended (the myeloma cells were combined) in ~12mls plain medium each. These were then centrifuged again at 300xg for 5 minutes. This wash procedure was repeated yet again for the myelomas, but the splenocytes were resuspended on this second occasion in 5mls Tris-ammonium chloride and incubated at 37°C for 5 minutes in order to disrupt any contaminating erythrocytes. Both the splenocytes and the myelomas were centrifuged again at 300xg for 5 minutes. After aspirating the supernatants, both cell types were resuspended in 15mls 5%(v/v) dimethyl-sulphoxide (DMSO) in plain medium. They were centrifuged again at 300xg for 5 minutes and finally, resuspended accurately in 5mls plain medium.

The cells were counted by the technique previously described in Section 2.14.3 and then mixed together well in the ratio 2.5:1 (splenocytes:myelomas), trying to use all the splenocytes. The mixed cells were then centrifuged at 300xg for 5 minutes and were ready for fusion as described below.

The universal tube containing the mixed cell pellet was exposed to vigorous vibrations followed by the addition of 1ml 1%(w/v) polyethylene glycol dropwise over a period of 1 minute with constant agitation. The agitation was continued for a further 1 minute. Five millilitres of plain medium was then added over a period of 3 minutes with constant agitation, followed by another one minute of agitation. Finally, 14ml of plain medium was added over a period of 2 minutes with constant agitation. The cell suspension was then centrifuged at 200xg for 5 minutes. Supernatant was removed by aspiration and the cells were suspended in 10mls (accurately) of HAT medium and then counted as described in Section 2.14.3. Based on the cell count, the cells were diluted with HAT medium to give about 30,000 cells/ml. These were then plated out in the 96-well plates which were previously loaded with the macrophage feeder layer (Section 2.14.3) with ~280µl/well (i.e. 8000 cells/well) and transferred to the incubator.

2.14.5 Screening for Immunogenic Response and Positive Hybridomas

Two techniques were used for screening. The first procedure was based on an ELISA (Enzyme Linked ImmunoSorbent Assay) using a mouse hybridoma detection kit (Boehringer Mannheim). Ninety-six well microtitration plates pretreated at the manufacturers (Flow Laboratories Inc.) to enhance protein binding were filled with 50µl partially purified

5'Nase in a 0.5M sodium bicarbonate buffer, pH 9.4 and left overnight for maximal binding. Unbound protein was removed the next day by at least three washes with a 0.9%(w/v) NaCl, 0.1%(v/v) Tween 20 solution. A non-specific protein solution supplied with the kit was used to fill up the wells and incubated for 15 minutes with shaking to prohibit nonspecific binding of immunoglobulins directly to the well surface. This solution was washed out at least 3 times with the aforementioned wash solution. Fifty microlitres of either blood plasma for testing immunogenic response, or hybridoma supernatant was introduced to the wells and incubated with shaking for 30 minutes. The test samples were washed out three or more times and replaced with 50µl of a secondary antibody conjugated to peroxidase. This was again washed out and finally exposed to the highly sensitive (≥10ng) ABTS®-perborate substrate. After an incubation period of 30 minutes, positive wells (i.e. wells in which antibodies to the partially purified 5'Nase had been incubated) showed a dark green colour. Absorbance was read in the microtitration plates at a wavelength λ=405nm using a Titertek Multiskan® MCC/340 plate reader.

When positive samples had been identified by the above ELISA method using partially purified 5'Nase, a more labour intensive, but more accurate enzyme activity inhibition technique was used to confirm the former results. This involved incubating 25µl (~5µg) purified 5'Nase with ~50µl test sample, or medium as a control, at room temperature with

shaking for 3 hours. To this mixture 100µl of a commercial sheep anti-mouse antibody was added and incubated at room temperature with shaking for a further hour. Ten milligrams of Protein A-acrylic beads were preweighed in microfuge tubes and the protein-double antibody mixture was added, vortexed for 20 seconds and spun in a microfuge at 2,000xg for 15 minutes. The supernatants were then removed from the immuno-precipitations, diluted ten-fold with 0.1%(v/v) Tween 20 in 50mM Tris.Cl^-, pH7.4 and subsequently used in radiochemical 5'Nase assay (as in Section 2.11.1).

2.14.6 Expanding Positive Clones and Freezing Cells

Hybridomas which were positive for antibodies specific to 5'Nase as indicated by the screens in Section 2.14.5 were transferred to 24-well plates, allowed to grow up in a hypoxanthine/thymidine environment and then screened again by the above procedures. Those cells which still showed positive on both screens were grown up further in 25ml flasks and finally frozen down by the following procedure. The cells were suspended in the culture medium in which they were growing and then mixed in a 1:1 ratio with freezing medium (20%(v/v) dimethyl sulphoxide, 80%(v/v) foetal calf serum) in a sterile centrifuge tube. The cells were then centrifuged for 5 minutes at 300xg. Supernatant was removed by aspiration and the cells resuspended in exactly 0.5ml of the hypoxanthine/thymidine culture medium in which they had been growing. Over a period of 10 seconds, 0.5ml of freezing

medium (as above) was added with gentle agitation and then thoroughly mixed. This mixture was transferred to a vial suitable for long-term storage, frozen initially at -70°C for 48 hours and then transferred to storage under liquid nitrogen.

2.15 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.

CHAPTER 3

RESULTS: METABOLIC STATUS

RESULTS: METABOLIC STATUS

3.1.1 Alteration of Membrane-Bound 5'Nase Activity with Metabolic Status: Streptozotocin-Diabetes

The work carried out for this study concerns a membrane-bound 5'Nase from white adipose tissue. Either adipocytes or crude membranes derived from white epididymal adipose tissue were used as the source of this membrane-bound activity. It was believed that the membrane-bound 5'Nase under investigation was the ecto-enzyme and as such will be referred to as ecto-5'Nase (e5'Nase) throughout the following chapters.

Existing reports of alterations in e5'Nase activity with varying hormonal/physiological states suggest that the changes are tissue-specific. In the streptozotocin-diabetic state e5'Nase activities have been shown to decrease in hepatic membranes (Chandramouli & Carter, 1975), whilst in white adipocytes activities were increased by two to four fold (Jamal & Saggerson, 1987; Karnieli *et al*, 1987) and no effect at all was observed in brown adipose tissue (Jamal & Saggerson, 1987). Other metabolic states which are characterised by changes in hormone levels have also been described as having variable e5'Nase activities in different tissues. For example in PTU-induced hypothyroidism, brown and white adipose tissue show ~50% decreases in 5'Nase activities (Jamal & Saggerson, 1987), whilst synaptosomal membrane

e5'Nase is increased in some brain regions (Mazurkiewicz & Saggerson, 1989). In this present study these and other metabolic states, including hyperthyroidism and fasting, were investigated with respect to e5'Nase activity levels in white adipose tissue (see also Section 3.5).

In 7-day streptozotocin-diabetic rats the e5'Nase activity in membrane preparations from whole white epididymal adipose tissue was measured as 350.0 ± 24.8 nmol/min/mg protein (or 4.63 ± 0.16 nmol/min/μg DNA) (n=3) as compared to control animals in which it was measured as 191.3 ± 29.6 nmol/min/mg protein (or 1.94 ± 0.07 nmol/min/μg DNA) (n=3). These results agree with the reports that e5'Nase activity roughly doubles in white adipose tissue in the diabetic state. In white adipose tissue the expression of enzyme activity with respect to micrograms of DNA, as opposed to the conventional milligrams of protein, can reveal more about real changes which occur because unlike other cell types, the adipocyte undergoes large size fluctuations. The adipocyte expands and contracts to accomodate its fat storage and a corresponding increase or decrease in cell surface area can dramatically alter the quantity of protein without changing the cell number. Surprisingly, in the diabetic state when lipolysis significantly reduces cell size, 5'Nase measurements showed a 1.8 fold increase when expressed per milligram of protein, whilst there was a 2.4 fold increase in activity when expressed against DNA. This unexpected result may be explained by the fact that the measurements were made in

membranes derived from whole white adipose tissue and therefore the smaller change in specific activity observed when expressed per milligram of protein may reflect the incresed membrane protein due to increased content of blood cells in the tissue in diabetics. Nevertheless, when subjected to statistical analysis by using the student's t-test, these activities showed a significant difference both when expressed per milligram of protein ($P<0.02$) and when expressed per microgram of DNA ($P<0.001$).

3.1.2 Further Analysis of Altered E5'Nase Activities in White Adipose Tissue During Streptozotocin-Diabetes

The doubling of e5'Nase activity noted in Section 3.1.1 resulting from induction of a diabetic state by a streptozotocin injection was investigated further. The question which needed to be addressed was whether the noted changes were due to an increase in enzyme abundance, or possibly to modifications to existing enzyme protein. A series of membrane preparations were made from whole adipose tissue taken from both control and diabetic animals (n=4 for each metabolic state). The preparations were assayed for e5'Nase activity and as in Section 3.1.1, the activity was found to double in the diabetic state (see Table 3.1). The samples were then run out on a polyacrylamide gel (100µg per track) and Western blotted onto nitrocellulose. The nitrocellulose was exposed to a polyclonal rabbit anti-rat e5'Nase antiserum provided by Dr.J.P.Luzio. The photograph

of the developed blot is shown in Figure 3.1. Despite the large amounts of protein loaded into each track, only two major bands cross-reacted with the antiserum, at 76kDa and 72kDa. Reports of mammalian e5'Nase subunit molecular size from SDS-PAGE of purified enzyme have generally been in the 70-74kDa range (Harb *et al*, 1983; Wada *et al*, 1986; Thompson *et al*, 1987; Klemens *et al*, 1990; Misumi *et al*, 1990 and 1990a; Lai & Wong, 1991; for review see Zimmermann, 1992). Overall, immunoblotting showed increased abundance of protein in the diabetic tracks, suggesting a quantitative change in 5'Nase rather than a modification of the integral activity. Studying the photograph in Figure 3.1, it is clear that greater quantities of anti-e5'Nase cross-reacted with the 76kDa protein both from the control and from the diabetic rats. Furthermore, it is the more intense 76kDa band which increased in abundance in the diabetic tracks, whilst the 72kDa band remained unaltered. This was confirmed by densitometric analysis.

Quantitation by densitometry provided comparative values for each band which are displayed in Table 3.1, with a sample densitometry trace shown in Figure 3.2. When considering the two cross-reacting bands individually, the intensity of the 76kDa band increased 2.5-fold in the diabetic tracks as compared to the control tracks, whilst there was no apparent change in abundance of the 72kDa band. However, the combined abundance of the two bands increased by 2-fold in the diabetic samples which correlates very well

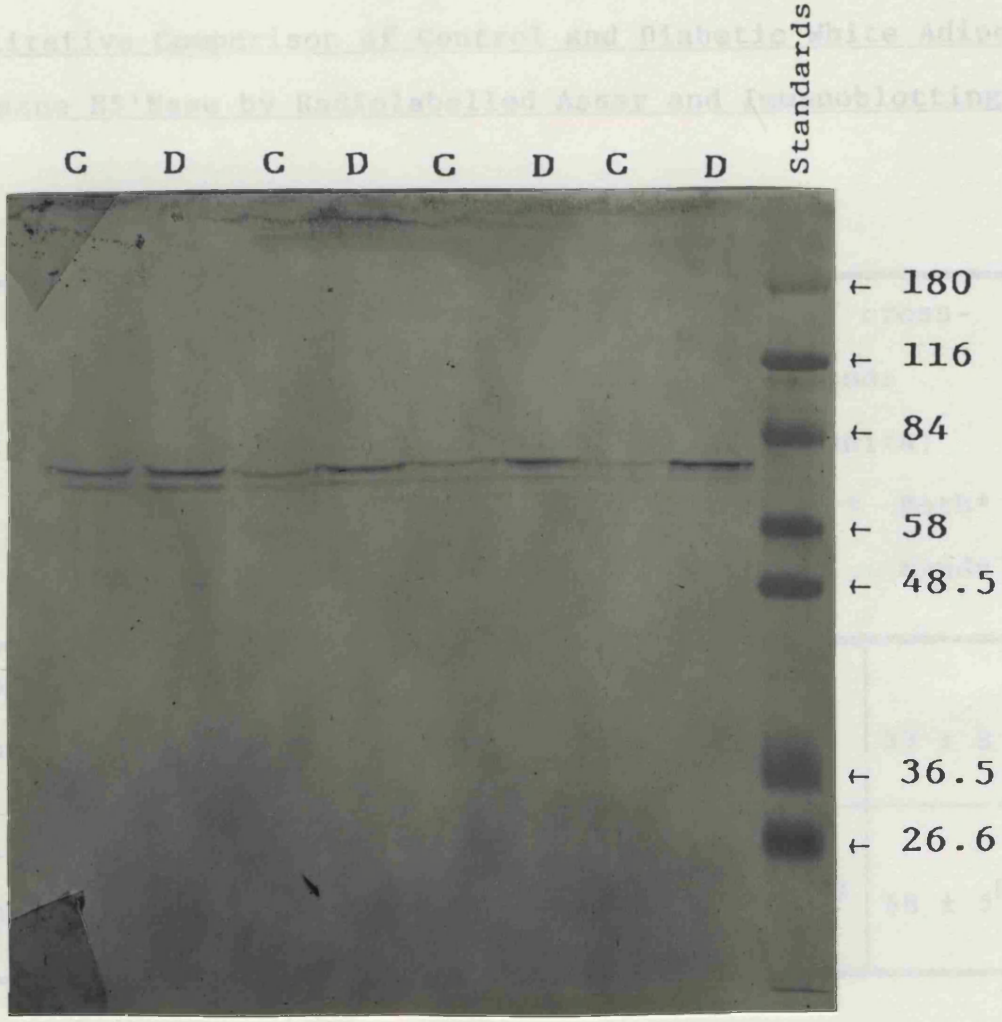


FIGURE 3.1: Western Blot of Control and Diabetic Rat White Adipose Tissue Membranes Exposed to Rabbit Anti-Rat E5'Nase

100µg crude membranes per track were resolved in 10% polyacrylamide gel as described in Materials and Methods.

Comparison to standard molecular weight markers indicated that the upper band is 76kDa and the lower band is 74kDa.

TABLE 3.1

Quantitative Comparison of Control and Diabetic White Adipose Tissue E5'Nase by Radiolabelled Assay and Immunoblotting

State	5'Nase Activity (nmol/min/mg protein)	Abundance of cross-reacting bands (arbitrary units)		
		Lower* band	Upper* band	Both* bands
Control (n=4)	171 ± 17	11 ± 2	22 ± 6	33 ± 8
Diabetic (n=4)	328 ± 26[A]	13 ± 3	55 ± 5[A]	68 ± 5[B]

Notes: * Lower = 72 kDa; Upper = 76 kDa; Both = 72 & 76 kDa bands combined (as calculated from Figure 3.2)

[A] denotes $P<0.01$ versus the control

[B] denotes $P<0.02$ versus the control

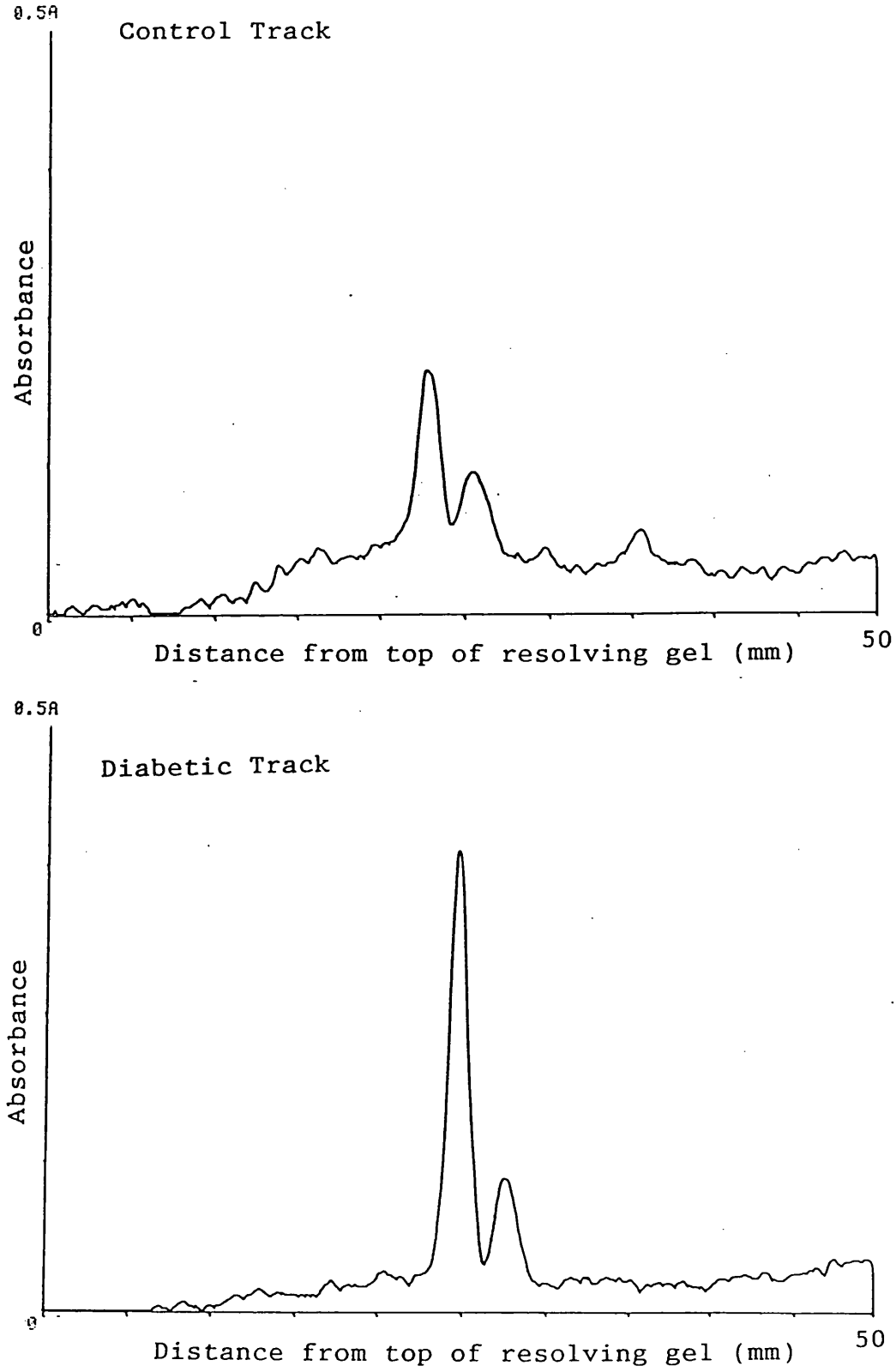


FIGURE 3.2: Sample Densitometry Trace of One Control Track and One Diabetic Track from an Immunoblot

The blot scanned for these traces is shown in Figure 3.1

with the 1.9-fold increase in 5'Nase activity measured by radiochemical assay as shown in Table 3.1. The implications of these two cross-reacting bands and their variations of abundance in control and diabetic membrane must be addressed. Despite the densitometric analysis providing values from the quantitation of the two bands combined which parallel the changes observed when measuring e5'Nase activity by radio-labelled assay, there is no direct evidence that either or both bands are indeed e5'Nase. Nevertheless, these results do raise the question as to whether the two cross-reacting bands may represent two distinct molecular forms of e5'Nase within white adipose tissue. From these results it would seem that the two forms could consist of a constitutive form and an adaptive form, arising either from the different cell types within the adipose tissue or from a single cell type. In the latter case, two different species of e5'Nase within a single cell type could support the concept of a PI-G-anchored form and a transmembrane form which has been suggested by several authors for e5'Nase (Dieckhoff *et al*, 1987; Grondal & Zimmermann, 1987; Thompson *et al*, 1987; Stochaj *et al*, 1989; Klemens *et al*, 1990) and which has already been demonstrated for the LFA-3 and N-CAM adhesion proteins (Dustin *et al*, 1987; Gower *et al*, 1988). Indeed, this reasoning may also provide a means by which one form could be constitutive and the other adaptive as the varying anchoring mechanisms would be affected differently by physiological changes such as the activation of an endogenous PI-PLC. Conversely, the two cross-reacting bands may

represent differing degrees of glycosylation or protein isoforms, with the two visualised on this blot simply representing the most abundant within the sample. Certainly, purification of e5'Nase from human placental e5'Nase showed thirteen isoforms when electrophoretically separated on two dimensional gels (Buschette-Brambrink & Gutensohn, 1989). Further investigation would be required for any definitive conclusions. However, regardless of whether the two cross-reacting bands are indeed both forms of e5'Nase or not, it is clear that the abundance of protein cross-reacting with the anti-e5'Nase antibodies is significantly greater in the diabetic tracks. This finding supports the idea of increased e5'Nase concentration rather than a modification of the integral e5'Nase activity.

3.2 Localisation of E5'Nase Within White Adipose Tissue

The measurements reported in Section 3.1 above were made in membranes isolated from whole white adipose tissue. As discussed in Chapter 1, the product of the reaction catalysed by e5'Nase is adenosine which not only promotes some of the actions normally attributed to insulin in the adipocyte (Ohisalo *et al*, 1981; Green, 1983; Honeyman *et al*, 1983; Wong *et al*, 1985), but also contributes to the regulation of blood flow in adipose tissue (Sollevi & Fredholm, 1981). Previous studies on parametrial and epididymal fat showed only ~32% of the 5'Nase activity was associated with the adipocytes (Green & Newsholme, 1981;

Vernon *et al*, 1983). It was therefore considered important to this present study to identify whether the e5'Nase activity was predominantly associated with the adipocytes or with other cell types present in the adipose tissue. Furthermore, studies were undertaken to determine whether the variations in 5'Nase activity which occur in streptozotocin-diabetes (see Section 3.1 above) were limited to one cell type or whether they occur universally within the white adipose tissue.

Whilst isolating adipocytes, both the pellet and the infranatant from the post-collagenase digest washing steps were collected as described in Chapter 2. These samples, together with the adipocytes, were used to obtain membrane fractions. E5'Nase activities were measured as before. The adipocyte membranes were found to have the lowest levels of e5'Nase, only 1.3% when expressed as a proportion of the total activity. Membranes obtained from the collagenase-wash "pellet" contained 27.8% of the total e5'Nase activity; whilst the collagenase-wash "infranatant" contained the remaining 70.9% e5'Nase activity. These proportions are displayed in Figure 3.3 overleaf.

From these data it is clear that only a very small proportion of 5'Nase is associated with those cells that have a sufficiently low density to be recovered above the infranatant. Presumably this layer will contain very few non-adipocytes, but the possibility that the infranatant and/or the

Fraction Description	Specific Activity (nmol/min/mg protein)
Adipocytes	15.08
Infranatant	99.52
Pellet	136.70

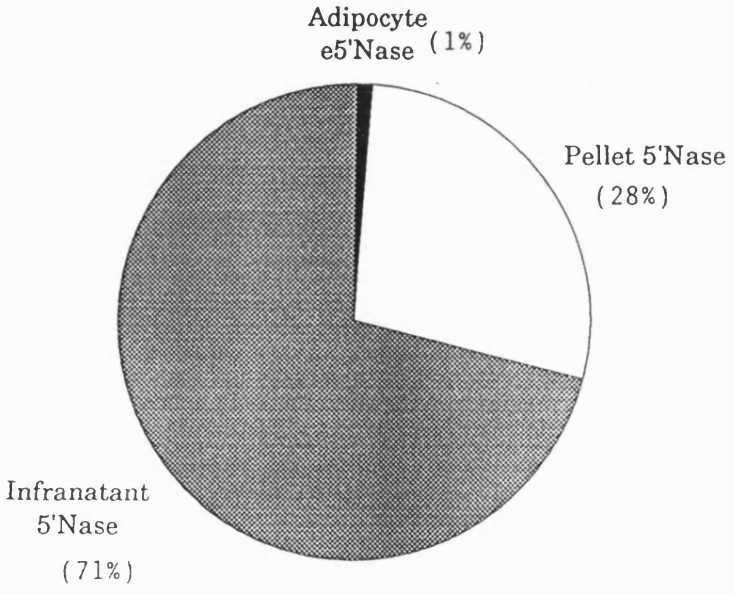


FIGURE 3.3: Ratios of e5'Nase Activity in Different Cellular Locations of White Adipose Tissue

Total Activity Profile, i.e. percentage of total e5'Nase in different parts of the adipose tissue.

Total activities measured:
Adipocytes = 19.3 nmol/min
Infranatant = 1074.8 nmol/min
Pellet = 421.0 nmol/min

lower pellet contain some very small, more dense, adipocytes and pre-adipocytes cannot be excluded. The samples are obtained after the collagenase digestion and consequently the connective tissue has been removed from the preparation. Non-adipocytes, such as vasculature-related cells for example, are therefore likely to form the majority of remaining cell types in these "infranatant" and "pellet" membranes. The report of Sollevi & Fredholm (1981) in which adenosine was implicated in the physiological regulation of adipose tissue blood flow, combined with the demonstrations by Madsen & Malchow-Mϕller (1983) and Jamal & Saggerson (1988) that insulin status could also affect adipose tissue blood flow, need to be considered in light of these findings that a large proportion of adipose tissue e5'Nase is associated with non-adipocyte material. Providing there were no physiological inhibitors such as ATP or ADP in the vicinity, the effect of supplying substrate to e5'Nase would be to raise adenosine concentrations locally. However, rapid clearance of the adenosine makes it questionable whether the paracrine effect would be limited to the site of formation (e.g. the vasculature) or whether the adenosine could also interact with receptors on the adipocytes.

Further investigations were carried out in this study to determine the location within white adipose tissue of the altered e5'Nase activities previously reported in the streptozotocin-diabetic state (Jamal & Saggerson, 1987; Karnieli *et al*, 1987). Whilst preparing white adipocytes

from 7-day streptozotocin-diabetic rats and from control rats, both the post-collagenase wash pellet and infranatant were combined. Membrane fractions were prepared from this combined sample as well as from the adipocytes and e5'Nase was measured in all samples. No variation in e5'Nase activity was found between the control and diabetic adipocyte membranes, with specific activities being measured at 11.05 ± 2.22 nmol/min/mg protein (controls) and 10.21 ± 1.61 nmol/min/mg protein (diabetics). However, the other cell types showed the type of increase previously measured in whole adipose tissue membranes, with specific activities being measured at 60.53 ± 5.61 nmol/min/mg protein (controls) and 134.80 ± 14.45 nmol/min/mg protein (diabetics). A summary of these values is depicted in Figure 3.4. These findings are in direct contrast to the earlier report of Jamal & Saggerson (1987) in which the reported increase in diabetes of e5'Nase activity was measured in membranes from isolated white adipocytes. Furthermore, the overall 5'Nase activities measured in that report were higher than those of this study. It is important to note however, that in the study of Jamal & Saggerson (1987) the induction of diabetes was achieved with a higher dose of streptozotocin (100mg/kg) and the development of diabetes, often leading to ketosis, was restricted to a shorter period of time (three days) than the seven days used in this study. The breeding stocks used also appear to have a noticeable effect on 5'Nase activity as will be reported in Section 3.5 concerning the investigation into the effects of fasting. Repetition of these experiments

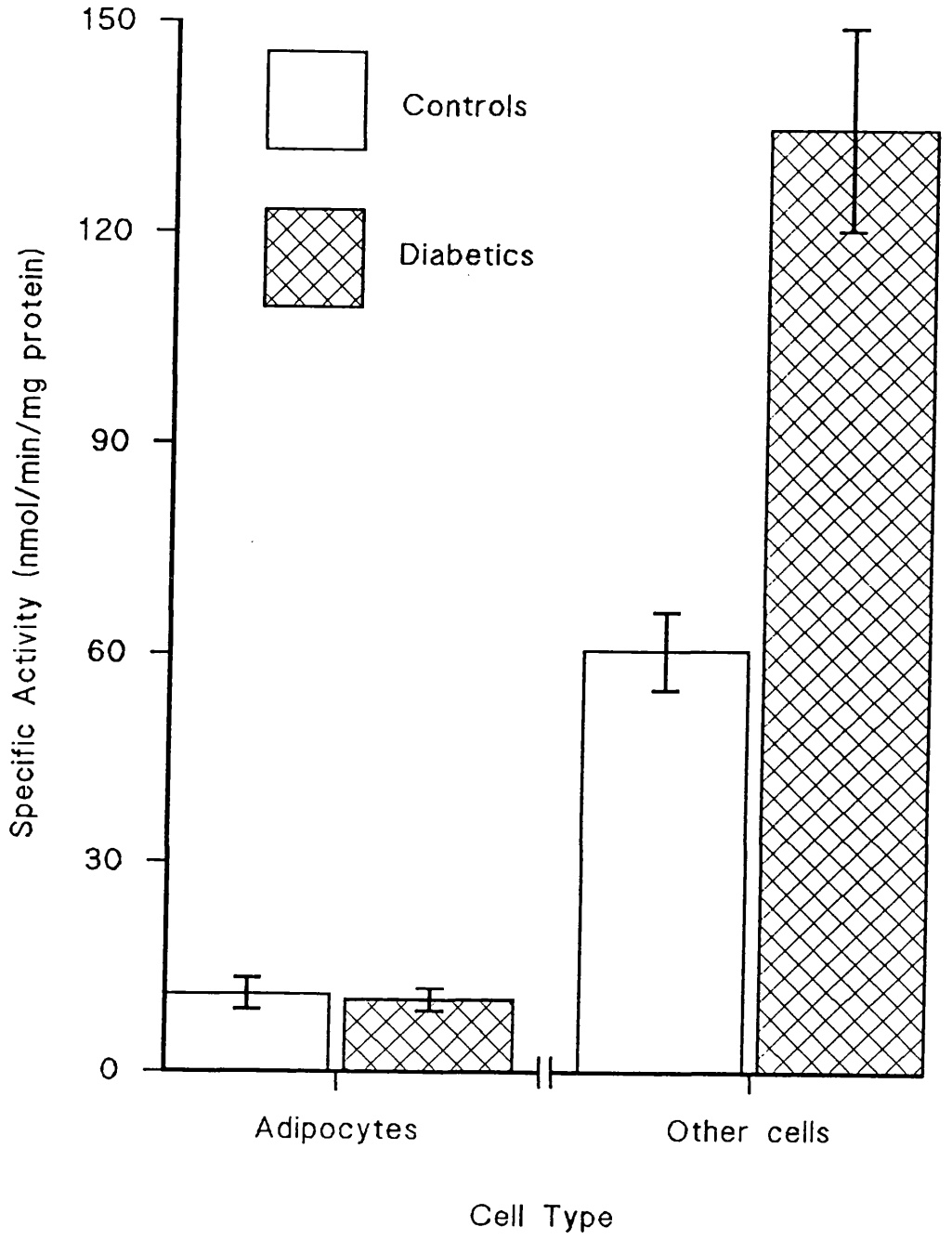


FIGURE 3.4: Cellular Location of Changes in E5'Nase which Occur in White Adipose Tissue of Streptozotocin-Diabetic Rats

Other cells = infranatant and pellet membranes combined from the adipocyte isolation procedure. The probability that the diabetic e5'Nase activity in the "Other cells" differs from the control activity is significant, $P<0.001$, where $n=6$ for controls and $n=3$ for diabetics, both for Adipocytes and Non-Adipocyte cells.

using varying diabetes-induction schedules could well produce some interesting results in this area.

3.3 The Effect on E5'Nase Activities of *In Vivo* Insulin Treatment in Streptozotocin-Diabetic Rats

As described above, it has been shown that adipose tissue blood flow increases in the diabetic state (Madsen & Malchow-Møller, 1983; Jamal & Saggerson, 1988). Of further interest in their study is that they also demonstrated that *in vivo* insulin administration correspondingly decreased the blood flow and they hypothesized that this is achieved by decreased production of adenosine. Jamal & Saggerson (1987) and Karnieli *et al* (1987) both found evidence to support this hypothesis by showing that whilst e5'Nase activity in white adipocytes was raised by inducing streptozotocin-diabetes, a schedule of *in vivo* insulin administration brought the activity back down to levels marginally lower than in the control animals.

In the present study the time course for insulin action was investigated in whole adipose tissue as it was considered that overall changes in e5'Nase activity were more likely to be relevant to whole tissue changes in lipolysis and blood flow. Diabetes was induced with streptozotocin in two groups of rats. At the given time point both the control animals and one group of diabetics received saline injections whilst insulin was administered to the second group of diabe-

tics. E5'Nase activity was measured in membrane preparations from each group: controls, diabetics, and insulin-treated diabetics. The data from these measurements are presented in Table 3.2. Interestingly, the 0.25 hour time point animals all showed significantly raised specific activities over the remainder of the time points. This phenomenon may reflect acute changes which result from stress, induced by the animal handling and injection. For all the other time points, there was a minimum of 2 hours between injection and killing during which time an acute change may have had time to subside. To facilitate comparative analysis, the activities have therefore been expressed as relative specific activities in Table 3.2 and Figure 3.5. Whilst e5'Nase activities remained relatively constant for both the control group and the diabetic group over the range of 2 to 48 hours, the insulin-treated diabetic group showed a dramatic drop in e5'Nase activity to a level marginally lower than control levels at a point between 24 to 48 hours, but not earlier. Statistical analyses of these results (as shown in Table 3.2) confirmed a consistent significant difference between the saline-treated diabetic group when compared to the control group at each time point up to and including 48 hours. However, the insulin-treated diabetic group remained significantly different from the control group at each time point up to and including 24 hours, whilst at 48 hours there ceased to be any statistical difference between this group and the control group.

TABLE 3.2

E5'Nase Activity in White Adipose Tissue Membranes Prepared from Control, Diabetic, and Insulin-Treated Diabetic Rats Over a Range of Time Points Between 0.25 to 48 Hours

Hours	0.25	2	6.5	16	24	48
CONTROLS & SALINE						
Specific Activity - nmol/min/mg protein ± S.E. [A]	170.5 ±15.8	120.7 ±10.3	116.1 ±6.3	105.2 ±6.0	116.3 ±11.8	112.2 ±15.5
Relative Specific Activity [B]	1	1	1	1	1	1
DIABETICS & SALINE						
Specific Activity - nmol/min/mg protein ± S.E. [A]	[C] 251.1 ±8.9	[D] 173.1 ±9.0	[C] 178.0 ±13.4	[F] 153.9 ±18.1	[D] 169.6 ±8.4	[G] 152.4 ±18.3
Relative Specific Activity [B]	1.47	1.43	1.54	1.47	1.47	1.36
DIABETICS & INSULIN						
Specific Activity - nmol/min/mg protein ± S.E. [A]	[D] 233.5 ±10.0	[E] 167.1 ±13.2	[C] 163.7 ±8.6	[F] 141.0 ±13.2	[D] 164.2 ±6.7	[H] 107.3 ±6.9
Relative Specific Activity [B]	1.37	1.38	1.41	1.34	1.41	0.96

[A] denotes n=6 for each time point except the Diabetic & Saline, 48 hour, where n=4

[B] denotes that relativity is to corresponding time matched control group

[C] denotes $P<0.002$ as compared to the time matched controls

[D] denotes $P<0.01$ as compared to the time matched controls

[E] denotes $P<0.02$ as compared to the time matched controls

[F] denotes $P<0.05$ as compared to the time matched controls

[G] denotes $P<0.2$ as compared to the time matched controls

[H] denotes $P>>>0.3$ as compared to the time matched controls

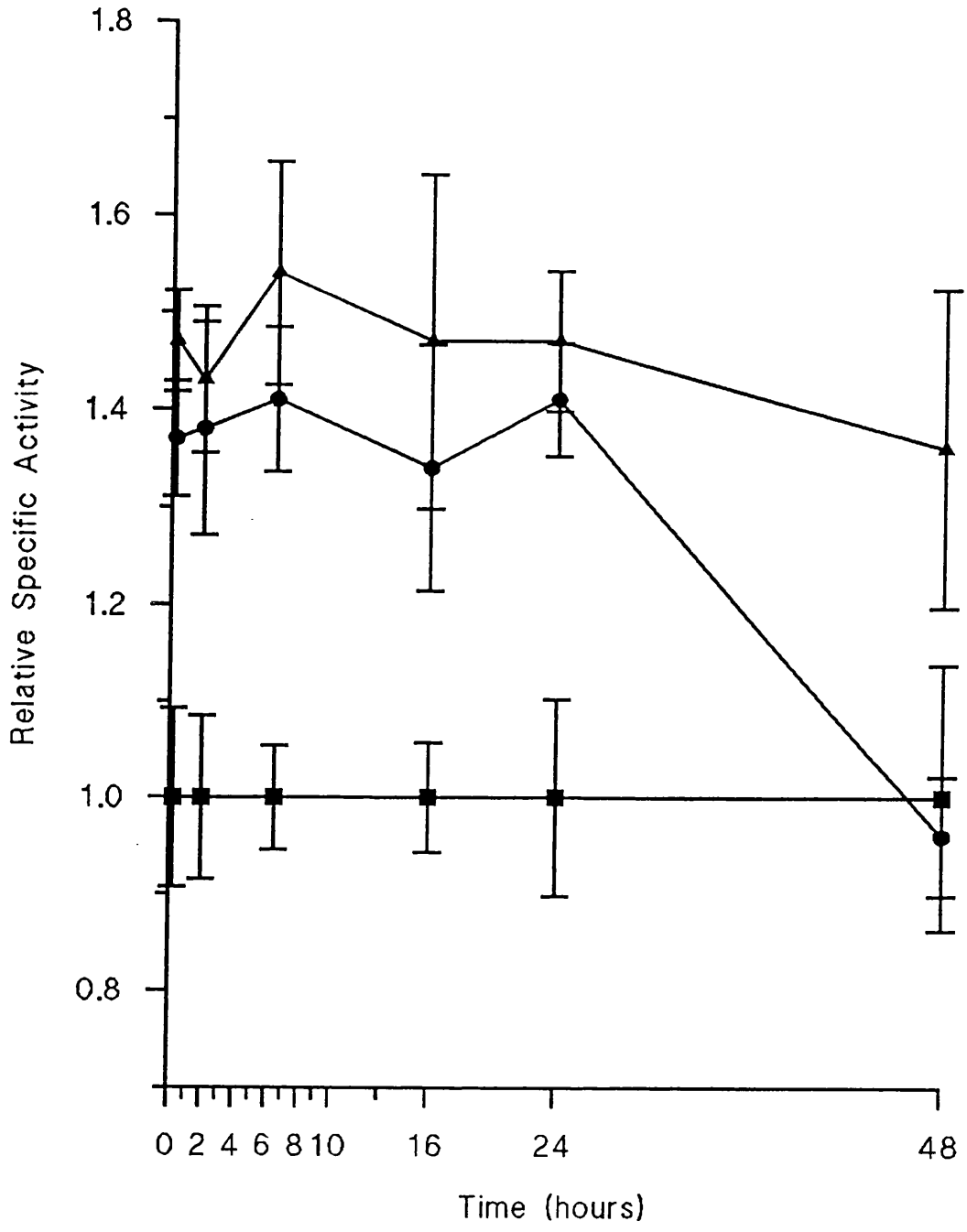


FIGURE 3.5: Relative Specific Activities of E5'Nase Measured in Control, Diabetic, and Insulin-Treated Diabetic Rats Over a Range of Time Points Between 0.25 to 48 Hours

■ = Control groups (n=6 at each time point)
▲ = Diabetic, saline treated groups (n=6 at each time point except 48 hours, when n=4)
● = Diabetic, insulin treated groups (n=6 at each time point)

The studies reported by Sollevi & Fredholm (1981) and Madsen & Malchow-Møller (1983) both demonstrated that alterations in blood flow occurred rapidly (20-80 minutes) in response to changes in plasma concentrations of adenosine and insulin. It would therefore appear that changes in e5'Nase resulting from *in vivo* insulin administration are not responsible for regulating adipose tissue blood flow.

Several mechanisms of action could be postulated for the insulin-mediated reduction of e5'Nase levels. The finding of this present study, showing a relatively slow response to *in vivo* insulin treatment, appears to eliminate the possibility that regulation in this instance is by insulin-activation of the PI-G-specific phospholipase C thus cleaving the e5'Nase anchor. Nevertheless, this mechanism is further addressed in Section 3.4.

There have been several reports of e5'Nase exchange between the cell surface and smooth intracellular vesicles via membrane internalisation (Stanley *et al*, 1980; Widnell *et al*, 1982; Van den Bosch *et al*, 1988). However, this mechanism also appears to be redundant in terms of insulin's regulation of e5'Nase as the cellular redistribution is a bidirectional event and the cycle is completed within a few hours. This bidirectional exchange occurs even in the presence of the protein synthesis inhibitor cycloheximide. The half-time for total cell surface exchange was calculated at approximately four hours when measured in cultured rat

fibroblasts (Widnell *et al*, 1982), whilst experiments in isolated rat hepatocytes, adipocytes and lymphocytes showed similar rates (Stanley *et al*, 1980).

A third mechanism which could explain long-term effects on protein abundancy would be that regulation was at a transcriptional or translational level. Certainly insulin has been shown to act in such a down-regulatory role, as in the case of hepatic phosphenolpyruvate carboxykinase in which expression is decreased at the level of mRNA transcription (Forest *et al*, 1990). This regulatory mechanism by insulin would need to be investigated further to determine whether e5'Nase is controlled in a similar fashion.

3.4 Attempted *In Vitro* Release of E5'Nase from Isolated Adipocytes and from Membrane Preparations - Incubations with Insulin and with PI-PLC

As discussed in Section 3.3 above, two groups have independently demonstrated that the raised e5'Nase levels which result in white adipocytes from the induction of streptozotocin-diabetes in rats, are similarly reduced to below control levels on insulin treatment *in vivo* (Jamal & Saggerson, 1987; Karnieli *et al*, 1987). The mechanism by which insulin controls e5'Nase levels remains unclear. It has been shown that e5'Nase is covalently bound to the plasma membrane via the PI-G anchor (Low & Finean, 1978; Shukla *et al*, 1980; Panagia *et al*, 1981; Baron *et al*, 1986; Bailyes *et*

al, 1990). Furthermore, the PI-G anchor has been shown to be susceptible to insulin-sensitive PI-G PLC release (Larner *et al*, 1974; Mato *et al*, 1987; Chan *et al*, 1988; Romero *et al*, 1988; Alverez *et al*, 1988). The possibility that insulin released e5'Nase from white adipocytes into the extracellular space was therefore investigated.

Isolated adipocytes and adipose tissue membranes from control and diabetic animals were incubated for up to 2 hours with 1mU/ml insulin (~7.5nM). During the incubations, in order to satisfy any requirements of the potentially mediating enzymes such as PI-G PLC, different combinations of additions were made including $MgCl_2$, ±ATP, and ±GTP(γ)S. Incubation media were collected by removal of the infranatants from the adipocytes and passing the samples through 0.45µm filters. The filtrates were dialysed overnight to remove the incubation additives, especially the ATP which is a potent inhibitor of e5'Nase activity.

As can be seen in Figures 3.6 and 3.7 there is no evidence that *in vitro* incubations of either whole adipocytes or adipose tissue membranes with insulin results in the release of e5'Nase. There was no difference between the control and diabetic values with respect to the effect of insulin and therefore all results are presented together as relative activities. The addition of ATP and GTP(γ)S similarly had no effect on promoting insulin-stimulated release of e5'Nase from white adipocytes or the corresponding

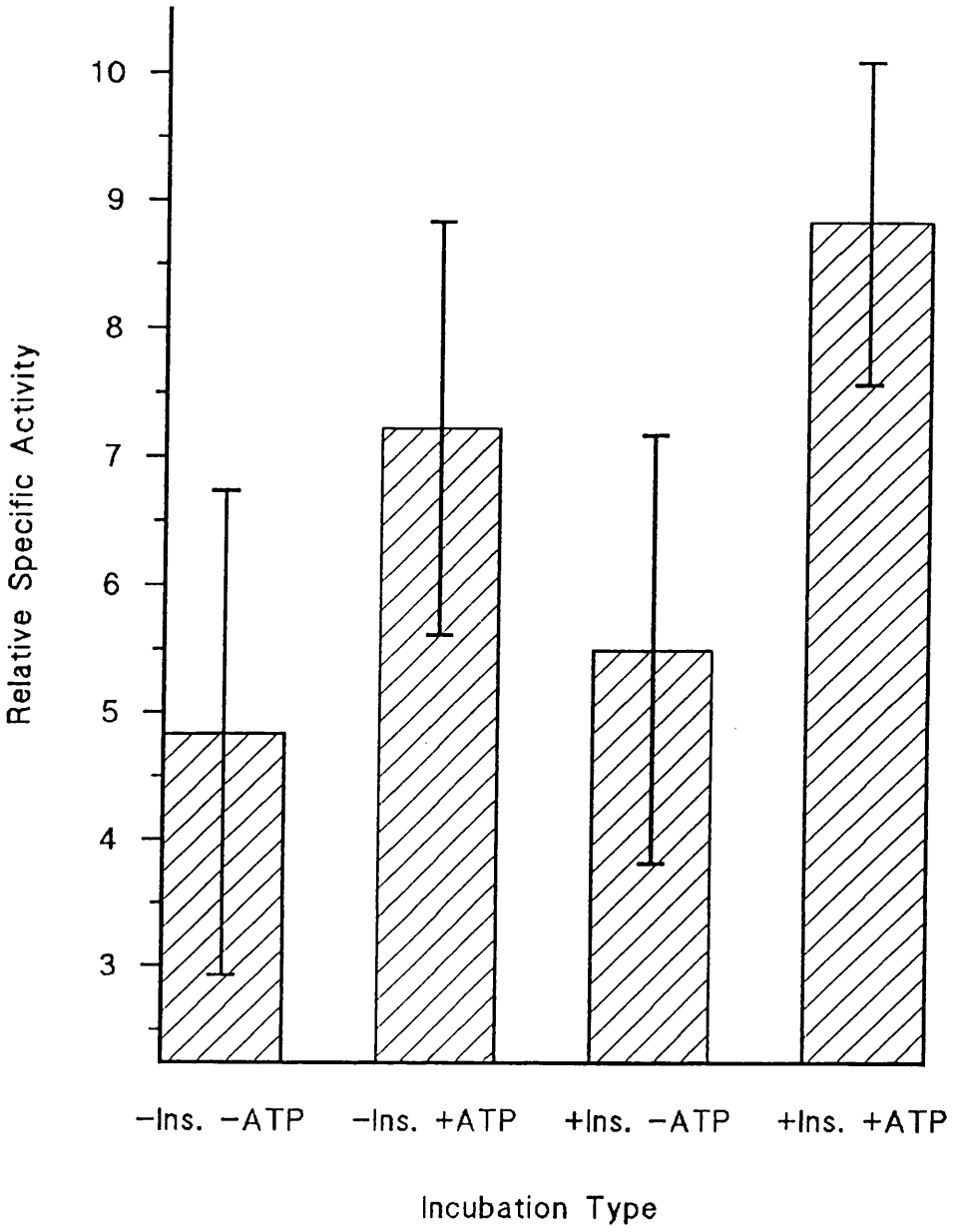


FIGURE 3.6: Effect of *In Vitro* Insulin Addition to Intact Whole White Adipocytes on E5'Nase Release

[Activities are relative to incubation blanks in which no additions were made, to facilitate direct comparisons.]
The relative specific activities shown above represent the e5'Nase activity measured in the incubation medium after a 30 minute incubation with the indicated additions.

±Ins. = ±1mU/ml Insulin
±ATP = ±3mM ATP
} n=2 for each bar
All incubations included 1mM $MgCl_2$

Dialysis overnight ensured no inhibition by any additive.

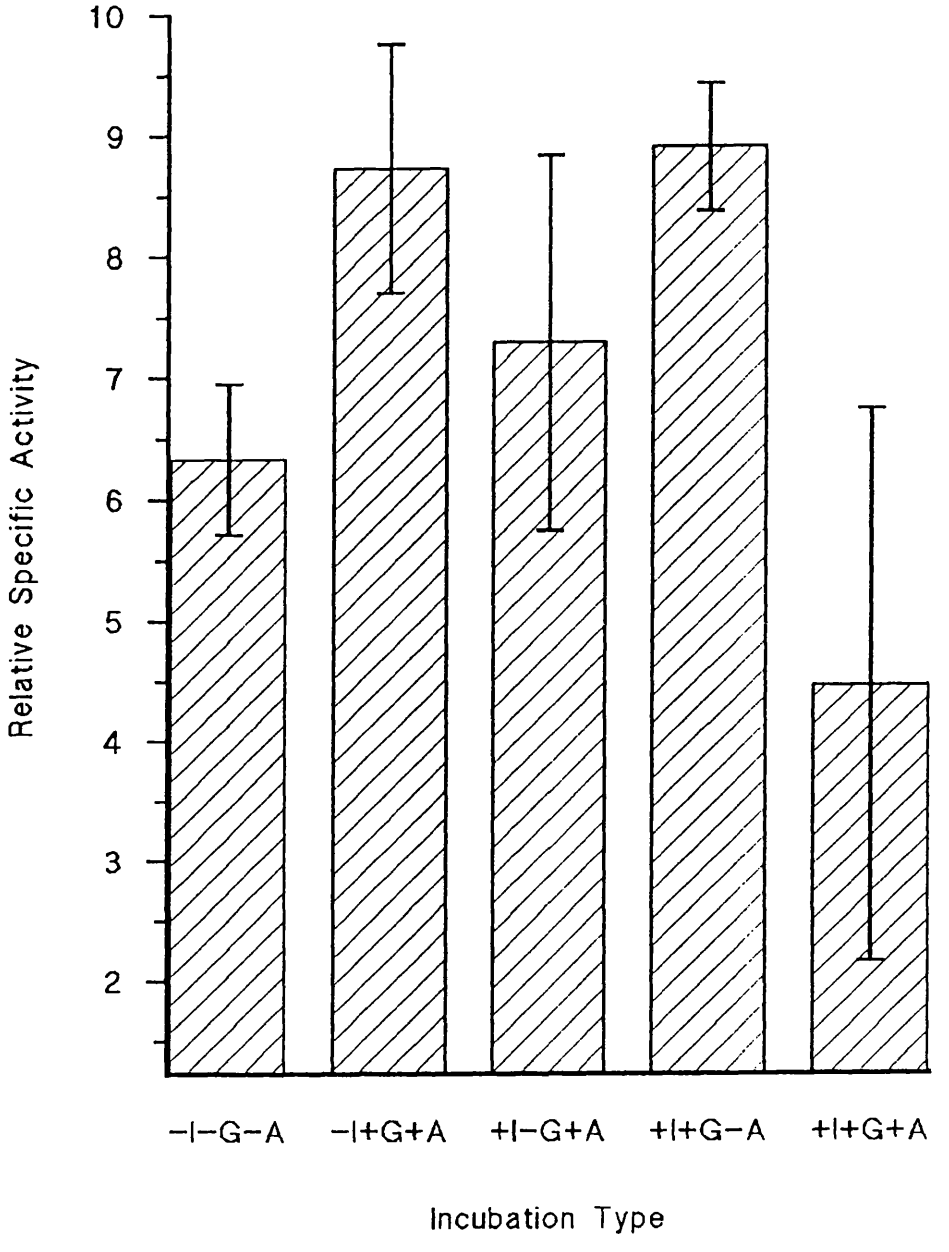


FIGURE 3.7: **Effect of *In Vitro* Insulin Addition to White Adipose Tissue Membranes on E5'Nase Release**

[Activities are relative to incubation blanks in which no additions were made, to facilitate direct comparisons.]
The relative specific activities shown above represent the e5'Nase activity measured in the incubation medium after a 2 hour incubation with the indicated additions.

±I = ±1mU/ml Insulin
±G = ±10μM GTP(γ)S
±A = ±3mM ATP
All incubations included 2mM $MgCl_2$

} n=2 for each bar

Dialysis overnight ensured no inhibition by any additive.

membranes. These findings appear to be at variance with those of Karnieli *et al* (1987) in which they found a 46% decrease of 5'Nase specific activity within thirty minutes of exposure to insulin. However, it is important to note that they measured the activity in purified plasma membrane fractions after the incubations, whilst here I measured release of activity into the surrounding medium. Alternatively, internalised e5'Nase would also not have been identified in their study, nor indeed from this experiment. The effect of insulin *in vivo*, as discussed in section 3.3, only became apparent after 24 hours of administration to diabetic animals. The short-term incubations in this case therefore cannot be directly comparable to the *in vivo* insulin treatments in order to determine the fate of e5'Nase.

There are several potential sites of insulin action to reduce the e5'Nase activity of diabetics *in vivo.* Figure 3.8 illustrates a few possibilities which divide into two general areas. Either the synthesis/degradation cycle of the 5'Nase may be affected or insulin-induced cleavage of the enzyme from the membrane by PI-G PLC may be responsible. During an insulin-deprived state such as diabetes, synthesis of e5'Nase may up-regulate thus producing the larger quantities of enzyme observed in Sections 3.1 and 3.2. Subsequent re-introduction of insulin may then reduce levels of further synthesis, allowing normal rates of degradation to lower the levels of e5'Nase in the natural course of events. Alternatively, degradation of e5'Nase may be directly affected by

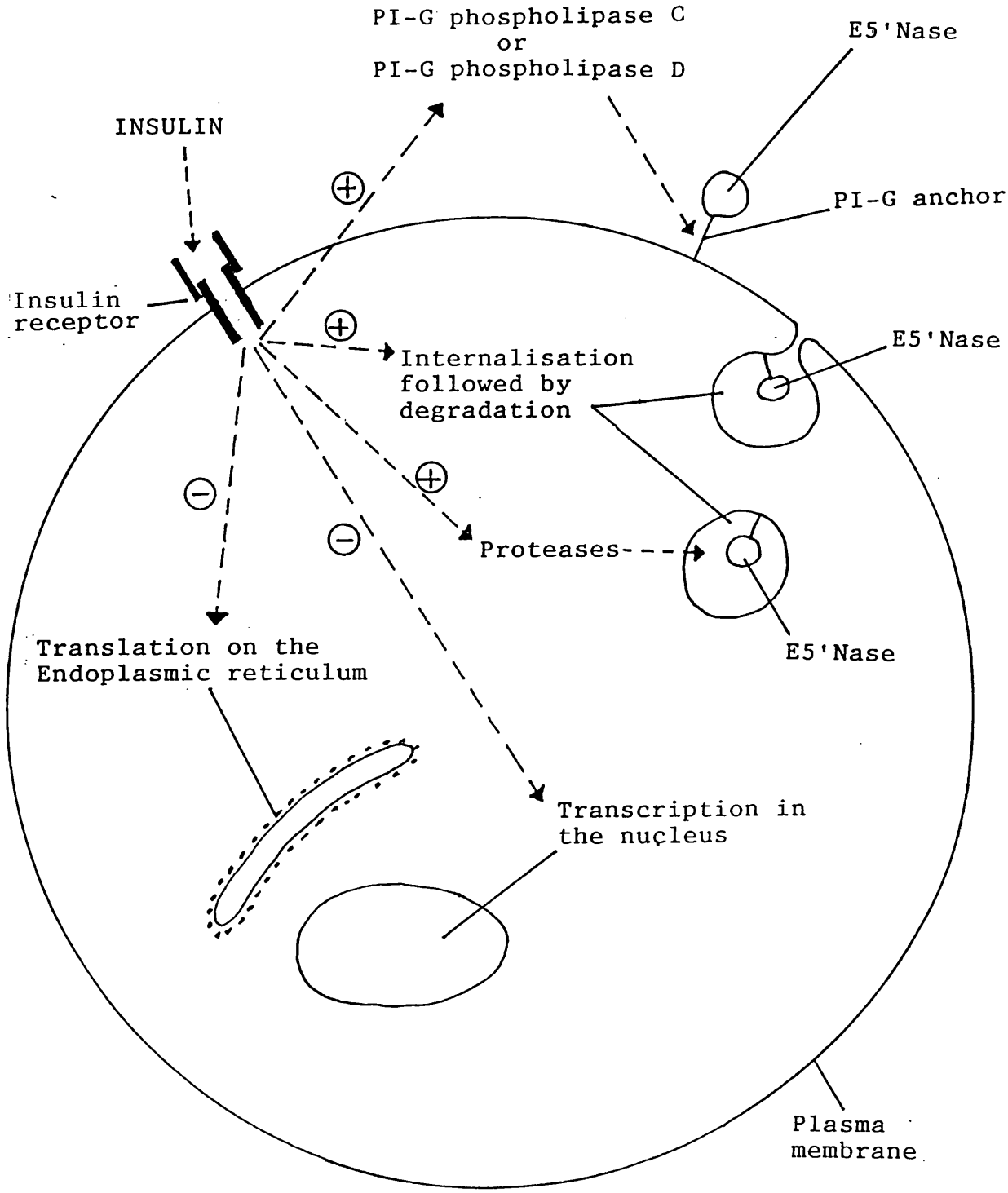


FIGURE 3.8: **Schematic Illustration of Potential Sites of Insulin Action for the Regulation of E5'Nase Levels**

⊖/⊕ Inhibitory/Stimulatory action of insulin.

insulin stimulation of the degradative enzyme(s), or possibly both the synthetic and degradative processes may be regulated simultaneously.

Another possibility could involve the activation of PI-G PLC, including the series of events from insulin interaction with its receptor through to the actual PLC activation which may be interrupted during insulin-deprivation. This line of reasoning could explain the increases in PI-G-anchored e5'Nase concentrations in the membrane. Furthermore, reintroduction of insulin *in vivo* would then re-establish the activation of PI-G PLC thereby lowering the levels of PI-G-PLC-cleavable proteins.

In order to test white adipose tissue e5'Nase susceptibility to PLC-cleavage, exogenous PI-PLC was added to control and diabetic adipose tissue membranes which were subsequently exposed to a Triton X-114 temperature-induced phase separation. This technique was used successfully for other PI-G-anchored proteins (Hooper & Bashir, 1991). However, it is important to note that these *in vitro* studies are not ideal for comparison with *in vivo* events as the exogenous PLC added is PI-PLC as opposed to PI-G-PLC and as such, its activity is associated with slightly different substrates. Pre-treatment with hydroxylamine, as has been used for the acetylcholinesterase anchor (Toutant *et al*, 1989), was tried in an attempt to overcome the PI-PLC cleavage resistance previously noted for e5'Nase.

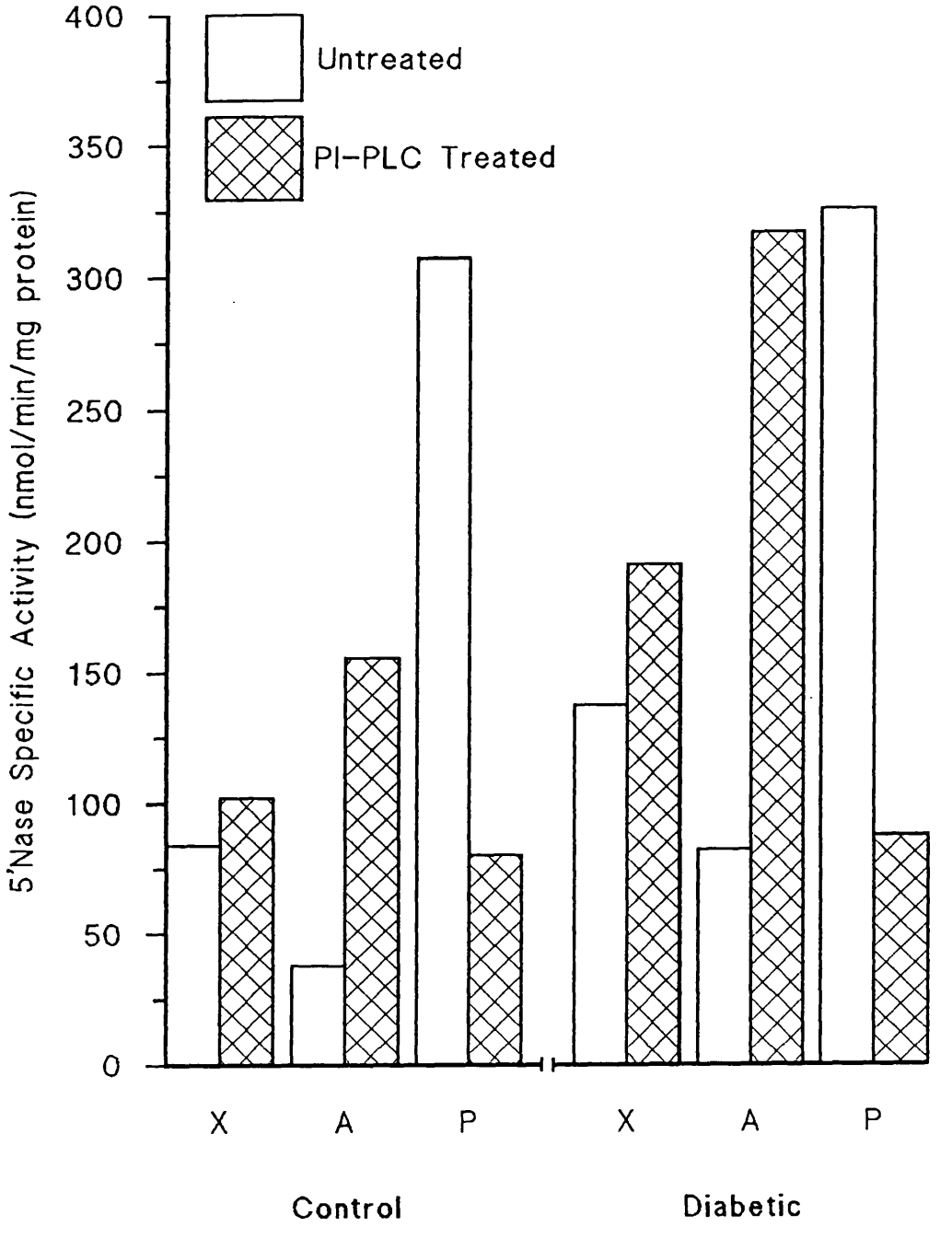


FIGURE 3.9: Percentage of E5'Nase Cleaved from White Adipose Tissue Membranes with Exogenous PI-PLC Application - Specific Activities

Representative experiment (n=1)
X = Samples not subjected to TX-114 phase separation
A = Aqueous portion after TX-114 phase separation
P = Pellet, hydrophobic portion after TX-114 phase separation

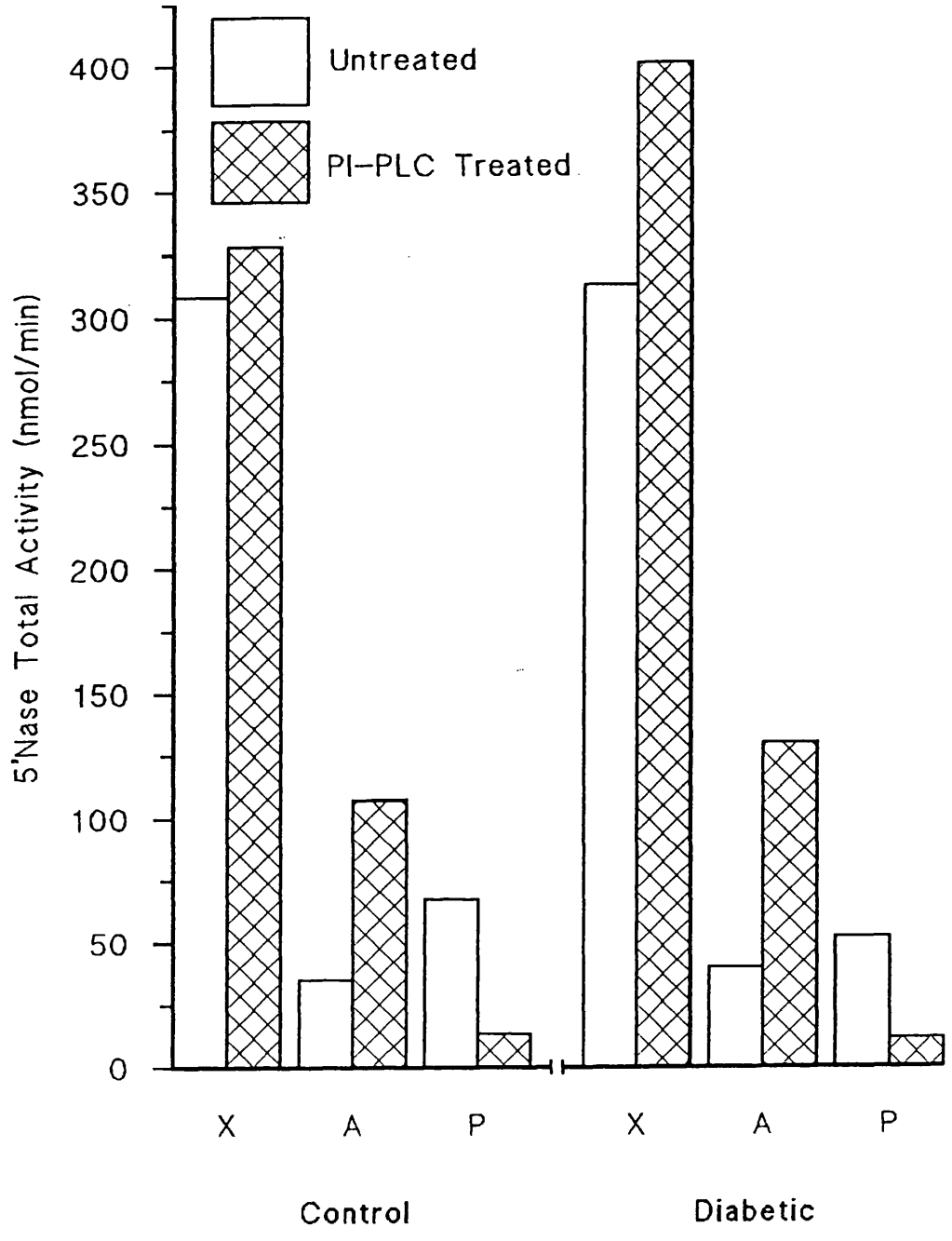


FIGURE 3.10: Percentage of E5'Nase Cleaved from White Adipose Tissue Membranes with Exogenous PI-PLC Application - Total Activities

Representative experiment (n=1)
X = Samples not subjected to TX-114 phase separation
A = Aqueous portion after TX-114 phase separation
P = Pellet, hydrophobic portion after TX-114 phase separation

Unfortunately the pH changes required for the technique destroyed all e5'Nase activity even after a return to neutral pHs. With regard to phase separation, some success at PI-PLC cleavage was achieved by using the Triton X-114 two-phase technique increasing the hydrophilic (cleaved) 5'Nase activity from 11% to 66% for the control membranes after a 30 minute incubation (see Figure 3.9). Similar cleavage was obtained in the diabetic membranes where PI-PLC incubation resulted in an increase in hydrophilic 5'Nase activity from 20% to 78% (also shown in Figure 3.9). It is possible that the slightly higher percentage of cleaved 5'Nase activity seen in the diabetic membranes could explain the return of e5'Nase levels to control levels noted after *in vivo* insulin treatment. However, there are still insufficient data to draw any strong conclusions regarding the *in vivo* events.

Total recovered 5'Nase activities are shown in Figure 3.10, in which it can be seen that PI-PLC treatment increases the proportion of hydrophilic 5'Nase activity in the aqueous phase by about three-fold. Nevertheless, when comparing total recovered 5'Nase activity in the pellet and aqueous phases combined against pre-phase separation levels, only a third of the activity remains. This lack of stability in 5'Nase activity was also apparent during purification (see Chapter 4).

3.5 E5'Nase Levels in Other Metabolic States: Thyroid Hormone Imbalance and Fasting

When thyroid states were altered in the rat models, changes in e5'Nase activity were not so apparent. Hypothyroidism was induced as described in Chapter 2, by the introduction of 6-n-propylthiouracil into the drinking water combined with an iodine-deficient diet. When e5'Nase activity was measured in the white epididymal adipose tissue it was found to be 279.5 ± 31.5 nmol/min/mg protein, as compared to the euthyroid control animals in which the activity was measured at 234.5 ± 15.6 nmol/min/mg protein. Similarly, the e5'Nase activity in the triiodothyronine-induced hyperthyroid animals was measured at 322.8 ± 15.9 nmol/min/mg protein, whilst in the euthyroid animals it was 311.7 ± 19.4 nmol/min/ mg protein. Statistical analysis by the student's t-test showed that neither the hypothyroid e5'Nase activities, nor the hyperthyroid activities differed significantly from their euthyroid controls ($P>0.2$ and $P>0.3$ respectively). These hypothyroid results do not agree with the previous findings from this laboratory (Jamal & Saggerson, 1987) in which a 50% decrease of e5'Nase activity was measured. However, it is important to note that the measurements in that report were made in membranes derived from isolated adipocytes, whilst the values given above from this study were made in whole white adipose tissue membranes.

The final metabolic state investigated in this study

was fasting (48 hours). The animals used in these experiments were from a different breeding group than the other studies. E5'Nase activities measured in the control animals from this breeding stock were approximately five-fold lower than the previous breeding stock. Thus, e5'Nase activity in control animals was 51.96 ± 4.15 nmol/min/mg protein, whilst it rose to 75.21 ± 8.03 nmol/min/ mg protein in the fasted animals. Statistically, using the student's t-test, fasting produced a significant increase in e5'Nase activity ($P<0.05$). When repeated with animals from the same breeding stock as in the diabetic studies, e5'Nase activities were still found to increase, but only marginally from 147.15 ± 11.44 nmol/min/mg protein for control animals to 177.80 ± 18.69 nmol/ min/mg protein for fasted animals. These values were not significantly different when analysed by the student's t-test ($P<0.2$).

A summary of these e5'Nase activities in the different metabolic states is provided in Figure 3.11. The measurements are expressed as relative specific activities to facilitate direct comparison. In the previous sections of this chapter the activities of membrane-bound 5'Nase in streptozotocin-diabetes and insulin administration were reported. It is not clear however, whether the changes observed were directly or indirectly affected by circulating insulin concentrations. Other hormonal changes have been implicated in alterations of e5'Nase activity, including decreased e5'Nase in brown and white adipocyte membranes

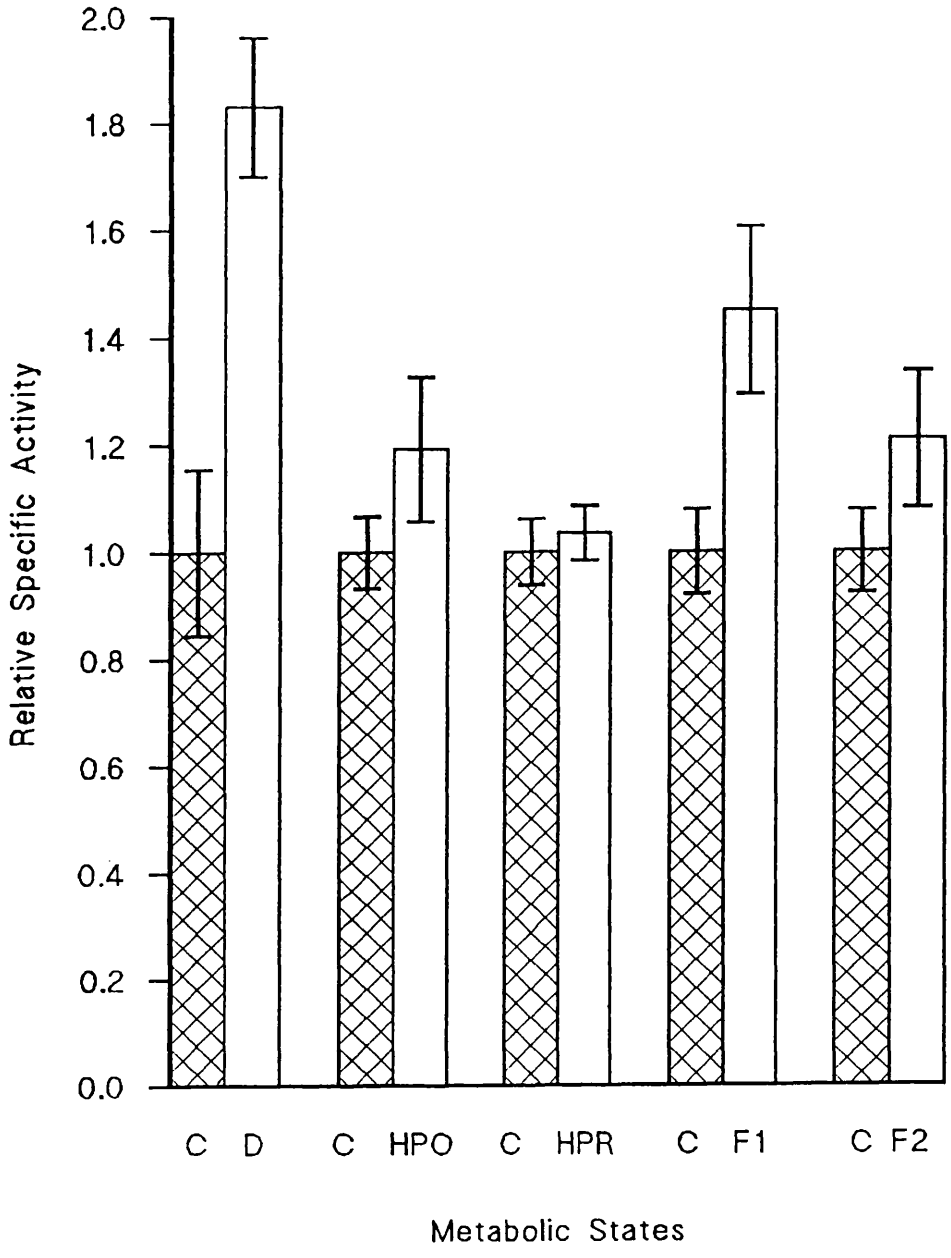


FIGURE 3.11: **Summary of E5'Nase Changes in Different Metabolic States**

C = Controls (n=same number as corresponding experimental group); D = Diabetics (n=3); HPO = Hypothyroids (n=6); HPR = Hyperthyroids (n=6); F1 = Fasted, group 1 (n=4); F2 = Fasted, group 2 (n=8).

Probability that the experimental group differs from their respective control groups:
Diabetics, $P<0.02$ (significant); Hypothyroids, $P>0.2$ (not significant); Hyperthyroid, $P>0.3$ (not significant), Fasted 1, $P<0.05$ (significant); Fasted 2, $P<0.2$ (not significant).

(Jamal & Saggerson, 1987) and increased e5'Nase in synaptosomal membranes (Mazurkiewicz & Saggerson, 1989), both resulting from PTU-induced hypothyroidism. In this present study neither of the thyroid hormone changes appeared to result in altered e5'Nase activity levels in whole white adipose tissue membranes. The 48 hour fasting state, which would produce reduced levels of circulating insulin, was the only metabolic state other than streptozotocin-diabetes that appeared to have an effect on e5'Nase levels in the adipose tissue. These findings, together with the *in vivo* insulin administration returning the e5'Nase levels to those of the non-diabetic control animals, suggest that insulin is directly implicated in the regulation of e5'Nase levels, although the mechanism through which regulation is achieved remains unresolved.

CHAPTER 4

RESULTS: PARTIAL PURIFICATION & CHARACTERIZATION OF 5'NASE FROM WAT

4.1.1 Partial Purification of E5'Nase from WAT - Solubilization

E5'Nase has been purified both partially and to apparent homogeneity from several tissue types including rat liver (Widnell, 1974; Bailyes *et al*, 1982; Wada *et al*, 1986; Misumi *et al*, 1990), bovine liver (Harb *et al*, 1983; Suzuki *et al*, 1993), bovine thyroid (Peeters *et al*, 1988), chicken gizzard smooth muscle (Dieckhoff *et al*, 1985), rat brain synaptic membranes (Lai & Wong, 1991) and human placenta (Thompson *et al*, 1987; Klemens *et al*, 1990; Misumi *et al*, 1990a). Detergent solubilizations have been used with some success (Widnell, 1974; Bailyes *et al*, 1982; Harb *et al*, 1983; Dieckhoff *et al*, 1985; Peeters *et al*, 1988; Misumi *et al*, 1990a; Lai & Wong, 1991), but the PI-G-anchor tends to be resistant to detergent solubilization and in most of the aforementioned purifications the e5'Nase was removed from the membrane via PI-G-specific PLC-cleavage. However, e5'Nase has been noted as being only partially susceptible to PLC-cleavage in several reports (Shukla *et al*, 1980; Thompson *et al*, 1987; Stochaj *et al*, 1989; Zekri *et al*, 1989). Results from this study (see section 3.4), using high levels of PI-PLC activity on e5'Nase in WAT, support those previous findings of e5'Nase PI-PLC-cleavage resistance. An early report by Bailyes *et al* (1982) demonstrated high solubilization of e5'Nase from rat liver plasma membranes with the

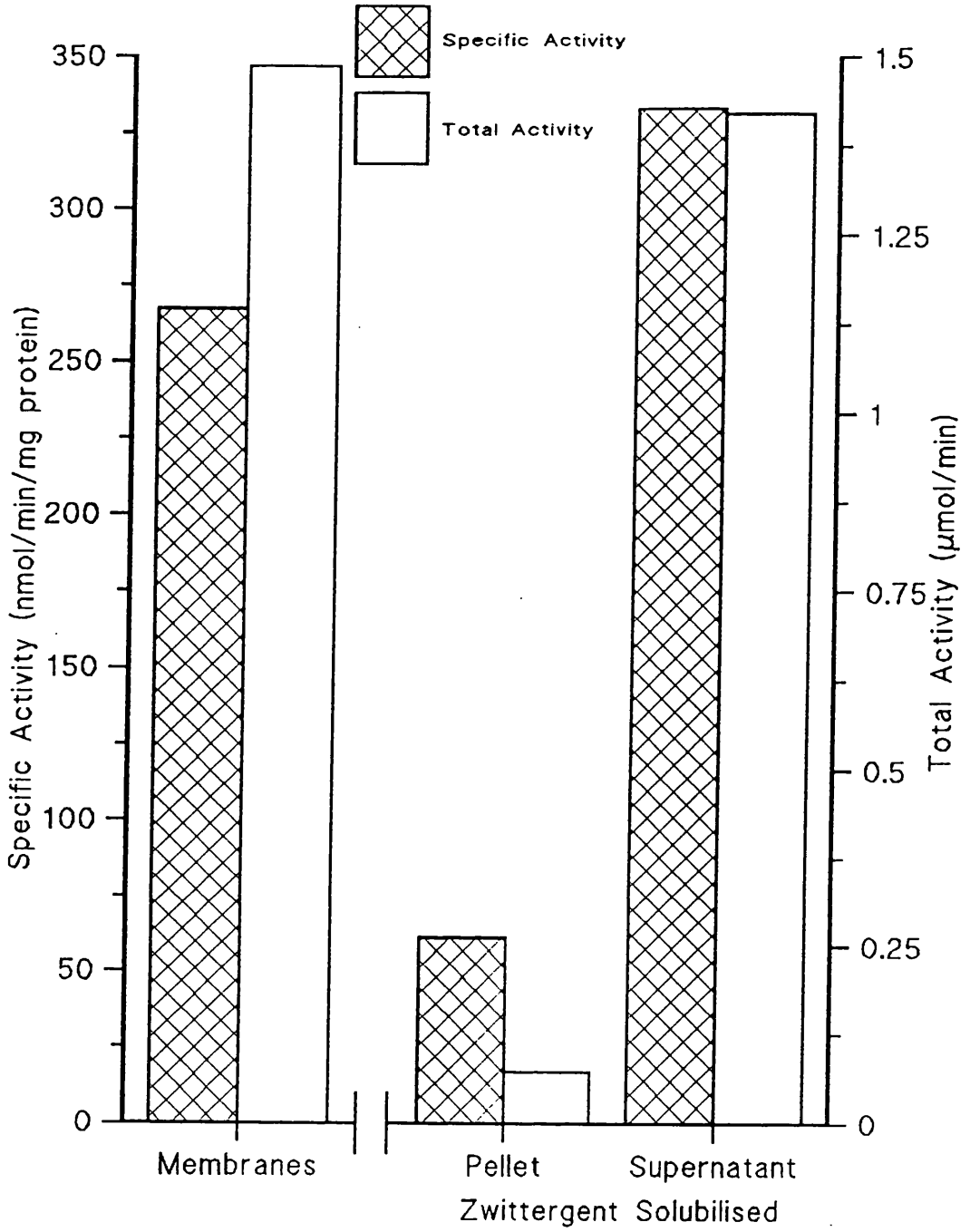


FIGURE 4.1: Extent of Zwittergent E5'Nase Solubilization from White Adipose Tissue

Protein determinations of these three samples indicated an apparent 2-fold increase in protein after solubilization (where Membranes = 1.03mg/ml, Pellet = 1.18mg/ml, Supernatant = 0.87mg/ml). Obviously this is an artifact created by the interference of the detergent with the protein assay. Independently the detergent appears to slightly enhance e5'Nase activity. Nevertheless, this graph shows that 95% of the total activity was coverted into a soluble form which transferred to the "solubilised" supernatant.

zwitterionic detergent sulphobetaine-14 (Zwittergent TM_{3-14} from Calbiochem). Their procedure of Zwittergent solubilization was satisfactorily carried out using WAT crude membranes in this study as shown in Figure 4.1. The figure shows that approximately 95% of the 5'Nase was solubilized, with no apparent loss of activity.

4.1.2 Purification - Chromatography

The soluble fraction from the above section was passed through a Concanavalin A-sepharose 4B affinity column to separate the glycoproteins. Subsequently the fractions identified as containing 5'Nase activity were passed through both an AMP-sepharose 4B affinity column and a Superose 12 gel filtration column. The relevant traces and corresponding 5'Nase activities in each fraction can be found in Section 2.9 of the Materials & Methods chapter. Specific and total activities were measured for each step to determine the extent of purification and yield of e5'Nase. As can be seen in Table 4.1 overleaf, purification of e5'Nase from WAT by this method gives a 78-fold purification, but the yield is very low at 4%. The yield could be increased by collecting and pooling fractions on the fringe of the measured 5'Nase activity from the chromatographic steps, but the purity was significantly reduced when this was attempted. During purification SDS-PAGE was used to check for purity, but as purity was increased a component in the 5'Nase-containing fractions progressively interacted with the gels such that

TABLE 4.1

Purification Table for E5'Nase

Sample Description	Specific Activity (µmol/min /mg protein)	Total Activity (µmol/min)	Yield (%)	Purification Factor
Homogenate	0.04	14.26	100	1
Crude Membranes	0.11	3.15	22	2.8
Detergent Solubilised Membranes	0.21[A]	3.55[A]	25[A]	5.3[A]
Post-Con A Affinity Column	1.42	3.27	23	36
Post-AMP Affinity Column	[B]	1.23	9	[B]
Post-Superose 12 Filtration Column	3.13	0.58	4	78

Notes:

[A] There is a slight activation of e5'Nase activity by the detergent Zwittergent-14 (see Figure 4.1) which produces the apparent increase.

[B] Values unobtainable as protein determination was not accurate despite several different assay types tried. An unidentified component in the samples at this stage of the purification caused extensive precipitation in all the different protein assays tried.

the samples did not travel through the gel smoothly. The physical appearance of the gel after electrophoresis was as if a component was dragged through the gel and the dye front was restrained. If very low protein was loaded, the effect was limited. Nevertheless, the final post-Superose-12 fraction was subjected to SDS-PAGE. As can be seen in Figure 4.2, two bands can be detected after silver staining. From comparative immunoblots (see Figure 3.2 in Section 3.1.2), the band identified as e5'Nase corresponded to a molecular weight of 76kDa as compared to molecular weight standards run alongside. Published e5'Nase purifications from other tissues including rat and bovine liver (Widnell, 1974; Bailyes *et al*, 1982; Harb *et al*, 1983), rat forebrain (Lai & Wong, 1991) and human placenta (Thompson *et al*, 1987) reported purification factors ranging from 1360-fold to 16,040-fold with final specific activities between 60.5 - 257 µmol/min/mg protein and yields from 7% to 24% from the original tissue homogenates. Comparison of the purification obtained in this study is clearly not as great when using specific activity as the determining factor, although the yield is only marginally lower than in those reported. However, when examining the corresponding SDS-PAGE gel separations, the purification in this study appears far more favourable. With the exception of Harb *et al* (1983), protein detection to check for contaminants was carried out by staining with Coomasie blue which is not a particularly sensitive technique and in at least one report (Bailyes *et al*, 1982) an abundant contaminant was present. When the more

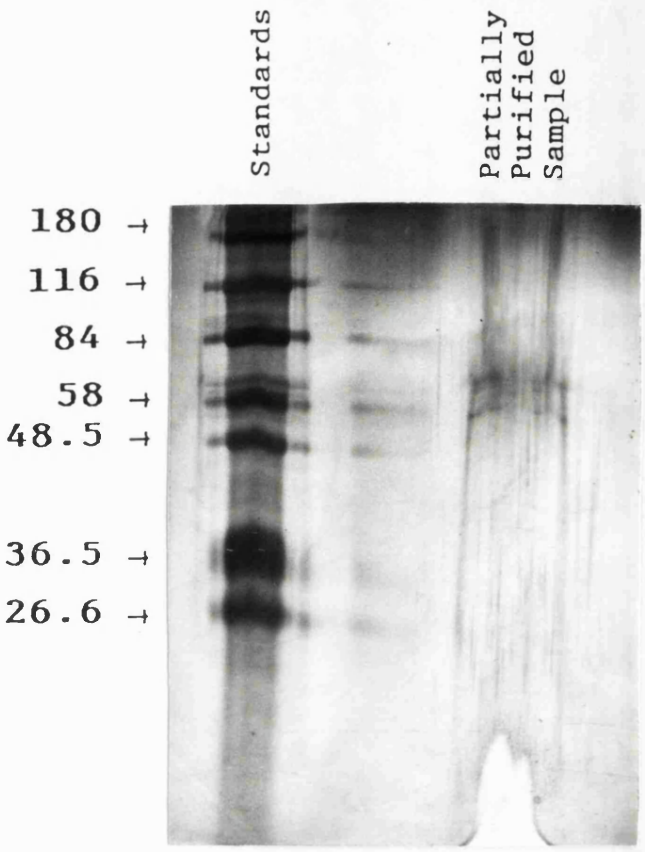


FIGURE 4.2: Silver Stained SDS-PAGE Gel of Partially Purified E'5Nase

The silver staining of acrylamide gels is a more sensitive technique than coomasie blue staining, but the Zwittergent detergent appeared to interfere with the silver staining to a limited degree. The gel shows that the e5'Nase has been purified to two bands: one at 76kDa which generally agrees with molecular weight determinations for other purified e5'Nases, whilst the second band is a probable contaminant at 63kDa.

sensitive silver staining technique was used (Harb *et al*, 1983) several contaminants could be seen in the published photograph. A number of rationalisations could be proposed to explain the apparent low purification factor achieved in this study as compared with other reported values despite the detection of only two bands on silver stained SDS-PAGE gel. An obvious suggestion is that extensive degradation of the enzyme has occured during the purification, thus reducing the apparent specific activity and incidentally may have resulted in the second protein band detected at 63kDa. Alternatively, the abundance of e5'Nase in WAT may be proportionately greater than in other tissues, especially when considering the nature of the adipocyte in which the central fat droplet constitutes a large volume of the cell. In this case yields should have been greater relative to the extent of purification when compared to e5'Nase purifications from other tissues.

For the purpose of this study the post-Superose-12 5'Nase activity was considered to be a partially purified ecto-5'Nase. With the exception of a small selection of the inhibition studies described in Section 4.3 (as indicated where appropriate), it was this partially purified e5'Nase which was used for the characterisation studies that constitute the remainder of the chapter. The partially purified e5'Nase was also used for attempting to raise monoclonal antibodies as discussed in the following chapter, Chapter 5.

4.2 K_m and V_{max} Measurements

Generally, the K_m measurements for e5'Nase which have been made fall in the range of 2-40μM as discussed in Chapter 1 (section 1.5.4). To confirm that the e5'Nase purified in this study conformed to the same basic kinetic parameters, activities were measured for the e5'Nase of this study over the substrate concentration range of 5-200μM AMP. The substrate-velocity curve from two representative experiments using measurements over a substrate concentration range of 5-50μM AMP is shown in Figure 4.3. The reciprocals of these values were used to draw a Lineweaver-Burke plot in Figure 4.4, in which the linearity of the line shows that the enzyme follows Michaelis-Menten kinetics. Both the K_m and V_{max} were derived from the Lineweaver-Burke plot. These were calculated as K_m = 11.1±0.6μM and V_{max} = 2.67±0.12μmol/min (n=2). This K_m agrees with measurements made with e5'Nase from other tissues.

4.3 Activation and Inhibition Studies

Various reports have been made regarding all the 5'Nases as to both activators and inhibitors of their hydrolytic activities in attempts to compare and contrast the different forms of this enzyme. Most studies of the ectoenzyme which have been undertaken have shown that unlike the cytosolic enzymes, it is unaffected by phosphate ions whilst being strongly inhibited by ADP, ATP, their corresponding

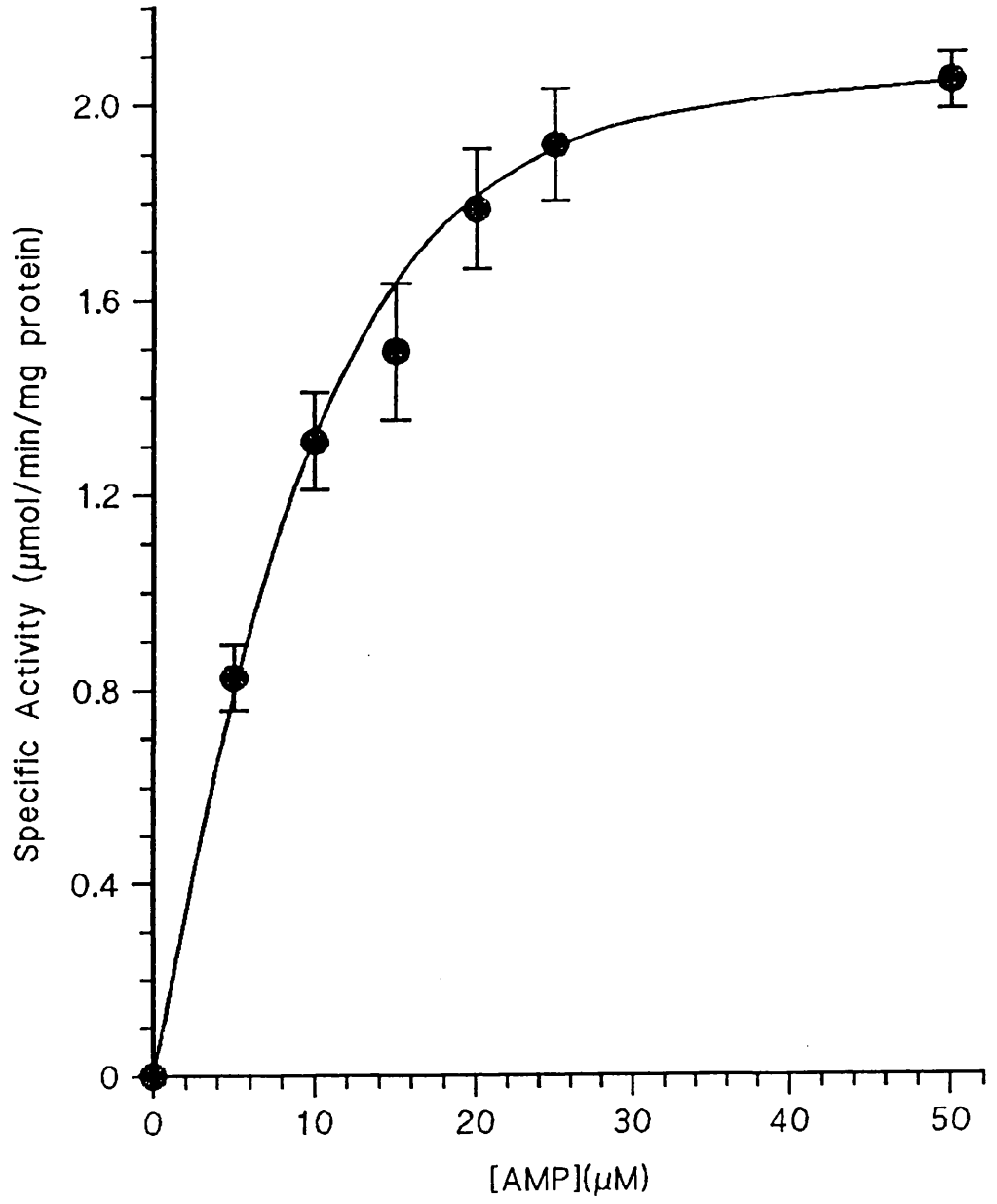


FIGURE 4.3: Substrate-Velocity Plot for E5'Nase Using AMP as Substrate

The partially purified e5'Nase was used at a concentration of 0.12µg/ml and again at 0.23µg/ml in these assays. This range of dilution was found to be necessary in order to maintain linearity of the assay with time. This plot shows the average values ±S.E. of 5'Nase specific activity when measured at these two protein concentrations (i.e. n=4).

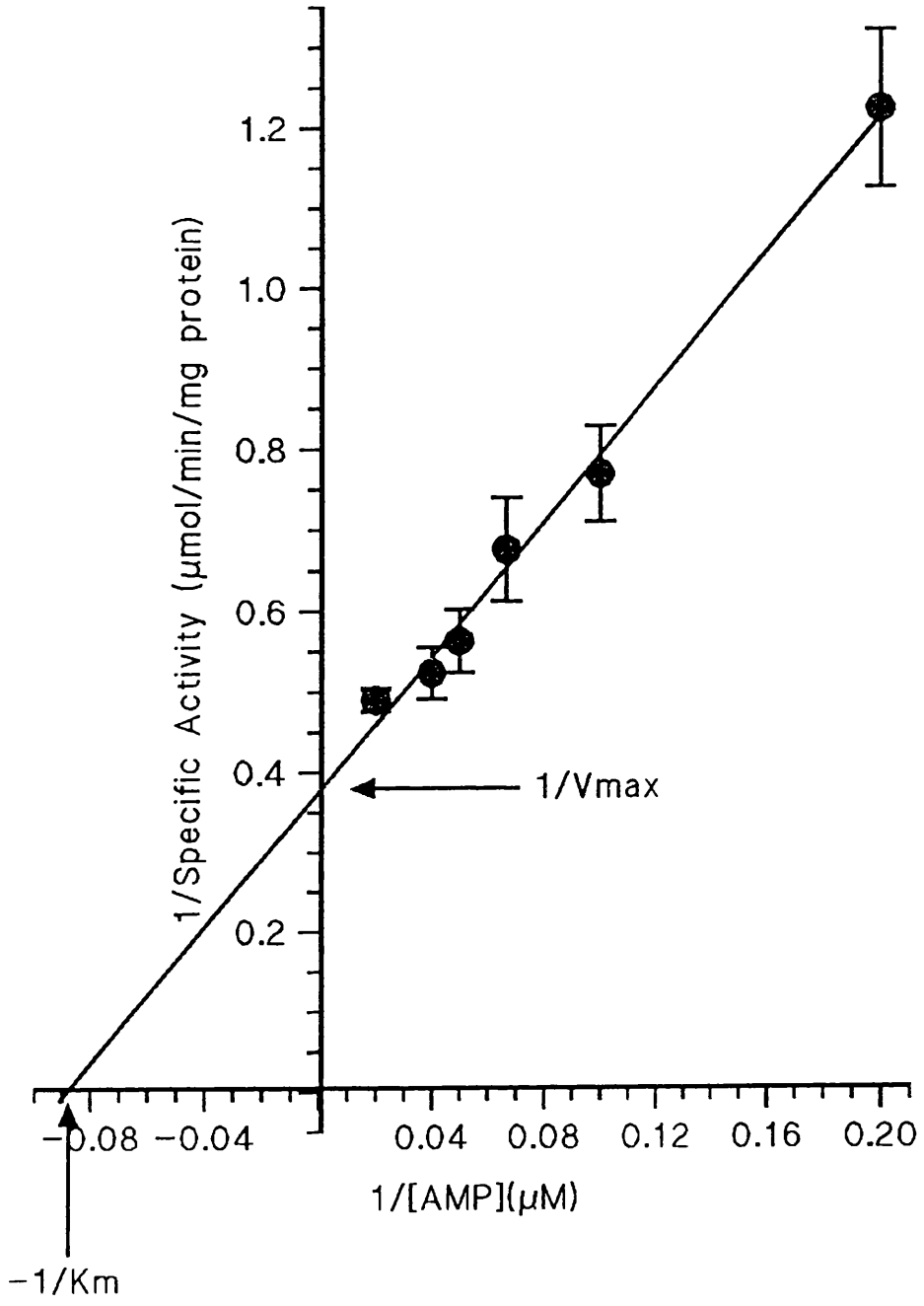


FIGURE 4.4: Lineweaver-Burke Plot for E5'Nase Activity to Determine K_m and V_{max} Values Using AMP as Substrate

The K_m was calculated as 11.1±0.6µM from this plot, whilst V_{max} was 2.67±0.12µmol/min. The correlation coefficient, as determined by linear regression, was 0.99. This plot shows the average ±S.E. reciprocal values for velocity measured at two protein concentrations as indicated in Figure 4.3 (n=4).

α,β-methylene-analogues (ADP>ATP) (Collinson *et al*, 1987; Lai & Wong, 1991), lectins such as Concanavalin A (Harb *et al*, 1983), and DTT (dithiothreitol) (Harb *et al*, 1983; Fini *et al*, 1985). There is some dispute as to whether magnesium ions activate the e5'Nase as for the cytosolic enzymes. Certainly it has been shown that after inhibition with EDTA, magnesium ions can restore activity (Naito & Lowenstein, 1981; Harb *et al*, 1983; Fini *et al*, 1985). Nevertheless, this is more probably due to EDTA chelation of the zinc ions which serve to form the metalloenzyme (Fini *et al*, 1990), followed by a partial restoration of activity by an alternative divalent cation - in this instance Mg^{2+}. The lack of effect by magnesium ions in the absence of prior EDTA inhibition would support this concept.

In this study several of these activators and inhibitors were tested to determine whether the e5'Nase purified from white adipose tissue possesses the same properties as those described for the enzyme purified from other tissues. Additionally, inhibition by DTT was studied, the pH optimum was determined, and the effects of an unidentified, ATP-sensitivity-conferring factor were investigated. All of these analyses are reported in the following sections 4.3.1-4.3.6.

4.3.1 Determination of pH Optimum

A pH profile ranging between pH 5.5 to pH 10.5 was

determined for the purified e5'Nase by radiochemical assay using [^{3}H]-AMP as substrate. It was necessary to assay blanks at each pH as their levels rose significantly in direct relation to alkalinity above pH 8.5. As can be seen in Figure 4.5, the pH optimum is at ~pH 7.5 with a reasonably broad pH range. Although the optimum agrees with most other reports, Harb *et al* (1983) demonstrated a markedly reduced tolerance to pH changes in the bovine liver e5'Nase once removed from the membrane. It can therefore be concluded that either the white adipose tissue e5'Nase behaves differently in this context, or the cleavage by PI-PLC (as used by Harb and colleagues) influenced the pH profile.

4.3.2 α,β-Methylene-ADP and -ATP Inhibition

During purification of e5'Nase from white adipose tissue, it was found that only some of the activity bound to the AMP-sepharose 4B column (see Chapter 2, section 2.9.3). Inhibition studies with α,β-methylene-ADP and -ATP were undertaken with the pooled fractions labelled "Bound"- and "Unbound"-e5'Nase. The inhibition constant (K_i) values for both samples were determined from Dixon plots (Dixon, 1953) as shown in Figures 4.6 - 4.9. The Dixon plot shows the relationship between inhibitor concentration and the inverse reciprocal of velocity at different substrate concentrations. With competitive or mixed inhibition the lines cross one another and at the point of intersection, the concentration of inhibitor is equal to $-K_i$. This was found to be the case

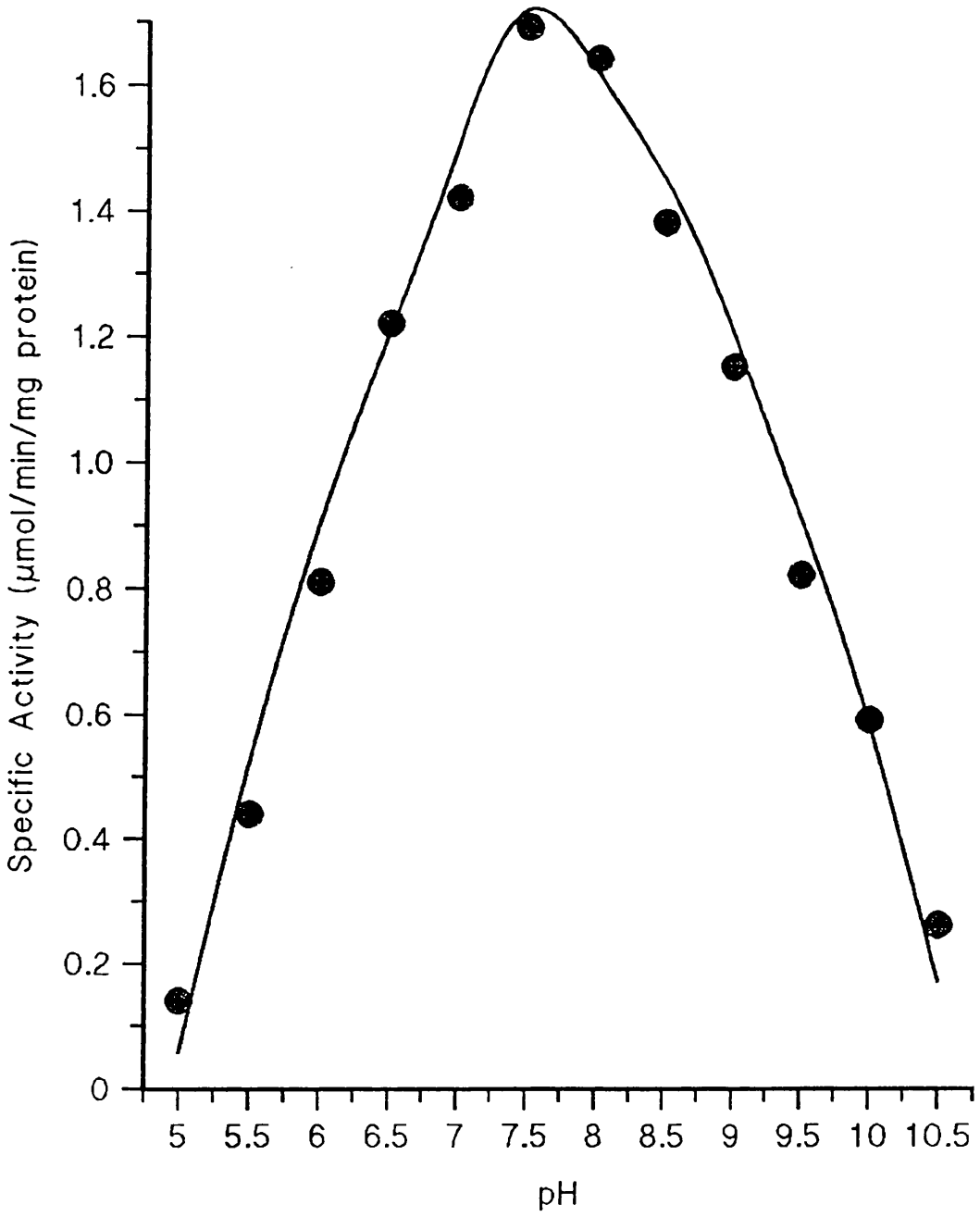


FIGURE 4.5: pH Profile of Purified E5'Nase from White Adipose Tissue

The optimal pH was measured at pH7.5, with 50% or greater activity being achieved between ~pH6.0 to ~pH9.5, as shown in the above profile.

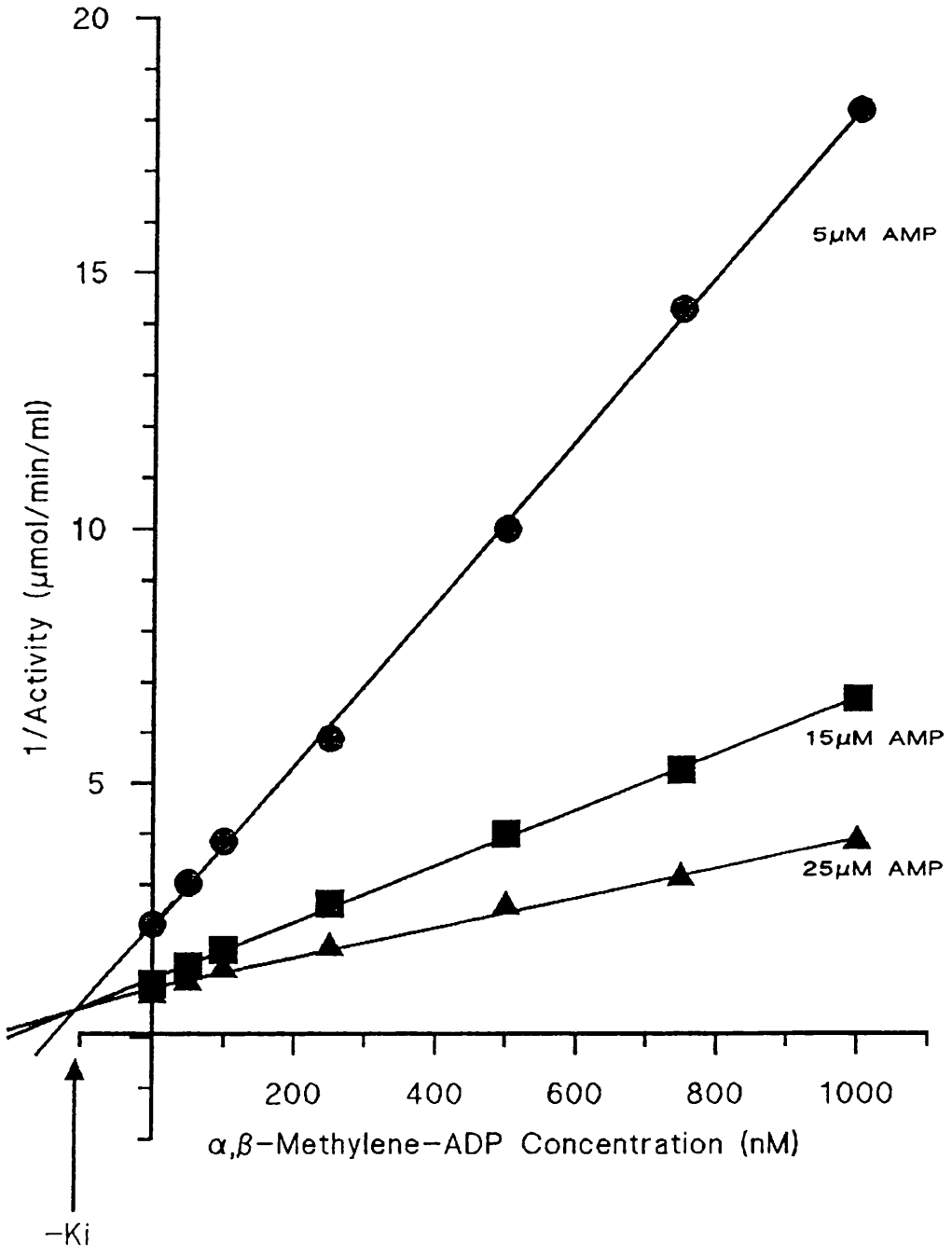


FIGURE 4.6: **Dixon Plot Showing α,β-Methylene-ADP Inhibition in "Bound"-E5'Nase**

The K_i from this plot was 105nM.
(Representative experiment, n=1)

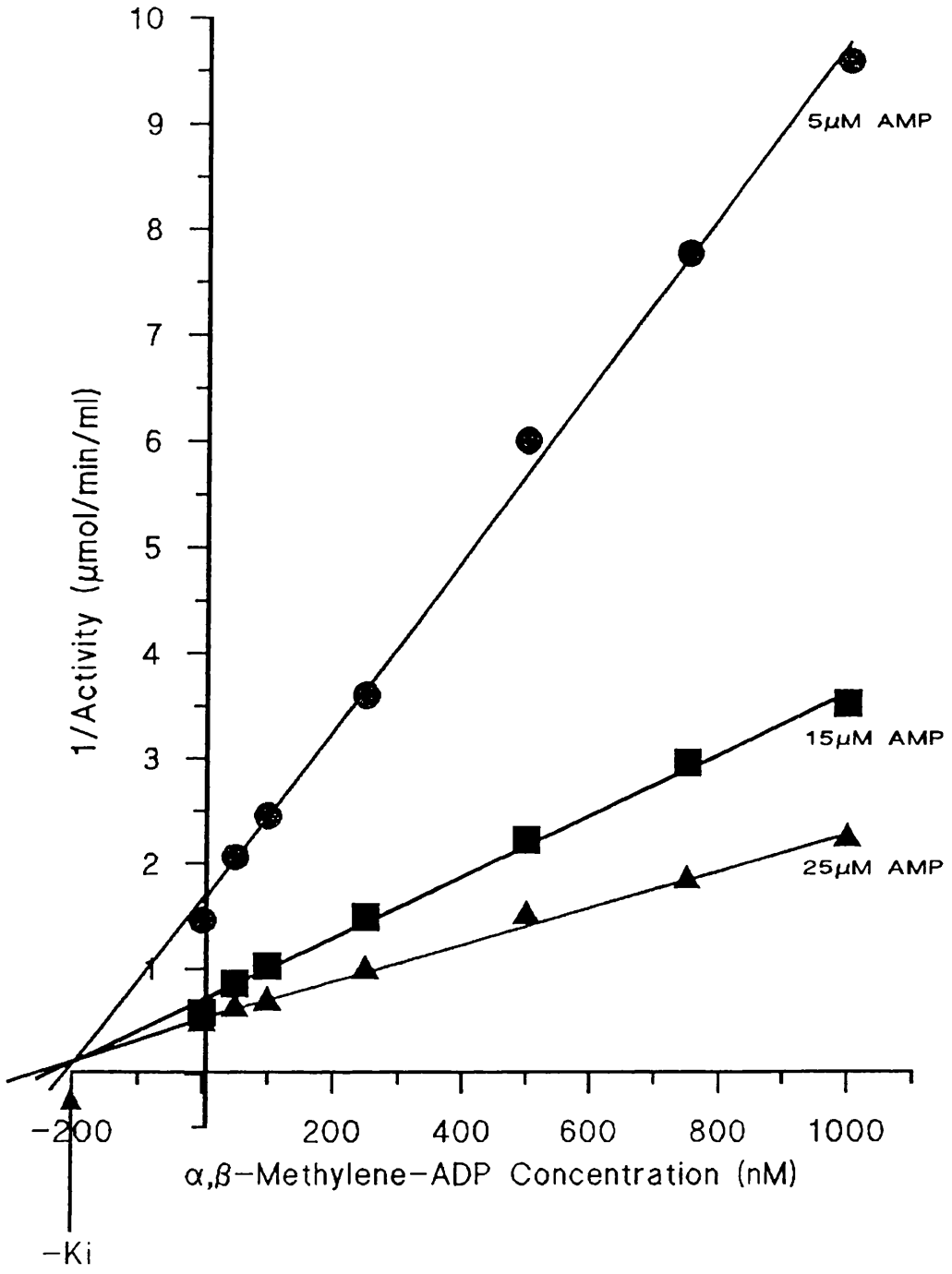


FIGURE 4.7: Dixon Plot Showing α,β-Methylene-ADP Inhibition in "Unbound"-E5'Nase

The K_i from this plot was 180nM.
(Representative experiment, n=1)

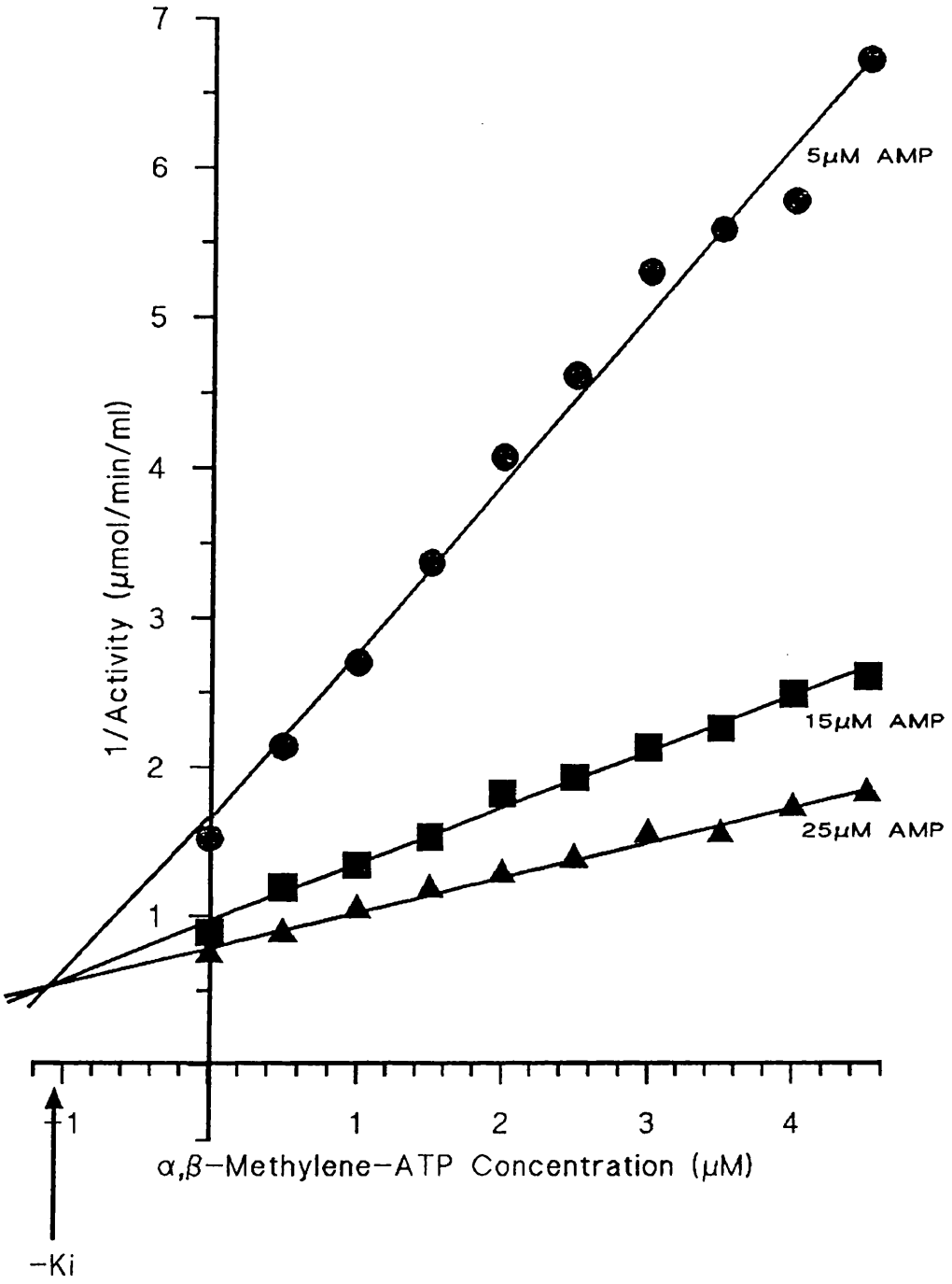


FIGURE 4.8: Dixon Plot Showing α,β-Methylene-ATP Inhibition in "Bound"-E5'Nase

The K_i from this plot was 1.1µM.
(Representative experiment, n=1)

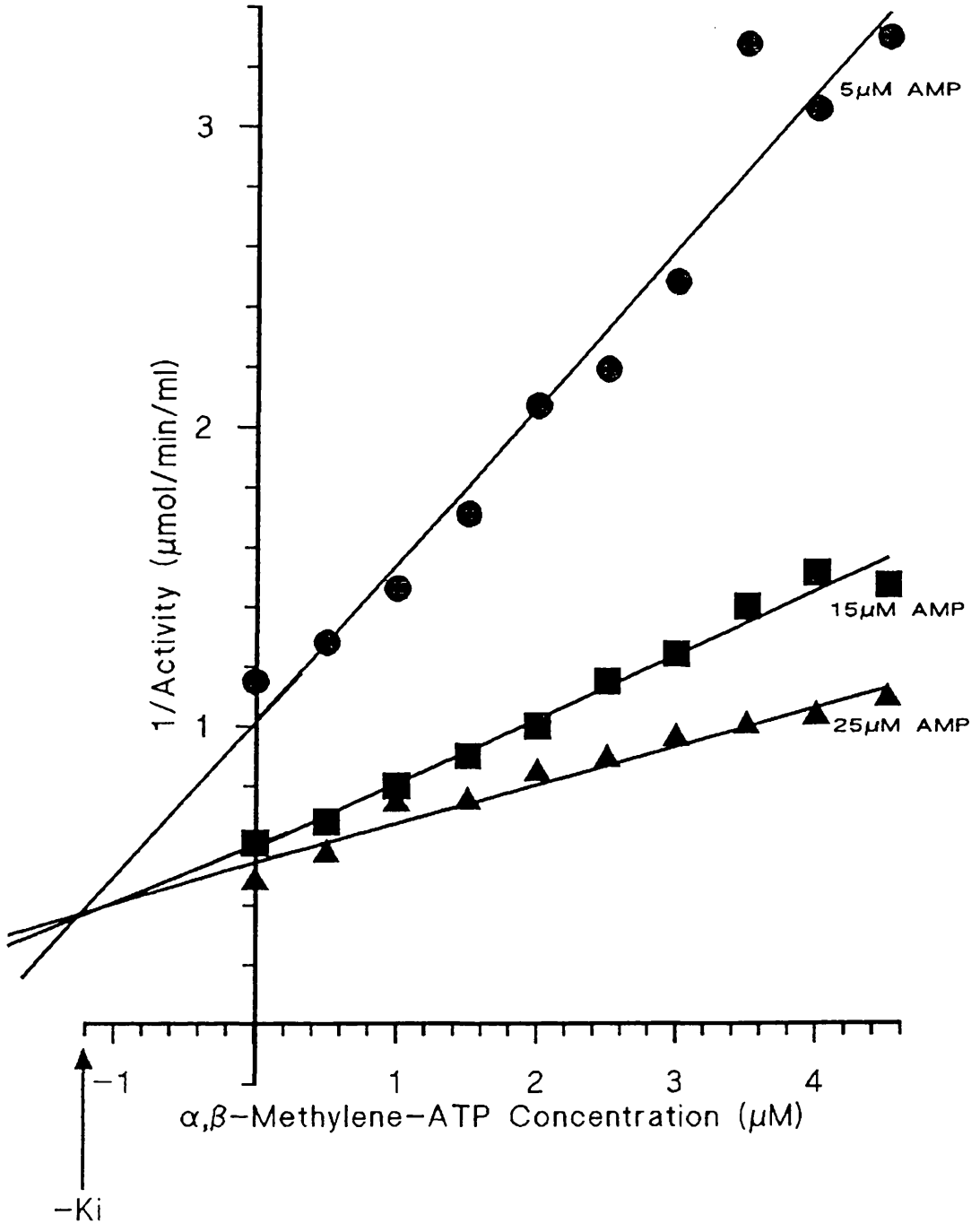


FIGURE 4.9: Dixon Plot Showing α,β-Methylene-ATP Inhibition in "Unbound"-E5'Nase

The K_i from this plot was 1.00-1.25μM. The absence of a clean intersection between all three lines prevents the assignment of a single value.
(Representative experiment, n=1)

for the post-AMP-Sepharose-e5'Nase samples in this study. The inhibition constants were calculated for the "Bound"-e5'Nase as $K_{i(\alpha,\beta\text{-me-ADP})}$=105nM and $K_{i(\alpha,\beta\text{-me-ATP})}$=1.1μM, whilst for the "Unbound"-e5'Nase the values were $K_{i(\alpha,\beta\text{-me-ADP})}$=180nM and $K_{i(\alpha,\beta\text{-me-ATP})}$=1.00-1.25μM. In order to distinguish between competitive and mixed type inhibition, graphs of substrate over velocity (s/v) against inhibitor concentration at different substrate concentrations, as described by Cornish-Bowden (1974), were plotted in Figures 4.10 and 4.11. With this type of plot, competitive inhibition is represented by a series of parallel lines, whilst mixed and uncompetitive inhibition lines cross one another (see Cornish-Bowden, 1979 for plot analyses). The type of inhibition observed in this study using this dual plot approach was shown to be competitive for both inhibitors in both "Bound" and "Unbound"-5'Nase samples. It is interesting to note however, that whilst there appears to be no difference in α,β-methylene-ATP inhibition between the "Bound" and "Unbound", the ADP-analogue exhibits stronger inhibition of "Bound"-e5'Nase activity.

4.3.3 Effect of Magnesium on Partially Purified E5'Nase

The effect of magnesium on the activity of partially purified e5'Nase was investigated over the range 0-20mM Mg^{2+}. This was carried out without prior chelation with either EDTA or EGTA in order to ensure that any effect which might be noted was not due simply to a divalent cation being substituted into the metalloenzyme complex as in prior studies

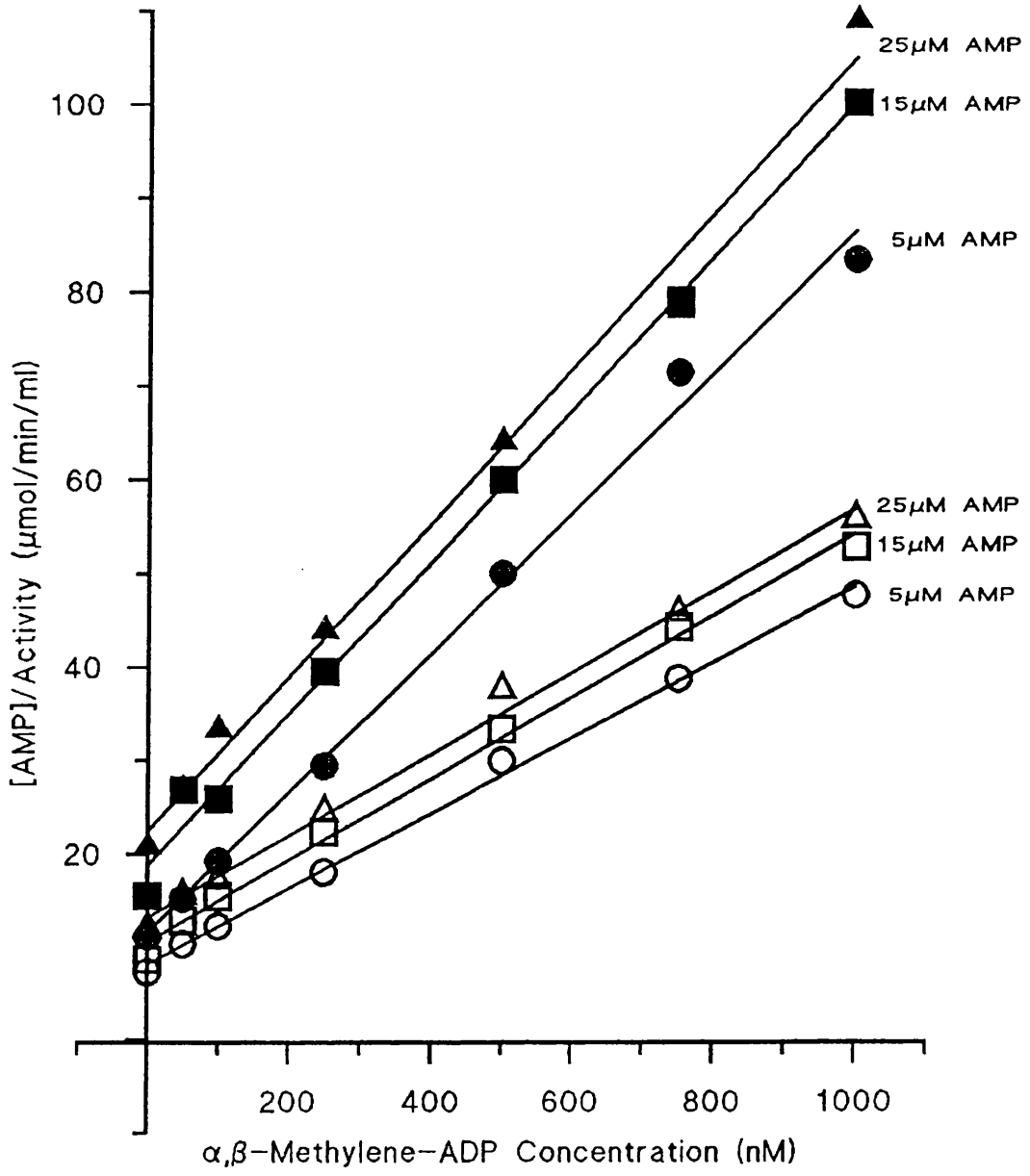


FIGURE 4.10: Plot Showing α,β-Methylene-ADP Inhibition in "Bound"- and "Unbound"-E5'Nase

Closed symbols = "Bound"; Open symbols = "Unbound"

Gradients:	Bound	Unbound
	5μM = 0.075	5μM = 0.040
	15μM = 0.081	15μM = 0.044
	25μM = 0.082	25μM = 0.044

The parallel lines between the three concentrations for the "Bound"-e5'Nase, and for the "Unbound"-e5'Nase, indicate that inhibition is competitive for both samples with this ADP-analogue (see Cornish-Bowden, 1979).

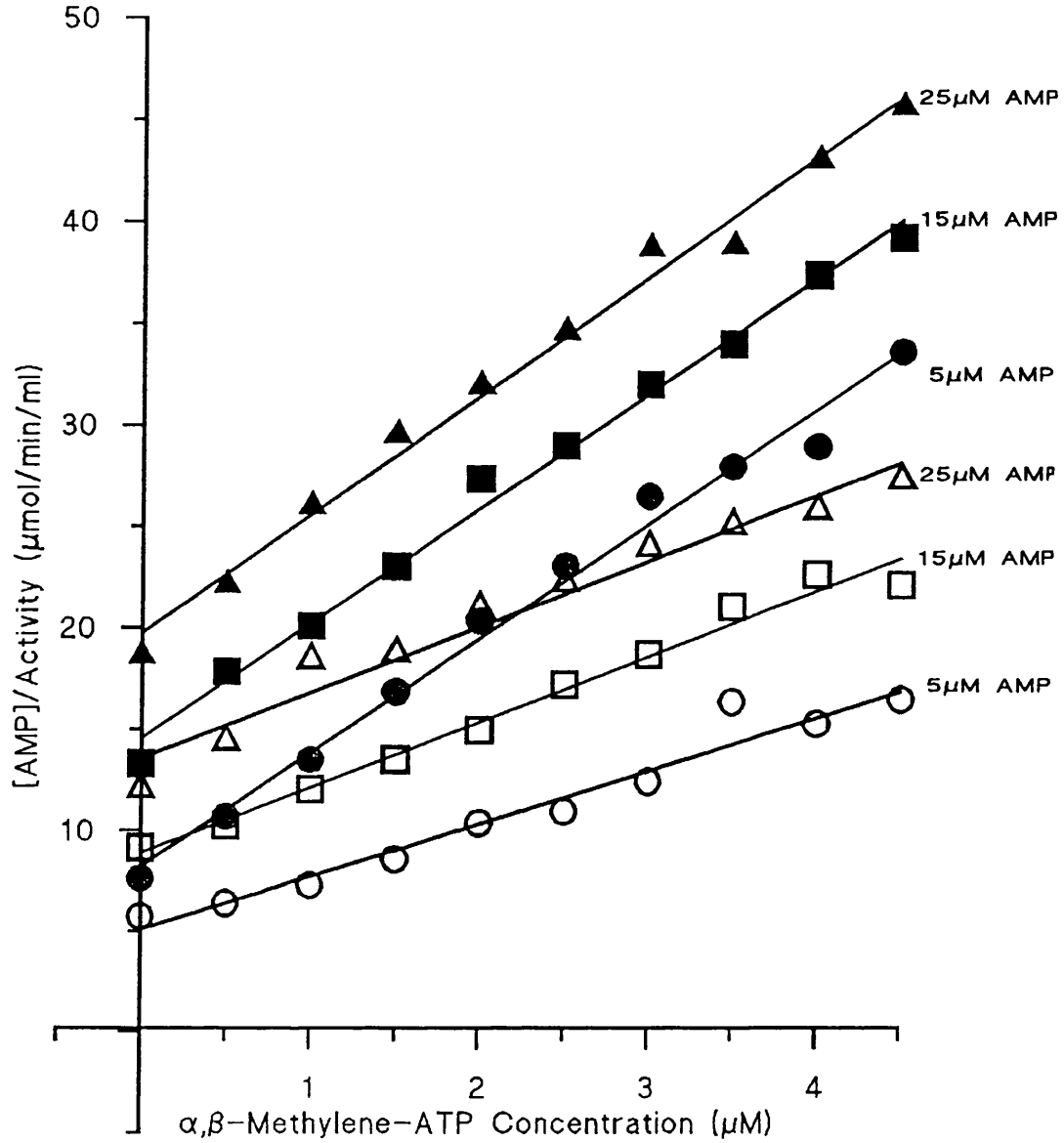


FIGURE 4.11: Plot Showing α,β-Methylene-ATP Inhibition in "Bound"- and "Unbound"-E5'Nase

Closed symbols = "Bound"; Open symbols = "Unbound"

Gradients:	Bound	Unbound
	5µM = 5.634	5µM = 2.613
	15µM = 5.660	15µM = 3.219
	25µM = 5.845	25µM = 3.231

The parallel lines between the three concentrations for the "Bound"-e5'Nase, and for the "Unbound"-e5'Nase, indicate that inhibition is competitive for both samples with this ATP-analogue (see Cornish-Bowden, 1979).

(Naito & Lowenstein, 1981; Harb *et al*, 1983; Fini *et al*, 1985). As can be seen in Figure 4.12, magnesium ions had no effect on the e5'Nase activity under these conditions even at concentrations above physiological magnesium.

4.3.4 Effect of DTT (Dithiothreitol) on Partially Purified E5'Nase

Several groups have demonstrated that the mature e5'Nase exists *in vivo* as a homodimer (Harb *et al*, 1983; Fini *et al*, 1985; Buschette-Brambrink & Gutensohn, 1989; Fini *et al*, 1990). Furthermore, the addition of dithiothreitol was shown to inhibit e5'Nase by 80% to 100% suggesting the importance of disulphide bridges for catalytic activity (Harb et al, 1983; Fini *et al*, 1985). However, it has not been made clear that disulphide bridges maintaining the homodimeric structure are critical to the e5'Nase activity. No significant difference in percentage inhibition was observed between the "Bound"- and "Unbound"-e5'Nases as shown in Figure 4.13, with an approximate 45% inhibition at 4mM DTT. By plotting the inverse of DTT concentration against the inverse of the inhibition quotient, the IC_{50}s were measured where the line crosses the X-axis (see inset of Figure 4.13). These were calculated as $IC_{50"Bound"}$ = 2.34mM and $IC_{50"Unbound"}$ = 2.25mM, suggesting that differences are negligible. Furthermore, maximum inhibitions could be determined from this plot where the lines cross the Y-axis. The inhibition quotients thus obtained were converted to percentages to give 65% and

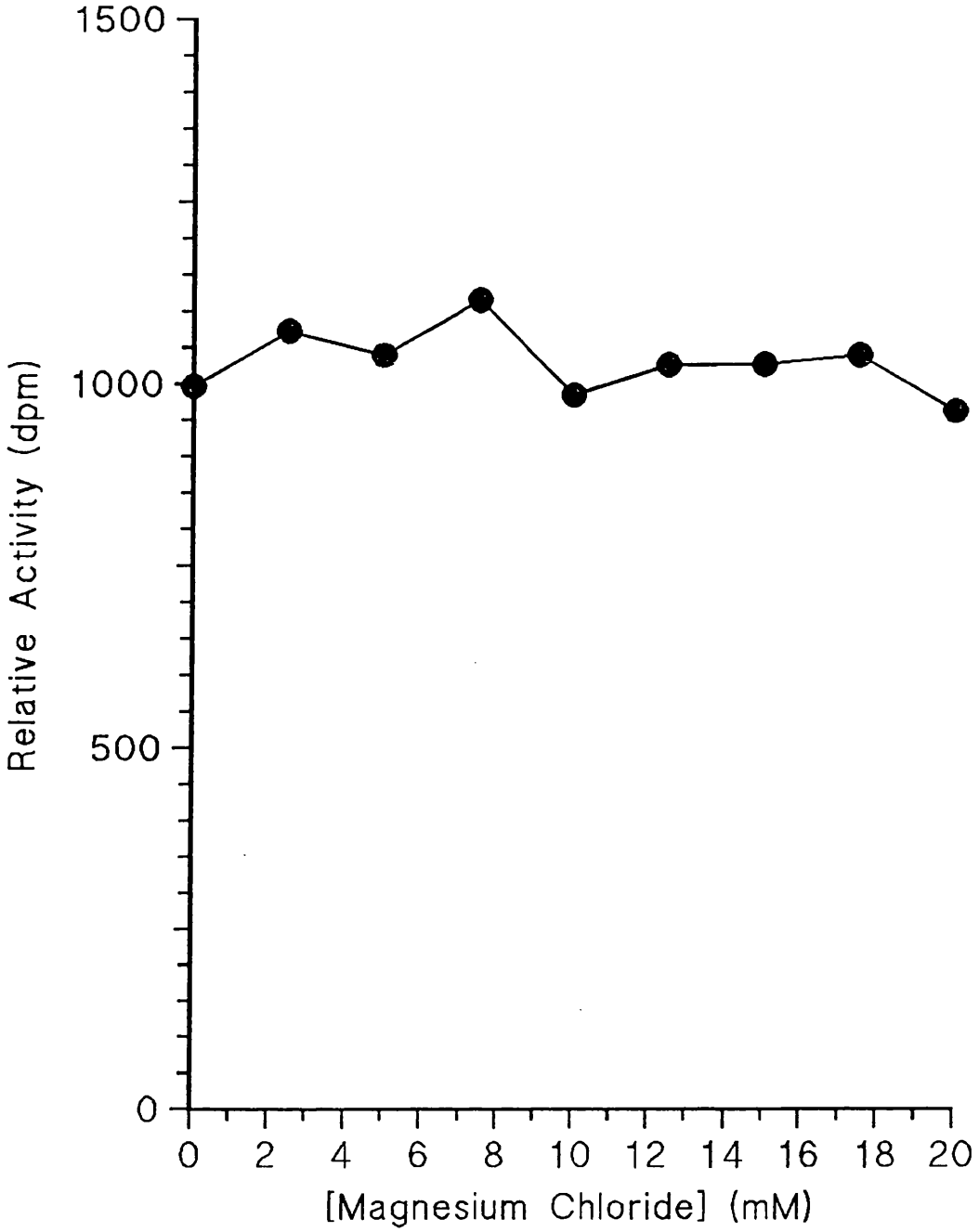


FIGURE 4.12: The Effect of Magnesium on Purified E5'Nase

Representative measurements, n=1.
No apparent effect of e5'Nase activity was observed over the full range of reported physiological concentrations of Mg^{2+}.

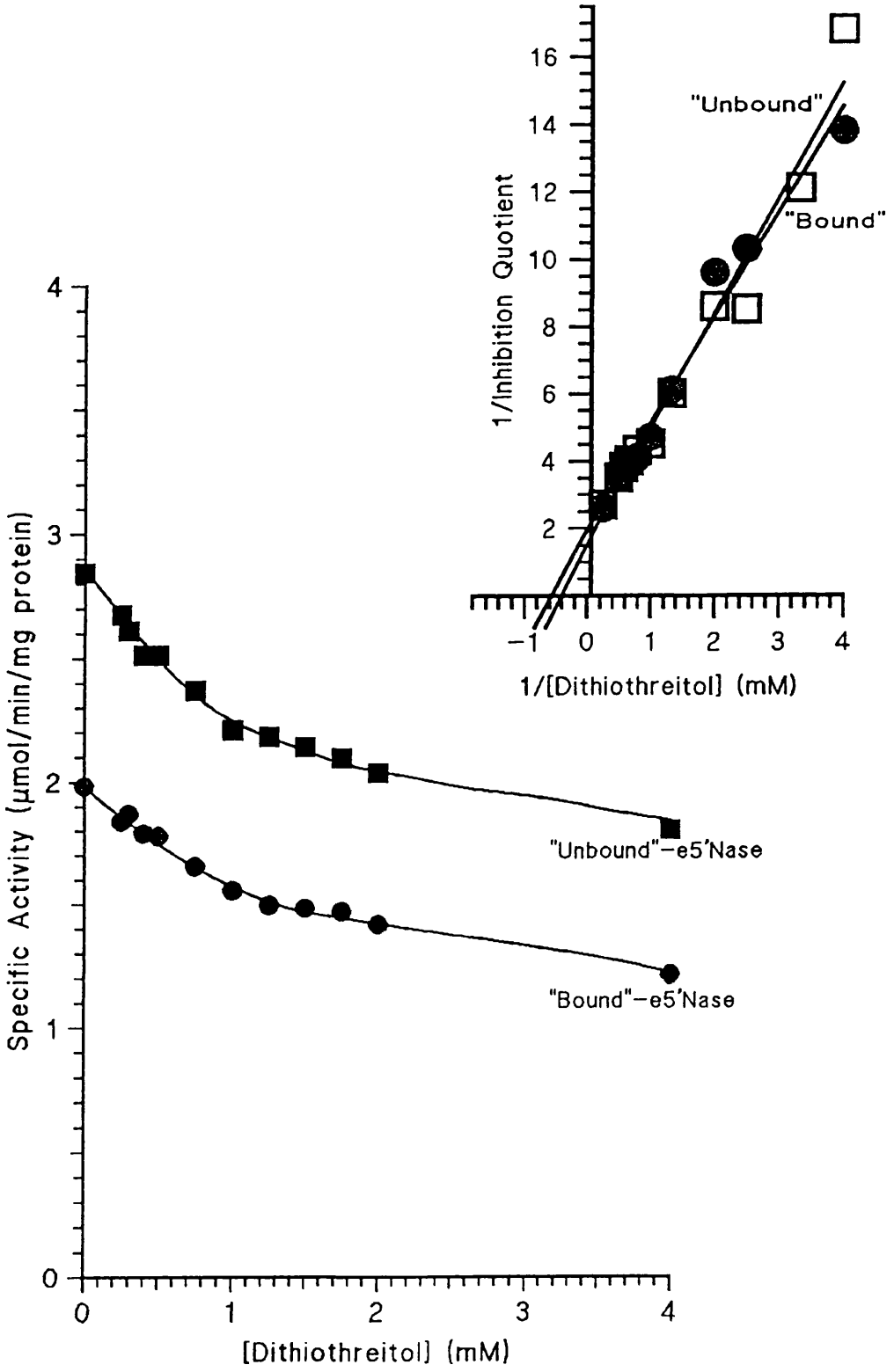


FIGURE 4.13: DTT Inhibition of Partially Purified "Bound"- and "Unbound"-E5'Nase

$IC_{50\text{"Bound"}}$ = 2.34mM and $IC_{50\text{"Unbound"}}$ = 2.25mM

66% for "Bound" and "Unbound" respectively.

4.3.5 Concanavalin A Inhibition

The ecto-enzyme is a highly glycosylated protein, with five potential glycosylation sites identified in rat liver (Van den Bosch *et al*, 1986; Wada *et al*, 1986; Misumi *et al*, 1990) and four sites in human placenta (Misumi *et al*, 1990a). The glycosylation appears to account for approximately 7kDa of the mature enzyme's molecular weight. As such, lectins in general, but specifically concanavalin A, are strong non-competitive inhibitors of e5'Nase. The extent of inhibition by concanavalin A on e5'Nase purified from white adipose tissue was measured in this study over a range of 0μM to 10μM lectin. The inhibition curve is shown in Figure 4.14. It can be seen that maximum inhibition is approached at 10μM concanavalin A. It is this strong inhibition which enables the use of this lectin for purification purposes via affinity chromatography. The binding is fully reversible by competition with α-methyl-D-mannoside which has a greater affinity for concanavalin A than the e5'Nase, thus enabling rapid elution of the protein.

The glycosylation of e5'Nase was investigated further by the use of a glycosidase and subsequent immunoblotting, but this topic will be covered in Section 4.4.

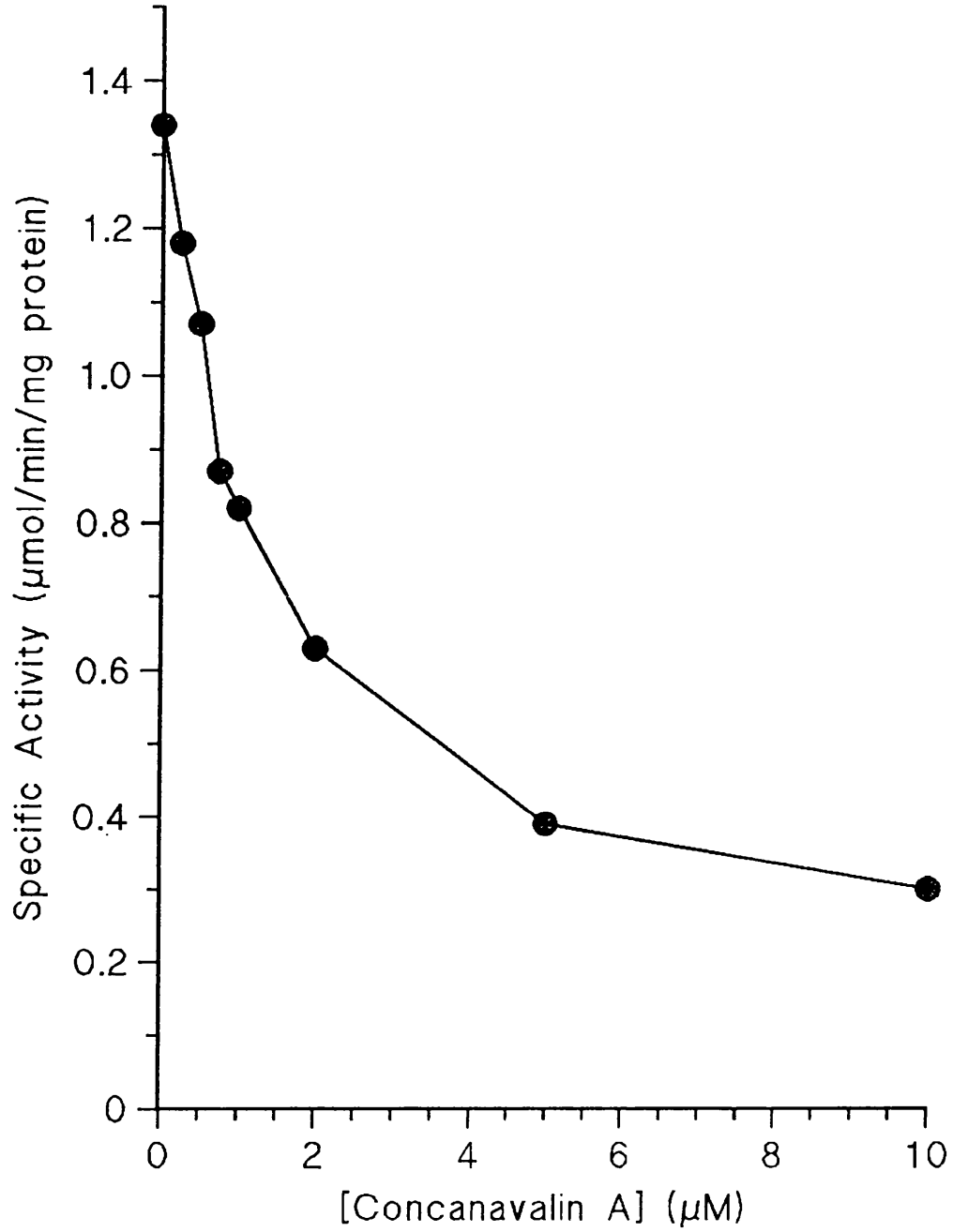


FIGURE 4.14: Inhibition of Partially Purified E5'Nase Activity by Concanavalin A

Representative measurements, n=1.

4.3.6 Effect of an ATP-Sensitivity-Conferring Factor on ATP Inhibition of E5'Nase Activity

The discovery of an ATP-sensitivity-enhancing factor which partially co-purified with an apparently soluble low K_m 5'Nase from rat brain (personal communication from Dr.M.Orford) led to many questions, not the least of which was whether the factor would have the same effect on ecto-5'Nase. This line of investigation was explored using the e5'Nase partially purified from white adipose tissue.

The factor was found to be equally effective at enhancing ATP-inhibition with the WAT e5'Nase as it had been with the "soluble" brain 5'Nase. Figure 4.15 shows inhibition curves obtained in the presence and absence of the factor. The ATP concentration required to produce 50% of the maximum inhibition was ~32.5μM in the absence of factor and ~0.8μM in the presence of factor, measured from the graph in Figure 4.15. Clearly ATP-sensitivity is enhanced by approximately 40-fold.

Buschette-Brambrink and Gutensohn (1989) showed from chemical cross-linking of the human placental ecto-enzyme that whilst it was still in the membrane, e5'Nase cross-linked with a species of approximately 30kDa. Their cross-linking studies were of interest in as much as the factor is known to be at least 30kDa in size and is labile once removed from the 5'Nase (Dr.M.Orford, personal communication).

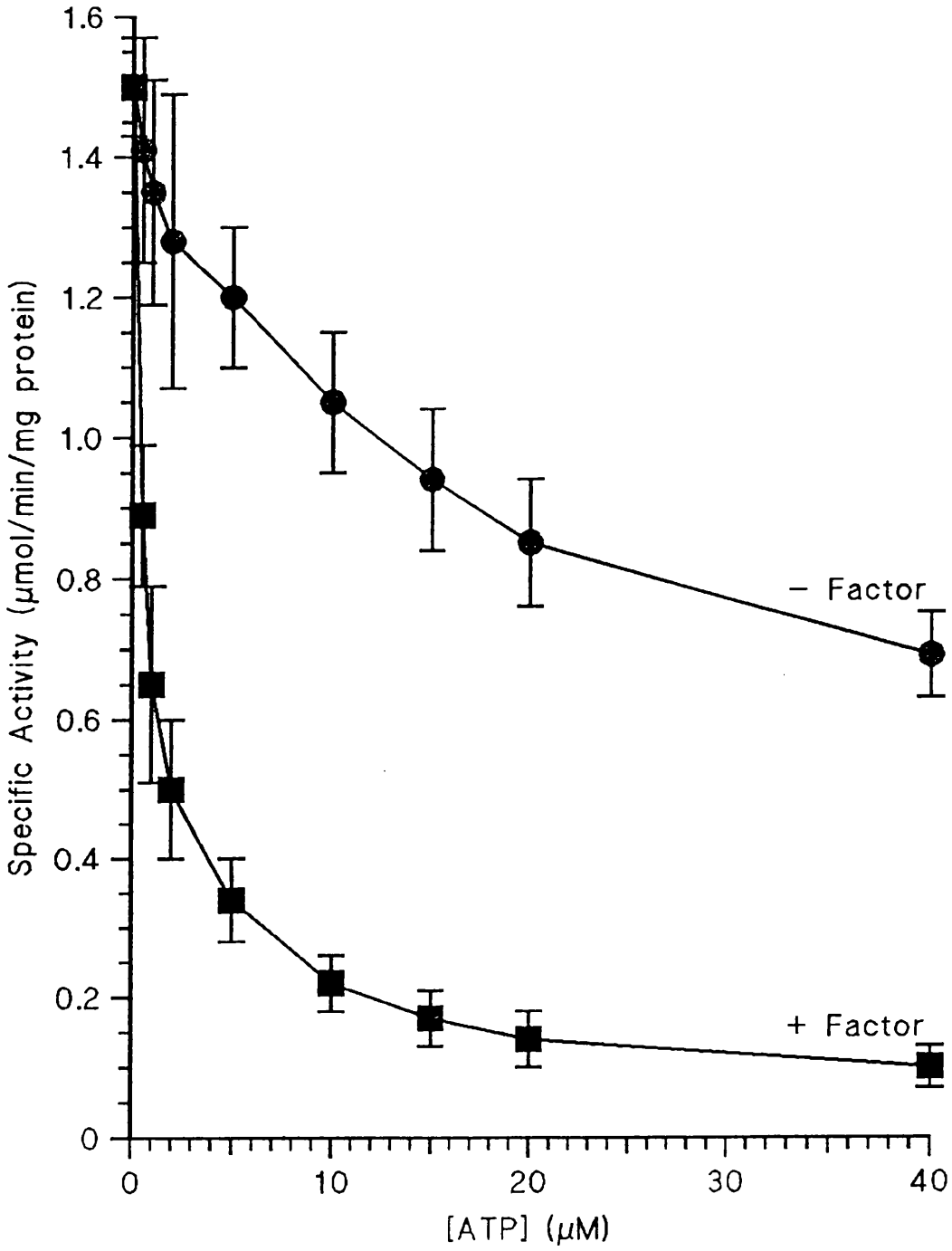


FIGURE 4.15: Inhibition of E5'Nase by ATP in the Presence and Absence of an ATP-Sensitivity-Enhancing Factor

For 0, 5-40µM ATP, n=3; for 0.5-2µM, n=2. The addition of 45µg of factor reduces the IC_{50} values from 2.9µM to 0.6µM.

In addition, purification of e5'Nase from rat liver carried out by Bailyes *et al* (1982) by two different procedures (ion exchange and immunoadsorbtion) produced a 38kDa contaminant in both cases. Chemical cross-linking prior to SDS-PAGE produced two new bands. One at 134kDa which they believed was the homodimeric form of e5'Nase, whilst the second was at 108kDa suggesting that the e5'Nase cross-linked with the 38kDa species. The possibility of an ATP sensitivity-enhancing factor existing in tissues other than brain and which may associate with the e5'Nase *in vivo*, would potentially alter the regulation of e5'Nase depending on the source of extracellular AMP and the concentrations of extracellular ATP and ADP. Indeed, the question of whether the factor may also enhance ADP inhibition needs to be addressed. Obviously the factor needs to be studied extensively and a priority should be to attempt purification and identification.

4.4 Deglycosylation of WAT E5'Nase

In Section 4.3.5 it was shown that concanavalin A strongly inhibits e5'Nase from white adipose tissue which is in accordance with other reports of extensive glycosylation. The extent to which the glycosylation contributes to the mature enzyme molecular weight was investigated in this study by the use of a glycosidase referred to as PNGase F (peptide-N-(N-acetyl-β-glucosaminyl)asparagine amidase). This enzyme, supplied by Boehringer, requires pretreatment with a

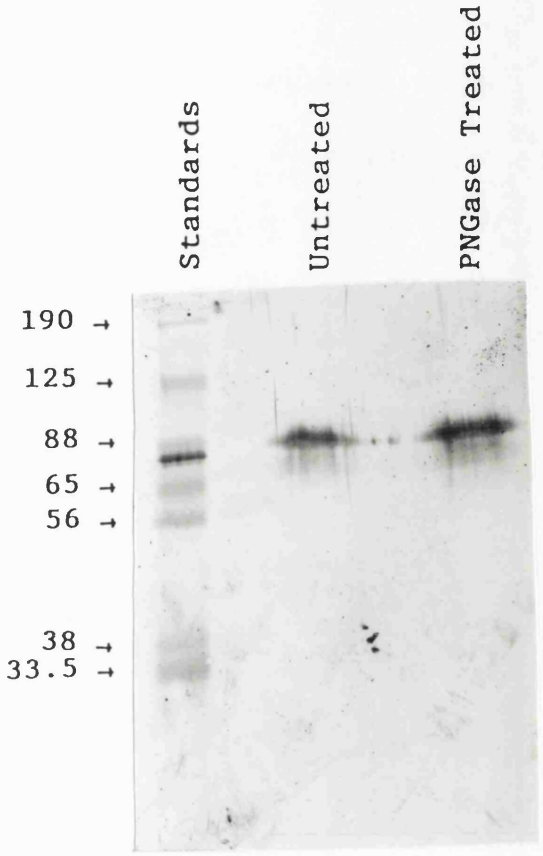


FIGURE 4.16: Immunoblot of E5'Nase Deglycosylation Attempt

PNGase treatment is described in the Materials & Methods chapter. Initial attempts to deglycosylate using Nonidet P-40 as the second detergent were unsuccessful, hence another attempt was made using MEGA-9 instead. This photograph shows the attempted deglycosylation with MEGA-9, using 60 IU of PNGase. Again, as can be seen in this photograph, deglycosylation did not take place.

non-ionic detergent after denaturation with SDS (sodium dodecyl-sulphate). Initial recommendations by Boehringer suggest the use of Nonidet P-40, but where that is ineffective or deglycosylation is limited, MEGA-9 (nonanoyl-N-methyl-gluca-amide) is suggested instead. This approach was used for the attempted deglycosylation in this study. However, as can be seen in the photograph of the immunoblot in Figure 4.16, even with MEGA-9 and using 60 IU PNGase F no change in the molecular weight could be detected. PNGase F is capable of deglycosylating all asparagine-linked glycans. The general consensus about e5'Nase glycosylation is that it is N-linked (see Zimmermann's review, 1992), but despite the numerous attempts with ever-increasing quantities of the PNGase F and varying the non-ionic detergent used, deglycosylation was not achieved. This would suggest that, at least in white adipose tissue, e5'Nase glycosylation is O-linked. However, no valid conclusions can be drawn without further evidence. Possibly attempting to deglycosylate with an O-glycosidase in conjunction with other glycohydrolases could provide some positive evidence.

CHAPTER 5

RESULTS: MONOCLONAL ANTIBODIES

RESULTS: MONOCLONAL ANTIBODIES

5.1 General

The concept of raising antibodies in this study for the detection and quantitation of adipose tissue e5'Nase was conceived from the beginning. However, purification of e5'Nase from white adipose tissue to absolute homogeneity, as required for the raising of polyclonal antibodies, proved to be difficult. The final sample referred to throughout Chapter 4 as partially purified e5'Nase, was in fact contaminated by a 63kDa species (see Figure 4.2). The raising of monoclonal antibodies (mAb) in this situation was deemed to be the ideal solution to this problem for two reasons. Not only would the raising of antibodies against a single epitope enable unique selection for e5'Nase from an impure starting point thus enabling further purification by use of an immunoaffinty chromatography column, but also selection of the mAbs against specific epitopes, such as the active site, could provide a useful tool for alternative studies.

Most mAbs used today are murine (generally using Balb/c mice), with several commercially available plasmacytoma cell lines available. The e5'Nase was purified from rat epididymal fat pads. Consequently, the raising of mAbs in mice appeared to be suitable. Initial manipulations of a Balb/c-derived plasmacytoma cell line called P3X63.Ag8.653

showed the cell line to be unfavourable under our conditions as the cells attached to the growing flasks too stringently. Subsequent trials with another cell line called Sp2/0-Ag14 were successful. The Sp2 cells are theoretically less stable, but fuse well and in this study, provided strong hybridomas.

5.2 Screening Serum Samples from Immunised Mice

Female balb/c mice were selected from a single litter whenever possible, to reduce potential variation between the control and immunised animals. Two mice were immunised simultaneously, but only one animal was ultimately selected on the basis of the strongest immunogenic response. Screening of blood serum was carried out by immunoprecipitation of purified rat e5'Nase by the serum samples, aided by a second anti-mouse antibody and insolubilised Protein A. After centrifugation, the resulting supernatant was assayed for 5'Nase activity to determine the extent of precipitation. Figure 5.1 shows that although the immunogenic response was not strong, it nevertheless existed to a limited degree. Further boosting during the immunisation schedule was attempted, but resulted in fatality. The mouse with the strongest response (giving ~10% immunoprecipitation) was therefore used for fusion. Interestingly, there is one publication in which a weak or non-existant immunogenic response against rat e5'Nase in balb/c mice was reported and success was only obtained on changing to C57BL/6 mice

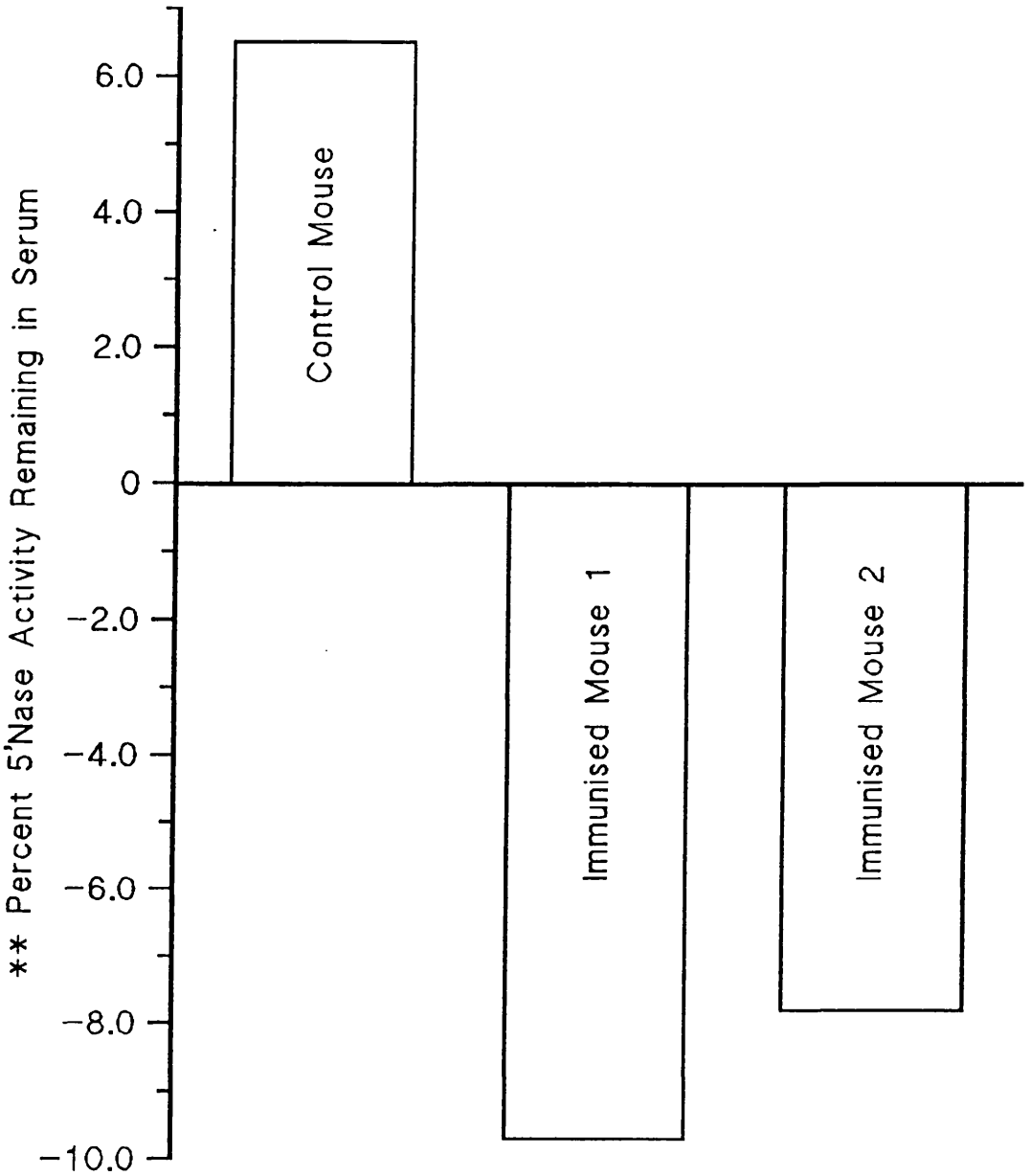


FIGURE 5.1: Percentage Immunoprecipitation of E5'Nase by Serum from both Immunised and Control Mice

** Indicates that the percentage of 5'Nase activity remaining is compared to the extent of 5'Nase activity measured when the partially purified e5'Nase was exposed only to PBS, combined with the activity naturally present in the serum without exogenous addition of 5'Nase.

(Thompson *et al*, 1989; Thompson, 1991). It therefore appears that e5'Nase may be highly conserved between the Sprague-Dawley rat from which it was purified and the Balb/c mouse, thereby limiting the immunogenic response.

5.3 Fusion and Screening of Hybridoma Supernatants

Fusion of the isolated splenocytes from the immunised mice and the Sp2-immortalised cell line was attempted on three occasions. Success, in so far as apparently stable hybridomas resulted from the fusion, was achieved on two of those occasions. However, the production and excretion of anti-5'Nase antibodies by the hybridomas was limited. Initial screening of the hybridomas for anti-5'Nase production was carried out using a commercially available mouse hybridoma detection kit (Boehringer Mannheim). The basis of detection relies on adhesion of the e5'Nase to a 96-well plate (pre-coated to enhance protein adhesion), followed by a double antibody (test hybridoma supernatant and anti-mouse antibody) system coupled to a colourimetric enzyme reaction. The absorbance was read in a specialised plate reader, thus revealing by a high absorbance, which hybridomas were producing and excreting anti-5'Nase. Many hybridomas from the original fusion were lost after a few weeks of growth. Some from bacterial contamination, whilst others ceased dividing after an initial growth period. Nevertheless, despite this loss and the poor immunogenic response, one well from the original fusion which had been substantially grown

up was identified as strongly positive from the initial screening. It was therefore necessary to carry out further screens to confirm that the antibody being produced was indeed anti-5'Nase.

An immunoprecipitation screen as used for screening the blood serums of the immunised mice, was then used on positively identified supernatants. This secondary screen was based on incubating the test supernatant with some 5'Nase, followed by the addition of a second IgG anti-mouse antibody. Protein A attached to acrylic beads was then added to the incubation ingredients to precipitate any IgG. The supernatant was removed and used in the radiolabelled 5'Nase assay to test for any remaining activity. This screen would not identify antibodies which interfered with activity (by binding to the active site for example), but removal of e5'Nase from the supernatant would certainly identify an anti-5'Nase antibody although the epitope recognised would not necessarily be specific to e5'Nase. The same hybridoma well was identified as positive, as with the commercial detection kit.

Finally, the aforementioned positively identified hybridoma supernatant was exposed to a third screen, to determine whether it could be used for Western blot analysis. This tertiary screen was carried out by a "slot-blot" analysis technique in which the partially purified e5'Nase was Western blotted onto nitrocellulose after SDS-PAGE in

which the sample was loaded in a single track which spread across the full width of the gel. The nitrocellulose sheet was then trapped in an apparatus consisting of slots which exposed the nitrocellulose sheet in vertical strips. Thus several different hybridoma supernatants were tested simultaneously by standard immunoblotting techniques. Unfortunately, the hybridoma supernatant identified positively above, did not show any signs of identifying e5'Nase immobilised on nitrocellulose.

The positively identified hybridoma was grown up and frozen for future use. On thawing it will have to be tested again as many hybrids cease producing the antibodies. If still identified as positive, cloning and subcloning will have to be carried out to select the individual hybrid cell which is producing the anti-5'Nase and thus form a monoclone.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

The regulation of e5'Nase activities may be an important step in the adjustment of white adipose tissue to conditions in which lipolysis is protracted. Adenosine is the product of the reaction catalysed by 5'Nase when AMP is the substrate. By interaction with specific purinergic receptors on the cell surface of both the adipocytes and the vasculature, adenosine can be a potent paracrine agent (i.e. locally acting hormone), causing a wide variety of effects in white adipose tissue normally attributed to insulin such as a strong antilipolytic response. Adenosine has also been shown to enhance the sensitivity of adipocytes to insulin (see Section 1.3). In contrast however, adenosine has a powerful vasodilatory effect in white adipose tissue, whilst insulin has been shown to decrease blood flow as discussed below.

Two independent findings were important to the rationale of this study. The first of these was the demonstration that insulin administration resulted in a decrease of adipose tissue blood flow in fasted rats (Madsen & Malchow-Mϕller, 1983) and in streptozotocin-diabetic rats (Jamal & Saggerson, 1988), whilst the second observation was that insulin status appeared to affect e5'Nase activity (Jamal & Saggerson, 1987; Karnieli *et al*, 1987). The hypothesis was that, providing there is a source of extracellular AMP, the increase in e5'Nase activity noted in the absence

of insulin, for example in streptozotocin-diabetes, would enhance adenosine production. This could in turn act as a metabolic signal to the vasculature to increase blood flow and remove products of lipolysis such as non-esterified fatty acids. In addition the increased levels of adenosine would partially alleviate the conditions resulting from the absence of insulin with its powerful antilipolytic effect in white adipocytes. Reintroduction of insulin *in vivo* has been shown to lower membrane-bound 5'Nase activity to control levels both by Jamal & Saggerson (1987) and in this study. The resulting reduction in adenosine production might be expected to explain the reduced adipose tissue blood flow discussed above. However, the time course of insulin action *in vivo* determined in this study (i.e. greater than 24 hours, but less than 48 hours) does not correspond to the relatively rapid (20-80 minutes) action of insulin on white adipose tissue blood flow previously reported (Madsen & Malchow-Møller, 1983; Jamal & Saggerson, 1988). Nevertheless, localisation of the postulated ecto-5'Nase within the adipose tissue indicated that a large proportion of the activity is associated with WAT non-adipocytes, including the vasculature, rather than the adipocytes. Furthermore, the measured increases in enzyme activity identified with the diabetic state were also associated with the non-adipocytes. These findings would conform with the idea of e5'Nase-related adipose tissue blood flow regulation in insulin-deficient states. Possibly there is a dual regulation of blood flow - the more immediate control exerted by insulin noted by Madsen

& Malchow-Møller (1983) in perfused tissue, and a longer term regulation via e5'Nase activity and adenosine levels in chronic states of insulin-deficiency.

Despite the finding referred to above in which most e5'Nase was associated with the non-adipocytes, surrounding adipocytes could also be exposed to adenosine produced on the non-adipocyte cells before cellular uptake and degradation occurs, due to the proximity of these other cell types to the adipocytes. This would be especially true in streptozotocin-diabetes when the reduced size of the adipocytes resulting from lipolysis would decrease the distances between the adipocytes and the non-adipocytes. Consequently, in the chronic insulin-deficient state, the adipocytes which are highly sensitive to adenosine, may be protected to a limited extent by exposure to locally produced adenosine. However, it should also be noted that potential increase in exposure to adenosine may be counter-balanced by a decreased sensitivity to adenosine in the diabetic animals as demonstrated by Saggerson *et al* (1991).

The relatively long time course mentioned above for insulin regulation of WAT membrane-bound 5'Nase, belies the concept that insulin may be able to regulate e5'Nase activity by stimulating the PI-G-anchor cleavage enzyme PI-G phospholipase C. Furthermore, the release of 5'Nase either from whole adipocytes or from isolated adipose tissue membranes by the exogenous addition of either insulin or PI-PLC in this

study showed both a resistance to cleavage overall and also no difference in release between diabetic and control membranes.

There have been reports of e5'Nase internalisation (Stanley *et al*, 1980; Widnell *et al*, 1982; Van den Bosch *et al*, 1988), but the time course for this process was shown to be approximately four hours which does not correspond to the 24 to 48 hour period of time required for insulin action *in vivo*. Furthermore, the 5'Nase activities in this study were measured in crude membranes and would therefore include internalised vesicles. One possibility that remains is that insulin affects the synthesis and/or degradation of e5'Nase, perhaps at the level of translation. Several proteins are regulated by insulin in this way, for example in the case of phosphenolpyruvate carboxykinase where synthesis is reduced by insulin (Forest *et al*, 1990). We may well be looking at a similar scenario for e5'Nase regulation.

Other metabolic states studied here appear to support the concept of direct insulin regulation of e5'Nase activity. Hyperthyroidism is characterised by increased lipolysis and increased plasma concentrations of non-esterified fatty acids - two conditions which are also characteristic of streptozotocin-diabetes, but in this study no significant difference was observed in WAT 5'Nase activity when hyper-thyroidism was induced. Conversely, hypothyroidism is characterised by an impairment of the adrenergic stimulation

of lipolysis which results in the same antilipolytic effect as insulin (Ohisalo *et al*, 1987). However, hypothyroidism has also been shown to be associated with markedly reduced adenosine concentrations in adipose tissue. Nevertheless, here too no significant difference was noted in whole WAT 5'Nase activity. The remaining metabolic state studied was fasting. This would be characterized by reduced circulating levels of insulin and although the e5'Nase measurements made were inconclusive, there did appear to be a tendency for raised e5'Nase activity. Perhaps this noted tendency would have become more pronounced over a longer period of fasting, but this idea would not be feasible to test.

Immunoblotting with the polyclonal anti-rat-e5'Nase donated by Dr.J.P.Luzio revealed two bands at 76kDa and 74kDa. The 76kDa band intensified in the streptozotocin-diabetic tracks, but only the combined intensities of the two bands correlated with measured activity changes of the membrane-bound 5'Nase. This finding led to the proposal that there may be two species of membrane-bound 5'Nase in white adipose tissue - one being constitutive, whilst the second could be adaptive. A situation in which there could be both a transmembrane 5'Nase as proposed by Zachowski *et al* (1981) and by Dieckhoff *et al* (1987) and the more accepted idea of a PI-G-anchored 5'Nase (Low & Finean, 1978; Shukla *et al*, 1980; Panagia *et al*, 1981; Baron *et al*, 1986; Bailyes *et al*, 1990; Ogata *et al*, 1990; Misumi *et al*, 1990; Misumi *et al*, 1990a), has been suggested by several groups (Dieckhoff *et*

al, 1987; Grondal & Zimmermann, 1987; Thompson *et al*, 1987; Stochaj *et al*, 1989; Klemens *et al*, 1990). Certainly this idea of two forms of anchorage could provide a mechanism by which a constitutive and an adaptive species of membrane-bound 5'Nase may co-exist. Purification by detergent solubilization did not reveal the 76kDa and 74kDa doublet. However, most if not all of the enzyme appeared to be solubilized with the sulphobetaine detergent. This suggests that if there were indeed a PI-G-anchored and transmembrane form of 5'Nase, both are susceptible to sulphobetaine solubilization, but do not have equal affinities for the AMP-Sepharose columns used in the purification. The attempt to raise monoclonal antibodies could have been used to try distinguishing between the two bands on the Western blots. However, this would be highly dependent on the epitope identified by each monoclonal antibody. Certainly the quantitation of 5'Nase bands on the Western blots could be made more accurate by use of the monoclonal antibodies. Furthermore, a distinction between the AMP-chromatography column "Bound" and "Unbound" samples may have been possible with monoclonal antibodies.

The WAT membrane-bound 5'Nase partially purified in this study was believed to be WAT e5'Nase based on the comparison of its molecular weight and properties against those reported for e5'Nases purified from other tissues. The potent inhibitory role of adenine di- and tri-phosphates and their corresponding α,β-methylene-analogues on e5'Nase

activity is well established. This begs the question as to the source of extracellular AMP. Both ATPases and ADPases have been identified as membrane-bound ecto-enzymes in brain cholinergic synaptic clefts (Richardson *et al*, 1987; Lai & Wong, 1991a; James & Richardson, 1993). Consequently, if such an ectophosphohydrolase system and an extracellular source of ATP are established in WAT - for example from neuronal terminal release - then theoretically it could be progressively dephosphorylated via a nucleotidase cascade. However, the build up of extracellular AMP would have to be immense to overcome the inhibition of ATP initially, and subsequently that of ADP which is an even more potent inhibitor of e5'Nase, before adenosine production could take place.

The identification of an ATP sensitivity-enhancing factor (>30kDa) which partially co-purifies with the brain "soluble" low K_m 5'Nase could further inhibit an ectophospho-hydrolase system at the point of e5'Nase. In this study the factor was shown to strongly enhance ATP inhibition of WAT membrane-bound 5'Nase. Other reports have been made of a 30kDa plasma membrane component (Buschette-Brambrink & Gutensohn, 1989) and a 38kDa co-purification polypeptide (Bailyes *et al*, 1982) which crosslinked with the liver e5'Nase. Further investigations may reveal the presence of this ~30kDa component in conjunction with the e5'Nase in other tissues. If this were found to be the case and it was further identified as an ATP sensitivity-enhancing factor

similar to that found in brain, then the concept of an extracellular adenine nucleotide cascade seems less likely.

An alternative source of extracellular AMP could be via nucleotide transport from intracellular stores. Under normal metabolic conditions insulin stimulates cAMP phosphodiesterase activity (Smith *et al*, 1991; Manganiello *et al*, 1992), thus increasing the formation of AMP from cAMP. Although cytosolic 5'Nase tends to prefer IMP as substrate it is also capable of catalysing the hydrolysis of AMP (Naito & Tsushima, 1976; Worku & Newby, 1983; Newby *et al*, 1987, Itoh & Yamada, 1991). AMP deaminase has an affinity constant in the 60μM to 2.5mM range (Nikiforuk & Colowick, 1956; Setlow & Lowenstein, 1967), although its apparent affinity from AMP is increased by the presence of ATP (Setlow & Lowenstein, 1967). Consequently even if a situation were to arise in which AMP levels increased, the presence of non-specific phosphatases would tend to prevent any large fluctuations. It is therefore difficult to envisage a situation induced either by the presence of absence of insulin, which would lead to a significant increase in AMP levels such that the transport of the nucleotide out of the cell would be encouraged.

One suggestion made for the role of WAT ecto-5'Nase was to "mop-up" ATP released from damaged cells (Kather, 1990). However, it seems equally unlikely that e5'Nase activities would alter significantly in chronic insulin-

deficient states such as streptozotocin-diabetes without a purpose. Investigations will need to be carried out to determine finally whether indeed there may be an extra-cellular source of AMP, potentially changing with insulin status. The possible role for the ATP sensitivity-enhancing factor also needs further study. Perhaps both determining its presence or absence in membrane fractions, and if present, identifying it via purification.

CHAPTER 7

REFERENCES

REFERENCES

Achterberg, P.W.; de Tombe, P.P.; Harmsen, E. and de Jong, J.W. (1985) Myocardial S-adenosylhomocysteine hydrolase is important for adenosine production during normoxia. Biochim.Biophys.Acta, 840: 393-400

Alemany, S.; Mato, J.M. and Strålfors, P. (1987) Phospho-dephospho-control by insulin is mimicked by a phospho-oligosaccharide in adipocytes. Nature, 330: 77-79

Alvarez, J.F.; Varela, I.; Ruiz-Albusac, J.M. and Mato, J.M. (1988) Localisation of the insulin-sensitive phosphatidylinositol glycan at the outer surface of the cell membrane. Biochem.Biophys.Res.Comm., 152: 1455-1462

Andy, R.J. and Kornfeld, R. (1982) The adenosine deaminase binding protein of human skin fibroblasts is located on the cell surface. J.Biol.Chem., 257: 7922-7925

Arch, J.R.S. and Newsholme, E.A. (1978) Activities and some properties of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from verte-brates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. Biochem.J., 174: 965-977

Auger, K.R.; Carpenter, C.L.; Shoelson, S.E.; Piwnica-Worms, H. and Cantley, L.C. (1992) Polyoma virus middle T antigen-pp60$^{c\text{-}src}$ complex associates with purified phosphatidylinositol 3-kinase *in vitro*. J.Biol.Chem., 267: 5408-5415

Augustine, J.A.; Schlager, J.W. and Abraham, R.T. (1990) Differential effects of interleukin-2 and interleukin-4 on protein tyrosine phosphorylation in factor-dependent murine T cells. Biochim.Biophys.Acta, 1052: 313-322

Avruch, J. and Wallach, D.F.H. (1971) Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. Biochim.Biophys. Acta, 233: 334-347

Baer, H.P.; Drummond, G.I. and Duncan, E.L. (1966) Formation and deamination of adenosine by cardiac muscle enzymes. Mol. Pharmacol., 2: 67-76

Bailyes, E.M.; Newby, A.C.; Siddle, K. and Luzio, J.P. (1982) Solubilization and purification of rat liver 5'-nucleotidase by use of a zwitterionic detergent and a monoclonal-antibody immunoadsorbent. Biochem.J., 203: 245-251

Bailyes, E.M.; Ferguson, M.A.J.; Colaco, C.A.L.S. and Luzio, J.P. (1990) Inositol is a constituent of detergent-

solubilized immunoaffinity-purified rat liver 5'-nucleotidase. Biochem.J., 265: 907-909

Ballard, K. and Rosell, S. (1971) Adrenergic neurohumoral influences on circulation and lipolysis in canine omental adipose tissue. Circ.Res., 28: 389-395

Ballard, K.; Malmfors, T. and Rosell, S. (1974) Adrenergic innervation and vascular patterns in canine adipose tissue. Microvasc.Res., 8: 164-171

Balsinde, J. and Mollinedo, F. (1990) Phosphatidylinositol hydrolysis by human plasma phospholipase D. FEBS Lett., 259: 237-240

Baltensperger, K.; Kozma, L.M.; Cherniack, A.D.; Klarlund, J.K.; Chawla, A.; Banerjee, U. and Czech, M.P. (1993) Binding of the ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science, 260: 1950-1952

Baron, M.D.; Pope, B. and Luzio, J.P. (1986) The membrane topography of ecto-5'-nucleotidase in rat hepatocytes. Biochem.J., 236: 495-502

Bell, P.M.; Firth, R.G. and Rizza, R.A. (1986) Assessment of insulin action in insulin-dependent diabetes mellitus using [6-^{14}C]glucose, [3-^{3}H]glucose, and [2-^{3}H]glucose. J.Clin.Invest., 78: 1479-1486

Berger, J.; Howard, A.D.; Brink, L.; Gerber, L.; Hauber, J.; Cullen, B.R. and Udenfriend, S. (1988) COOH-terminal requirements for the correct processing of a phosphatidylinositol-glycan anchored membrane protein. J.Biol.Chem., 263: 10016-10021

Bergh, C.-H.; Hjalmarson, Å; Sjögren, K.-G. and Jacobsson, B. (1988) The effect of diabetes on phosphatidyl-inositol turnover and calcium influx in myocardium. Horm.Metabol.Res., 20: 381-386

Berne, R.M. (1963) Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. Am.J. Physiol., 204: 317-322

Berne, R.M. and Rubio, R. (1974) Adenine nucleotide metabolism in the heart. Circ.Res., 34-35(Suppl.III): 109-120

Berne, R.M.; Imai, S. and Riley, A.L. (1963) Adenine nucleotides and their derivatives in anoxic cardiac and skeletal muscle. Physiologist, 6: 138-14

Bodansky, O. and Schwartz, M.J. (1963) Comparative effects of L-histidine on the activities of 5'-nucleotidase and alkaline phosphatase. J.Biol.Chem., 238: 3420-3427

Bodansky, O. and Schwartz, M.J. (1968) 5'-Nucleotidase. Adv. Clin.Chem., 11: 277-328

Bontemps, F.; Vincent, M.F.; Van den Bergh, F.; Van Waeg, G. and Van den Berghe, G. (1989) Stimulation by glycerate 2,3-bisphosphate: a common property of cytosolic IMP-GMP 5'-nucleotidase in rat and human tissues. Biochim. Biophys.Acta, 997: 131-134

Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J.Biol.Chem., 256: 1604-1607

Borthwick, A.C.; Edgell, N.J. and Denton, R.M. (1990) Protein kinase from rat epididymal adipose tissue which phosphorylates and activates acetyl-CoA carboxylase. Biochem.J., 270: 795-801

Boyle, J.M.; Hey, Y.; Guerts van Kessel, A. and Fox, M.(1988) Assignment of ecto-5'-nucleotidase to human chromosome 6. Hum.Genet., 81: 88-92

Brownsey, R.W.; Edgell, N.J.; Hopkirk, T.J. and Denton, R.M. (1984) Studies on insulin-stimulated phosphorylation of acetyl-CoA carboxylase, ATP citrate lyase and other proteins in rat epididymal adipose tissue. Biochem.J., 218: 733-743

Bulow, R. and Overath, P. (1986) Purification and characterization of the membrane-form variant surface glycoprotein hydrolase of *Trypanosoma brucei*. J.Biol. Chem., 261: 11918-11923

Burger, R.M. and Lowenstein, J.M. (1970) Preparation and -nucleotidase from smooth muscle of small intestine. J. Biol.Chem., 245: 6274-6280

Burnstock, G. (1972) Purinergic nerves. Pharmacol.Rev., 24: 509-581

Burnstock, G. (1978) A basis for distinguishing two types of purinergic receptor. In: L. Bolis and R.W. Straub (eds) Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. (Raven, N.Y.), pp.107-118

Buschette-Brambrink, S. and Gutensohn, W. (1989) Human placental ecto-5'-nucleotidase: isoforms and chemical crosslinking products of the membrane-bound and isolated enzyme. Biol.Chem. Hoppe-Seyler, 370: 67-74

Caldwell, A. and Fain, J.N. (1971) Triiodothyronine stimulation of cyclic adenosine 3',5'-monophosphate accumulation in fat cells. Endocrinology (Baltimore), 89: 1195-1204

Cantley, L.C.; Auger, K.R.; Carpenter, C.; Duckworth, B.; Graziani, A.; Kapeller, R. and Soltoff, S. (1991) Oncogenes and signal transduction. Cell, 64: 281-302

Caras, I.W. and Weddell, G.N. (1989) Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment. Science, 243: 1196-1198

Cardoso de Almeida, M.L.; Turner, M.I.; Stambuk, B.B. and Schenkman, S. (1988) Identification of an acid-lipase in human serum which is capable of solubilizing glycophosphatidylinositol-anchored proteins. Biochem.Biophys.Res.Comm., 150: 476-482

Caputto, R. (1962) Nucleoside kinases. The Enzymes, 6: 133-138

Chan, B.L.; Lisanti, M.P.; Rodriguez-Boulan, E. and Saltiel, A.R. (1988) Insulin-stimulated release of lipo-protein lipase by metabolism of its phosphatidyl-inositol anchor. Science, 241: 1670-1672

Chandramouli, V. and Carter, J.R. (1975) Cell membrane changes in chronically diabetic rats. Diabetes, 24: 257-262

Chohan, P.; Carpenter, C. and Saggerson, E.E. (1984) Changes in the anti-lipolytic action and binding to plasma membranes of N^6-L-phenylisopropyladenosine in adipocytes from starved and hypothyroid rats. Biochem.J., 223: 53-59

Ciaraldi, T.P. (1988) The role of adenosine in insulin action coupling in rat adipocytes. Mol.Cell.Endocrinol., 60: 31-41

Coddington, A. (1965) Some substrates and inhibitors of adenosine deaminase. Biochim.Biophys.Acta, 99: 442-451

Collinson, A.R.; Peuhkurinen, K.J. and Lowenstein, J.M. (1987) Regulation and function of 5'-nucleotidases. In: E.Gerlach & B.F.Becker (eds.) Topics and Perspectives in Adenosine Research (Springer-Verlag), pp.133-144

Cornish-Bowden, A.J. (1974) A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem.J., 137: 143-144

Cornish-Bowden, A.J. (1979) Fundamentals of Enzyme Kinetics (Butterworth, London-Boston), pp.78-82

Coughlin, S.R.; Escobedo, J.A. and Williams, L.T. (1989) Science, 243: 1191-1194

Cross, G.A.M. (1987) Eukaryotic protein modification and membrane attachment via phosphatidylinositol. Cell, 48: 179-181

Cusack, M.J.; Pearson, J.D. and Gordon, J.L. (1983) Stereoselectivity of ectonucleotidases on vascular endothelial cells. Biochem.J., 214: 975-981

Daddona, P.E. and Kelley, W.N. (1978) Human adenosine deaminase binding protein. Assay, purification and properties. J.Biol.Chem., 253: 4617-4623

Davitz, M.A.; Hereld, D.; Shak, S.; Krakow, J.; Englund, P.T. and Nussenzweig, V. (1987) A glycan-phosphatidylinositol-specific phospholipase D in human serum. Science, 238: 81-84

Davitz, M.A.; Hom, J. and Schenkman, S. (1989) Purification of a glycosyl-phosphatidylinositol-specific phospholipase D from human plasma. J.Biol.Chem., 264: 13760-13764

Debons, A.F. and Schwartz, I.L. (1961) Dependence of the lipolytic action of epinephrine *in vitro* upon thyroid hormone. J.Lipid Res., 2: 86-89

DePierre, J.W. and Karnovsky, M.L. (1974) Ecto-enzymes of the guinea pig polymorphonuclear leukocyte. II. Properties and suitability as markers for the plasma membrane. J.Biol. Chem., 249: 7121-7129

Deykin, D. and Vaughan, M. (1963) Release of free fatty acids by adipose tissue from rats treated with triiodothyronine or propylthiouracil. J.Lipid Res., 4: 200-203

Dieckhoff, J.; Knebel, H.; Heidemann, M. and Mannherz, H.G. (1985) An improved procedure for purifying 5'-nucleotidase from various sources. Eur.J.Biochem., 151: 377-383

Dieckhoff, J.; Mollenhauer, J.; Kühl, U.; Niggemeyer, B.; Von der Mark, K. and Mannherz, H.-G. (1986) The extracellular matrix proteins laminin and fibronectin modify the AMPase activity of 5'-nucleotidase from chicken gizzard smooth muscle. FEBS Lett., 195: 82-86

Dieckhoff, J.; Niggemeyer, B.; Lietzke, R. and Mannherz, H.G. (1987) Reconstitution of purified chicken gizzard 5'-nucleotidase in phospholipid vesicles. Evidence for its transmembraneous character and the existence of functional domains on both sides of the phospholipid bilayer. Eur.J.Biochem., 162: 451-459

Dixon, M. (1953) The determination of enzyme inhibitor contstants. Biochem.J., 55: 170-171

Doering, T.L.; Masterson, W.J.; Englund, P.T. and Hart, G.W. (1989) Biosynthesis of the glycosyl phosphatidylinositol membrane anchor of the Trypanosome variant surface glycoprotein. J.Biol.Chem., 264: 11168-11173

Dornand, J.; Bonnafous, J.-C. and Mani, J.-C. (1980) 5'-Nucleotidase - adenylate cyclase relationships in mouse thymocytes. FEBS Lett., 110: 30-34

Draye, J.-P.; Courtoy, P.J.; Quintart, J. and Baudhuin, P. (1987) Relations between plasma membrane and lysosomal membrane. Eur.J.Biochem., 170: 405-411

Drummond, G.I. and Yamamoto, M. (1971) Nucleotide phosphomonoesterases. In: P.D.Boyer (ed.) The Enzymes (Academic Press), pp.337-354

Drury, A.N. and Szent-Györgyi, A. (1929) The physiological activity of adenine compounds with especial reference to their action upon the mammalian hear. J.Physiol.Lond., 68: 213-237

Dustin, M.L.; Selvaraj, P.; Mattaliano, R.J. and Springer, T.A. (1987) Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. Nature, 329: 846-848

Ebert, R. and Schwabe, U. (1973) Studies on the antilipolytic effect of adenosine and related compounds in isolated fat cells. Naunyn-Schmeideberg's Arch.Pharmacol., 278: 247-259

Evans, W.H. and Gurd, J.W. (1973) Properties of a 5'-nucleotidase purified from mouse liver plasma membranes. Biochem.J., 133: 189-199

Fain, J.N. and Wieser, P.B. (1975) Effects of adenosine deaminase on cyclic adenosine monophosphate accumulation, lipolysis, and glucose metabolism of fat cells. J.Biol.Chem., 250: 1027-1034

Ferguson, M.A.J. and Williams, A.F. (1988) Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Ann.Rev.Biochem., 57: 285-320

Ferguson, M.A.J.; Low, M.G. and Cross, G.A.M. (1985) Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. J.Biol.Chem., 260: 14547-14555

Fernandez, B.M. and Saggerson, E.D. (1978) Alterations in response of rat white adipocytes to insulin, noradrenaline, corticotropin and glucagon after adrenalectomy. Biochem.J., 174: 111-118

Fini, C.; Minelli, A.; Camici, M. and Floridi, A. (1985) Effects of dithiothreitol, dithioerythritol and chelating agents on 5'-nucleotidase from bull seminal plasma. Biochim.Biophys.Acta, 827: 403-409

Fini, C.; Palmerini, C.A.; Damiani, P.; Stochaj, U.; Mannherz, H.G. and Floridi, A. (1990) 5'-Nucleotidase from bull seminal plasma, chicken gizzard and snake venom is a zinc metalloprotein. Biochim.Biophys.Acta, 1038: 18-22

Fisher, M.N. and Newsholme, E.A. (1984) Properties of rat heart adenosine kinase. Biochem.J., 221: 521-528

Forest, C.D.; O'Brien, R.M.; Lucas, P.C. and Granner, D.K. (1990) In: P.J.Randle, J.Bell and J.Scott (eds) Genetics and Human Nutrition. (John Libbey, London), pp.111-119

Fox, J.A.; Duszenko, M.; Ferguson, M.A.J.; Low, M.G. and Cross, G.A.M. (1986) Purification and character-ization of a novel glycan phosphatidylinositol-specific phospholipase C from *Trypanosoma brucei*. J.Biol.Chem., 261: 15767-15771

Fox, J.A.; Soliz, N.M. and Saltiel, A.R. (1987) Purification of a phosphatidylinositol-glycan-specific phospholipase C from liver plasma membranes: a possible target of insulin action. Proc.Natl.Acad.Sci.USA, 84: 2663-2667

Fredholm, B.B. (1974) Vascular and metabolic effects of theophylline, dibuturyl cyclic AMP and dibuturyl cyclic GMP in canine subcutaneous adipose tissue *in situ*: combination of lipolysis inhibition and increased re-esterification. Acta Pharmacol.Toxicol., 33: 300-307

Fredholm, B.B. (1976) Release of adenosine-like material from isolated perfused dog adipose tissue following sympathetic nerve stimulation and its inhibition By adrenergic α-receptor blockade. Acta Physiol.Scand., 96: 422-430

Freeman, R.M. Jr.; Plutzky, J. and Neel, B.G. (1992) Identi-fication of a human src homology-2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew. Proc.Natl.Acad.Sci.USA, 89: 11239-11243

Fritzon, P.; Haugen, T.B. and Tjernshaugen, H. (1986) The presence and activity in normal and regenerating rat liver postmicrosomal supernatant fraction of an enzyme with properties similar to those of membrane-bound 5'-nucleotidase. Biochem.J., 239: 185-190

Furchgott, R.F. (1984) The role of endothelium in the responses of vascular smooth muscle to drugs. Annu.Rev. Pharmacol.Toxicol., 24: 175-197

Gawler, D.; Milligan, G.; Spiegel, A.M.; Unson, C.G. and Houslay, M.D. (1987) Abolition of the expression of inhibitory guanine nucleotide regulatory protein G_i activity in diabetes. Nature, 327: 229-232

Gibson, W.B. and Drummond, G.I. (1972) Properties of 5'-nucleotidase from avian heart. Biochemistry, 11: 223-229

Goswami, A. and Rosenberg, I.N. (1978) Thyroid hormone modulation of epinephrine-induced lipolysis in rat adipocytes: a possible role of calcium. Endocrinology (Baltimore), 103: 2223-2233

Gower, H.J.; Barton, C.H.; Elsom, V.L.; Thompson, J.; Moore, S.E.; Dickson, G. and Walsh, F.S. (1988) Alternative splicing generates a secreted form of N-CAM in muscle and brain. Cell. 55: 955-964

Green, A. (1983) Catecholamines inhibit insulin-stimulated glucose transport in adipocytes, in the presence of adenosine deaminase. FEBS Letts., 152: 261-264

Green, A. (1987) Adenosine receptor down-regulation and insulin resistance following prolonged incubation of adipocytes with an A_1 adenosine receptor agonist. J.Biol.Chem., 262: 15702-15707

Green, A. and Newsholme, E.A. (1979) Sensitivity of glucose uptake and lipolysis of white adipocytes of the rat to insulin and effects of some metabolites. Biochem.J., 180: 365-370

Green, A. and Newsholme, E.A. (1981) Distribution of adenosine metabolising enzymes between adipocyte and stromal-vascular cells of adipose tissue. Biochim.Biophys.Acta, 676: 122-124

Grondal, E.J.M. and Zimmermann, H. (1987) Purification, characteriztion and celular localization of 5'-nucleotidase from *Torpedo* electric organ. Biochem.J., 245: 805-810

Gurd, J.W. and Evans, W.H. (1974) Distribution of liver plasma membrane 5'Nucleotidase as indicated by its reaction with anti-plasma membrane serum. Arch.Biochem.Biophys., 164: 305-311

Halperin, M.L. and Denton, R.M. (1969) Regulation of glyclysis and L-glycerol 3-phosphate concentration in rat epididymal adipose tissue *in vitro*. Biochem.J., 113: 207-214

Harb, J.; Meflah, K.; Duflos, Y. and Bernard, S. (1983) Purification and properties of bovine liver plasma

membrane 5'-nucleotidase. Eur.J.Biochem., 137: 131-138

Harlow, E. and Lane, D. (1988) Antibodies: a laboratory manual. (Cold Spring Harbour Laboratory, New York), p.653

Hashimoto, N.; Robinson, F.W.; Shibata, Y.; Flanagan, J.E. and Kono, T. (1987) Diversity in the effects of extracellular ATP and adenosine on the cellular processing and physiologic actions of insulin in rat adipocytes. J.Biol.Chem., 262: 15026-15032

Hayashi, H.; Kamohara, S.; Nishioka, Y.; Kanai, F.; Miyake, N.; Fukui, Y.; Shibasaki, F.; Takenawa, T. and Ebina, Y. (1992) Insulin treatment stimulates the tyrosine phosphorylation of the α-type 85-kDa subunit of phosphatidylinositol 3-kinase *in vitro*. J.Biol.Chem., 267: 22575-22580

Hedqvist, P. and Fredholm, B.B. (1976) Effects of adenosine on adrenergic neurotransmission; prejunctional inhibition and postjunctional enhancement. Naunyn-Schmiedeberg's Arch. Pharmacol., 293: 217-223

Hereld, D.; Krakow, J.L.; Bangs, J.D.; Hart, G.W. and Englund, P.T. (1986) A phospholipase C from *Trypanosoma brucei* which selectively cleaves the glycolipid on the variant surface glycoprotein. J.Biol.Chem., 261: 13813-13819

Hjemdahl, P.; Belfrage, E. and Daleskog, M. (1979) Vascular and metabolic effects of circulating epinephrine and norepinephrine. J.Clin.Invest., 64: 1221-1228

Hoffbrand, B.I. & Forsyth, R.P. (1973) Regional blood flow changes during norepinephrine, tyramine and methoxamine infusions in the unanaesthetized rhesus monkey. J.Pharmacol.Exp.Ther., 184: 656-661

Hoffman, B.B.; Chang, H.; Farahbakhsh, Z. and Reaven, G. (1984) Inhibition of lipolysis by adenosine is potentiated with age. J.Clin.Invest., 74: 1750-1755

Honeyman, T.W.; Strohsmitter, W.; Scheid, C.R. and Schimmel, R.J. (1983) Phosphatidic acid and phosphatidyl-inositol labelling in adipose tissue: relationship to the metabolic effects of insulin and insulin-like agents. Biochem.J., 212: 489-498

Hooper, N.M. and Bashir, A. (1991) Glycosyl-phosphatidyl-inositol-anchored membrane proteins can be distinguished from transmembrane polypeptide-anchored proteins by differential solubilization and temperature-induced phase separation in Triton X-114. Biochem.J., 280: 745-751

Houslay, M.D.; Pyne, N.J.; O'Brien, R.M.; Siddle, K.; Strassheim, D.; Palmer, T.; Spence, S.; Woods, M.; Wilson, A.; Lavan, B.; Murphy, G.J.; Saville, M.; McGregor, M.-A.; Kilgour, E.; Anderson, N.; Knowler, J.T.; Griffiths, S. and Milligan, G. (1989) Guanine nucleotide regulatory proteins in insulin's action and in diabetes. Biochem.Soc.Trans., 17: 627-629

Huang, K.-S.; Li, S.; Fung, W.-J.C.; Hulmes, J.D.; Reik, L.; Pan, Y.-C.E. and Low, M.G. (1990) Purification and characterization of glycosyl-phosphatidylinositol-specific phospholipase D. J.Biol.Chem., 265: 17738-17745

Ishihara, M.; Fedarko, N.S. and Conrad, N.E. (1987) Involvement of phosphatidylinositol and insulin in the co-ordinate regulation of proteoheparan sulphate metabolism and hepatocyte growth. J.Biol.Chem., 262: 4708-4716

Itoh, R. (1981) Purification and some properties of cytosol 5'-nucleotidase from rat liver. Biochim.Biophys.Acta, 657: 402-410

Itoh, R. and Tsushima, K. (1972) Changes in 5'-nucleotidase activity in chick liver during development and dietary treatment. Biochim.Biophys.Acta, 273: 229-235

Itoh, R. and Yamada, K. (1991) Determination of cytoplasmic 5'-nucleotidase which preferentially hydrolyses 6-hydroxypurine nucleotides in pig, rat and human tissues by immunotitration. Int.J.Biochem., 23: 461-465

Itoh, R.; Oka, J. and Ozasa, H. (1986) Regulation of rat heart cytosol 5'-nucleotidase by adenylate energy charge. Biochem.J., 235: 847-851

Jamal, Z. and Saggerson, E.D. (1987) Enzymes involved in adenosine metabolism in rat white and brown adipocytes. Biochem.J., 245: 881-886

Jamal, Z. and Saggerson, E.D. (1988) Factors influencing the altered thermogenic response of rat brown adipose tissue in streptozotocin-diabetes. Biochem.J., 249: 415-421

James, S. and Richardson, P.J. (1993) Production of adenosine from extracellular ATP at the striatal cholinergic synapse. J.Neurochem., 60: 219-227

Joost, H.G. and Steinfelder, H.J. (1982) Modulation of insulin sensitivity by adenosine. Effects on glucose transport, lipid synthesis, and insulin receptors of the adipocyte. Mol.Pharmacol., 22: 614-618

Karnieli, E.; Armoni, M.; Cohen, P.; Kanter, Y. and Rafaeloff, R. (1987) Reveral of insulin resistance in diabetic rat adipocytes by insulin therapy. Diabetes, 36: 925-931

Kather, H. (1990) Pathways of purine metabolism in human adipocytes. J.Biol.Chem., 265: 96-102

Kather, H.; Wieland, E.; Fischer, B. and Schlierf, G. (1985) Antilipolytic effects of N^6-phenylisopropyladeno-sine and prostaglandin E_2 in fat-cells of obese volunteers before and during energy restriction. Biochem.J., 231: 531-535

Kather, H.; Weiland, E.; Fischer, B.; Wirth and Schlierf, G. (1985a) Eur.J.Clin.Invest., 15: 30

Kelly, K.L.; Mato, J.M. and Jarett, L. (1986) The polar head group of a novel insulin-sensitive glycophospholipid mimics insulin action on phospholipid methyltransferase. FEBS Lett., 209: 238-242

Kelly, K.L.; Mato, J.M.; Merida, I. and Jarett, L. (1987) Glucose transport and antilipolysis are differentially regulated by the polar head group of an insulin-sensitive glycophospholipid. Proc.Natl.Acad.Sci.USA, 84: 6404-6407

Klemens, M.R.; Sherman, W.R.; Holmberg, N.J.; Ruedi, J.M.; Low, M.G. and Thompson, L.F. (1990) Characteri-zation of soluble vs. membrane-bound huma placental 5'-nucleotidase. Biochem.Biophys.Res.Comm., 172: 1371-1377

Klip, A.; Ramlal, T.; Douen, A.G.; Burdett, E.; Young, D.; Cartee, G.D. and Holloszy, J.O. (1988) Insulin-induced decrease in 5'-nucleotidase activity in skeletal muscle membranes. FEBS Lett., 238: 419-423

Kodukula, K.; Micanovic, R.; Gerber, L.; Tamburrini, M.; Brink, L. and Udenfriend, S. (1991) Biosynthesis of phosphatidylinositol glycan-anchored membrane proteins. J.Biol.Chem., 266: 4464-4470

Kuroda, M.; Honnor, R.C.; Cushman, S.W.; Londos, C. and Simpson, I.A. (1987) Regulation of insulin-stimulated glucose transport in the isolated rat adipocyte. J.Biol.Chem., 262: 245-253

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685

Lai, K.-M. and Wong, P.C.L. (1991) A comparison of the properties of 5'-nucleotidase purified from the cytosolic and synaptic plasma membrane fractions of rat forebrain. Int.J.Biochem., 23: 1123-1130

Lai, K.-M. and Wong, P.C.L. (1991a) Metabolism of extra cellular adenine nucleotides by cultured rat brain astrocytes. J.Neurochem., 57: 1510-1515

Lamphere, L. and Lienhard, G.E. (1992) Components of signaling pathways for insulin and insulin-like growth factor-I in muscle myoblasts and myotubes. Endocrinol., 131: 2196-2202

Larner, J.; Huang, L.C.; Brooker, G.; Murad, F. and Miller, T.B. (1974) Inhibitor of protein kinase formed in insulin treated muscle. Fed.Proc., 33: 261

Larner, J.; Huang, L.C.; Schwartz, C.F.W.; Oswald, A.S.; Shen, T.-Y.; Kinter, M.; Tang, G. and Zeller, K. (1988) Rat liver insulin mediator which stimulates pyruvate dehydrogenase phosphatase contains galactosamine and D-chiroinositol. Biochem.Biophys.Res.Comm., 151: 1416-1426

Lee, Y.P. (1960) Adenylic deaminase. The Enzymes, 4: 279-283

Le Hir, M. and Dubach, U.C. (1988) An ATP-inhibited soluble 5'-nucleotidase of rat kidney. Am.J.Physiol., 254: F191-195

Lewis, G.P. and Matthews, J. (1970) The mechanism of functional vasodilation in rabbit epigastric adipose tissue. J.Physiol., 207: 15-30

Lindberg, B.; Klenow, H. and Hansen, K. (1967) Some properties of partially purified mammalian adenosine kinase. J.Biol.Chem., 242: 350-356

Lindner, F. and Rigler, R. (1931) Über die Beeinflussung der Weita der Herzteranzgefasse durch Produkte des Zellkern stoffwechsels. Pfluegers Arch., 226: 697-708

Lisanti, M.P. and Rodriguez-Boulan, E. (1990) Glycophospholipid membrane anchoring provides clues to the mechanism of protein sorting in polarized epithelial cells. TIBS, 15: 113-118

Lisanti, M.P.; Sargiacomo, M.; Graeve, L.; Saltiel, A.R. and Rodriguez-Boulan, E. (1988) Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. Proc.Natl.Acad.Sci. USA, 85: 9557-9561

Lisanti, M.P.; Darnell, J.C.; Chan, B.L.; Rodriguez-Boulan, E. and Saltiel, A.R. (1989) The distribution of glycosyl-phosphatidylinositol-anchored proteins is differentially regulated by serum and insulin. Biophys.Biochem.Res.Comm., 164: 824-832

Londos, C. and Wolff, J. (1977) Two distinct adenosine-sensitive sites on adenylate cyclase. Proc.Natl.Acad. Sci.USA, 74: 5482-5486

Londos, C.; Cooper, D.M.F. and Wolff, J. (1980) Subclasses of external adenosine receptors. Proc.Natl.Acad.Sci. USA, 77: 2551-2554

Londos, C.; Cooper, D.M.F. and Rodbell, M. (1981) Receptor-mediated stimulation and inhibition of adenylate cyclase: the fat cell as a model system. Adv.Cyclic Nucleotide Res., 14: 163-171

Londos, C.; Honnor, R.C. and Dhillon, G.S. (1985) cAMP-dependent protein kinase and lipolysis in rat adipocytes: 3) Multiple modes of insulin regulation of lipolysis and regulation of insulin responses by adenylate cyclase regulators. J.Biol.Chem., 260: 15139-15145

Lönnroth, P.; Davies, J.I.; Lönnroth, I. and Smith, U. (1987) The interaction between the adenylate cyclase system and insulin-stimulated glucose transport. Biochem.J., 243: 789-795

Low, M.G. (1987) Biochemistry of the glycosyl-phosphatidyl-inositol membrane protein anchors. Biochem.J., 244: 1-13

Low, M.G. and Finean, J.B. (1978) Specific release of plasma membrane enzymes by a phosphatidylinositol-specific phospholipase C. Biochim.Biophys.Acta, 508: 565-570

Low, M.G. and Prasad, A.R.S. (1988) A phospholipase D specific for the phosphatidylinositol anchor of cell-surface proteins is abundant in plasma. Proc.Natl.Acad.Sci.USA, 85: 980-984

Low, M.G. and Saltiel, A.R. (1988) Structural and functional roles of glycosyl-phosphatidylinositol in membranes. Science, 239: 268-275

Lowenstein, E.J.; Daly, R.J.; Batzer, A.G.; Li, W.; Margolis, B.; Lammers, R.; Ullrich, A.; Skolnik, E.Y.; Bar-Sagi, D. and Schlessinger, J. (1992) The SH2 and SH3 domain-containing proteins GRB2 links receptor tyrosine kinases to *ras* signaling. Cell, 70: 431-442

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J.Biol.Chem., 193: 265-275

Lundberg, J.; Hökfelt, T.; Änggård, A.; Terenius, L.; Elde, R.; Markey, K.; Goldstein, M. and Kimmel, J. (1982)

Organizational principles in the peripheral sympathetic nervous system: subdivision by coexisting peptides (somatostatin-, avian pancreatic polypeptide-, and vasoactive intestinal polypeptide-like immunoreactive materials). Proc.Natl.Acad.Sci.USA, 79: 1030-1307

Macaulay, S.L. and Larkins, R.G. (1990) Isolation of insulin-sensitive phosphatidylinositol-glycan from rat adipocytes. Biochem.J., 271: 427-435

Machicao, F.; Mushack, J.; Seffer, E.; Ermel, B. and Häring, H.-U. (1990) Mannose, glucosamine and inositol monophosphate inhibit the effects of insulin on lipogenesis. Biochem.J., 266: 909-916

Madrid-Marina, V. and Fox, I.H. (1986) Human placental cytoplasmic 5'-nucleotidase. J.Biol.Chem., 261: 444-452

Madsen, J. and Malchow-Møller, A. (1983) Effects of glucose, insulin and nicotinic acid on adipose tissue blood flow in rats. Acta Physiol.Scand., 118: 175-180

Maguire, G.A. and Luzio, J.P. (1985) The presence and orientation of ecto 5'-nucleotidase in rat liver lysosomes. FEBS Lett., 180: 122-126

Malbon, C.C. and Gill, D.M. (1979) ADP-ribosylation of membrane proteins and activation of adenylate cyclase by cholera toxin in fat cell ghosts from euthyroid and hypothyroid rats. Biochim.Biophys.Acta, 586: 518-527

Malbon, C.C. and Graziano, M.P. (1983) Adenosine deaminase normalizes cyclic AMP responses of hypothyroid rat fat cells to forskilin but not β-adrenergic agonists. FEBS Lett., 155: 35-38

Malbon, C.C.; Moreno, F.J.; Cabelli, R.J. and Fain, J.N. (1978) Fat cell β-adrenergic receptor in the hypothyroid rat. J.Biol.Chem., 253: 671-678

Malbon, C.C.; Graziano, M.P. and Johnson, G.L. (1984) Fat cell β-adrenergic receptor in the hypothyroid rat. J.Biol.Chem., 259: 3254-3260

Malbon, C.C.; Rapiejko, P.J. and Mangano, T.J. (1985) Fat cell adenylate cyclase system. J.Biol.Chem., 260: 2558-2564

Mallol, J. and Bozal, J. (1983) Modification of 5'-nucleotidase activity by divalent cations and nucleotides. J.Neurochem., 40: 1205-1211

Manganiello, V.C.; Degerman, E.; Smith, C.J.; Vasta, V.; Tornqvist, H. and Belfrage, P. (1992) Mechanisms for activation of the rat adipocyte particulate cyclic-GMP-

inhibited cyclic AMP phosphodiesterase and its importance in the antilipolytic action of insulin. Adv.Sec.Messenger & Phosphoprotein Res., 25: 147-164

Mato, J.M.; Kelly, K.L.; Abler, A. and Jarett, L. (1987) Identification of a novel insulin-sensitive glycophospholipid from H35 hepatoma cells. J.Biol.Cheml., 262: 2131-2137

Mato, J.M.; Kelly, K.L.; Abler, A.; Jarett, L.; Corkey, B.E.; Cashel, J.A. and Zopf, D. (1987a) Partial structure of an insulin-sensitive glycophospho-lipid. Biochem. Biophys.Res.Comm., 146: 764-770

Mazurkiewicz, D. and Saggerson, E.D. (1989) Changes in the activities of adenosine-metabolizing enzymes in six regions of the rat brain on chemical induction of hypothyroidism. Biochem.J., 261: 667-672

Meghji, P.; Middleton, K.M. and Newby, A.C. (1988) Absolute rates of adenosine formation during ischaemia in rat and pigeon hearts. Biochem.J., 249: 695-703

Mentzer, R.M.; Rubio, R. and Berne, R.M. (1975) Release of adenosine by hypoxic canine lung tissue and its possible role in pulmonary circulation. Am.J.Physiol., 229: 1625-1631

Micanovic, R.; Kodukula, K.; Gerber, L.D. and Udenfriend, S. (1990) Selectivity at the cleavage/attachment site of phosphatidylinositol-glycan anchored membrane proteins is enzymatically determined. Proc.Natl. Acad.Sci.USA, 87: 7939-7943

Miller, R.L.; Adamczyk, D.L.; Miller, W.H.; Koszalka, G.W.; Rideout, J.L.; Beacham III, L.M.; Chao, E.Y.; Haggerty, J.J.; Krenitsky, T.A. and Elion, G.B. (1979) Adenosine kinase from rabbit liver. II. Substrate and inhibitor specificity. J.Biol.Chem., 254: 2346-2352

Milligan, G. and Saggerson, E.D. (1990) Concurrent up-regulation of guanine-nucleotide-binding proteins $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ in adipocytes of hypothyroid rats. Biochem.J., 270: 765-769

Milligan, G.; Spiegel, A.M.; Unson, C.G. and Saggerson, E.D. (1987) Chemically induced hypothyroidism produces elevated amounts of the α subunit of the inhibitory guanine nucleotide binding protein (G_i) and the β subunit common to all G-proteins. Biochem.J., 247: 223-227

Mills, I.; Garcia-Sainz, J.A. and Fain, J.N. (1986) Pertussis toxin effects on adenylate cyclase activity, cyclic AMP accumulation and lipolysis in adipocytes from hypothyroid, euthyroid and hyperthyroid rats. Biochim.Biophys.

Acta, 876: 619-630

Misumi, Y.; Ogata, S.; Hirose, S. and Ikehara, Y. (1990) Primary structure of rat liver 5'-nucleotidase deduced from cDNA. J.Biol.Chem., 265: 2178-2183

Misumi, Y.; Ogata, S.; Ohkubo, K.; Hirose, S. and Ikehara, Y. (1990a) Primary structure of human placental 5'-nucleotidase and identification of the glycolipid anchor in the mature form. Eur.J.Biochem., 191: 563-569

Montero, J.M. and Fes, J.B. (1982) Purification and characterization of bovine brain 5'-nucleotidase. J.Neurochem., 39: 982-989

Moran, P.; Raab, H.; Kohr, W.J. and Caras, I.W. (1991) Glycolipid membrane anchor attachment. J.Biol.Chem., 266: 1250-1257

Moreno, F.J.; Mills, I.; Garcia-Sainz, J.A. and Fain, J.N. (1983) Effects of pertussis toxin treatment on the metabolism of rat adipocytes. J.Biol.Chem., 258: 10938-10943

Myers, M.G. Jr. and White, M.F. (1993) The new elements of insulin signaling: insulin receptor substrate-1 and proteins with SH2 domains. Diabetes, 42: 643-650

Myers, M.G. Jr.; Backer, J.M.; Siddle, K. and White, M.F. (1991) The insulin receptor functions normally in Chinese hamster ovary cells after truncation of the C-terminus. J.Biol.Chem., 266: 10616-10623

Myers, M.G. Jr.; Sun, X.J.; Cheatham, B.; Jachna, B.R.; Glasheen, E.M.; Backer, J.M. and White, M.F. (1993) IRS-1 is a common element in insulin and insulin-like growth factor-I signaling to the phosphatidylinositol 3'-kinase. Endocrinol., 132: 1421-1430

Naito, Y. and Lowenstein, J.M. (1981) 5'-Nucleotidase from rat heart. Biochemistry, 20: 5188-5194

Naito, Y. and Tsushima, K. (1976) Cytosol 5'-nucleotidase from chicken liver purification and some properties. Biochim.Biophys.Acta, 438: 159-168

Newby, A.C. (1988) The pigeon heart 5'-nucleotidase responsible for ischaemia-induced adenosine formation. Biochem.J., 253: 123-130

Newby, A.C.; Luzio, J.P. and Hales, C.N. (1975) The properties and extracellular location of 5'-nucleotidase of the rat fat-cell plasma membrane. Biochem.J., 146: 625-633

Newby, A.C.; Worku, Y. and Meghji, P. (1987) Critical evalua-

tion of the role of ecto- and cytosolic 5'-nucleotidase in adenosine formation. In: E.Gerlach and B.F.Becker (eds.) Topics and Perspectives in Adenosine Research (Springer-Verlag), pp.155-169

Nielsen, S.L.; Bitsch, V.; Larsen, O.A.; Lassen, N.A. and Quaade, P. (1968) Blood flow through human adipose tissue during lipolysis. Scand.J.Clin.Lab. Invest., 22: 124-130

Nikiforuk, G. and Colowick, S.P. (1956) The purification and properties of 5-adenylic acid deaminase from muscle. J.Biol.Chem., 219: 119-129

Ogata, S.; Hayashi, Y.; Misumi, Y. and Ikehara, Y. (1990) Membrane anchoring domain of rat liver 5'-nucleotidase: identification of the COOH-terminal serine-523 covalently attached with a glycolipid. Biochemistry, 29: 7923-7927

Ohisalo, J.J. and Stouffer, J.E. (1979) Adenosine, thyroid status and regulation of lipolysis. Biochem.J., 178: 249-251

Ohisalo, J.J.; Strandberg, H.; Kostiaineu, E.; Kuusi, T. and Ehuholm, C. (1981) Stimulation of lipoprotein lipase activity of rat adipose tissue and post-heparin plasma by N^6-(phenylisopropyl)adenosine. FEBS Letts., 132: 121-123

Ohisalo, J.J.; Ranta, S. and Huhtaniemi, I.T. (1986) Attenuated adenosine R-site effect in adipocytes in obesity. Metabolism, 35: 143-146

Ohisalo, J.J.; Stoneham, S. and Keso, L. (1987) Thyroid status and adenosine content of adipose tissue. Biochem.J., 246: 555-557

Olansky, L.; Myers, G.A.; Pohl, S.L. and Hewlett, E.L. (1983) Promotion of lipolysis in rat adipocytes by pertussis toxin: reversal of endogenous inhibition. Proc.Natl. Acad.Sci.USA, 80: 6547-6551

Olsson, R.A. and Pearson, J.D. (1990) Cardiovascular purinoceptors. Physiol.Rev., 70: 761-845

Olsson, R.A.; Snow, J.A.; Gentry, M.K. and Frick, G.P. (1972) Adenosine uptake by canine heart. Circ.Res., 31: 767-778

Orford, M.; Mazurkiewicz, D. and Saggerson, D. (1991) Soluble 5'-nucleotidase activities in rat brain. J.Neurochem., 56: 141-146

Panagia, V.; Heyliger, C.E.; Choy, P.C. and Dhalla, N.S. (1981) Solubilization of rat heart sarcolemma 5'-

nucleotidase by phosphatidylinositol-specific phospholipase C. Biochim.Biophys.Acta, 640: 802-806

Pearson, J.D. and Coade, S.B. (1987) Kinetics of endothelial cell ectonucleotidase. In: E. Gerlach and B.F. Becker (eds.) Topics and Perspectives in Adenosine Research: Proc. 3rd Intl. Symp. on Adenosine, Munich, June 1986. (Springer-Verlag), pp.145-154

Peeters, C.; De Wolf, M.; Van Dessel, G.; Lagrou, A.; Hilderson, H. and Dierick, W. (1988) Topography, purification and characterization of thyroidal 5'-nucleotidase. Int.J.Biochem., 20: 409-419

Piec, G. and Le Hir, M. (1991) The soluble 'low-K_m' 5'-nucleotidase of rat kidney represents solubilized ecto-5'-nucleotidase. Biochem.J., 273: 409-413

Plagemann, P.G.W. and Wohlheuter, R.M. (1983) Nucleoside transport in mammalian cells and interaction with intracellular metabolism. In: R.M. Berne, R.W. Rall, and R. Rubio (eds.) Regulatory Function of Adenosine. (Nijhoff, The Hague), pp.179-201

Pryde, J.G. and Phillips, J.H. (1986) Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114. Biochem.J., 233: 523-533

Pull, I. and McIlwain, H. (1972) Adenine derivatives as neurohumoral agents in the brain. Biochem.J., 130: 975-981

Rapiejko, P.J. and Malbon, C.C. (1987) Short-term hyperthyroidism modulates adenosine receptors and catalytic activity of adenylate cyclase in adipocytes. Biochem.J., 241: 765-771

Rapiejko, P.J.; Watkins, D.C.; Ros, M. and Malbon, C.C. (1989) Thyroid hormones regulate G-protein β-subunit mRNA expression *in vivo*. J.Biol.Chem., 264: 16183-16189

Reddington, M. and Pusch, R. (1983) Adenosine metabolism in a rat hippocampal slice preparation: incorporation into S-adenosylhomocysteine. J.Neurochem., 40: 285-290

Richardson, P.J.; Brown, S.J.; Bailyes, E.M. and Luzio, J.P. (1987) Ectoenzymes control adenosine modulation of immunoisolated cholinergic synapses. Nature, 327: 232-234

Riemer, B.L. and Widnell, C.C. (1975) The demonstration of a specific 5'-nucleotidase activity in rat tissues. Arch.Biochem.Biophys., 171: 343-347

Roberts, W.L.; Myher, J.J.; Kuksis, A.; Low, M.G. and Rosenberry, T.L. (1988) Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. J.Biol. Chem., 263: 18766-18775

Rodbell, M. (1964) Metabolism of isolated fat cells. J.Biol. Chem., 239: 375-380

Rökeus, Å.; Tatemoto, K. and Mutt, V. (1983) Vascular actions of peptide YY(PYY) - a new putative gut hormone. Regulatory Peptides, suppl.2: 172

Romero, G.; Luttrell, L. Rogol, A.; Zeller, K.; Hewlett, E. and Larner, J. (1988) Phosphatidylinositol-glycan anchors of membrane proteins: potential precursors of insulin mediators. Science, 240: 509-511

Romero, G.; Gamez, G.; Huang, L.C.; Lilley, K. and Luttrell, L. (1990) Anti-inositolglycan antibodies selectively block some of the actions of insulin in intact BC_3H1 cells. Proc.Natl.Acad.Sci.USA, 87: 1476-1480

Ros, M.; Northup, J.K. and Malbon, C.C. (1988) Steady-state levels of G-proteins and β-adrenergic receptors in rat fat cells. J.Biol.Chem., 263: 4362-4368

Rosell, S. and Belfrage, E. (1979) Blood circulation in adipose tissue. Physiol.Rev., 59: 1078-1104

Rubio, R.; Berne, R.M. and Dobson, J.G. (1973) Sites of adenosine production in cardiac and skeletal muscle. Am.J.Physiol., 225: 938-953

Saggerson, E.D. (1980) Increased antilipolytic effect of the adenosine 'R-site' agonist N^6-(phenylisopropyl)-adenosine in adipocytes for adrenalectomized rats. FEBS Letts., 115: 127-133

Saggerson, E.D. (1986) Sensitivity of adipocyte lipolysis to stimulatory and inhibitory agonists in hyper-thyroidism and starvation. Biochem.J., 238: 387-394

Saggerson, E.D. (1992) Thyroid disorders. In: G. Milligan & M. Wakelam (eds.) G Proteins Signal Transduction and Disease (Academic Press), pp.157-190

Saggerson, E.D. and Carpenter, C.A. (1986) Carnitine palmitoyltransferase in liver and five extrahepatic tissues in the rat. Biochem.J., 236: 137-141

Saggerson, E.D. and Carpenter, C.A. (1987) Effects of streptozotocin-diabetes and insulin administration *in vivo* or *in vitro* on the activities of five enzymes in the adipose-tissue triacylglycerol-synthesis pathway.

Biochem.J., 243: 289-292

Saggerson, E.D. and Greenbaum A.L. (1970) The regulation of triglyceride synthesis and fatty acid synthesis in rat epididymal adipose tissue. Biochem.J., 119: 193-219

Saggerson, E.D.; Orford, M.; Chatzipanteli, K. and Shepherd, J. (1991) Diabetes decreases sensitivity of adipocyte lipolysis to inhibition by G_i-linked receptor agonists. Cellular Signalling, 3: 613-624

Saltiel, A.R. (1987) Insulin generates an enzyme modulator from hepatic plasma membranes: regulation of adenosine 3',5'-monophosphate phosphodiesterase, pyruvate dehydrogenase, and adenylate cyclase. Endocrinology, 120: 967-972

Saltiel, A.R. and Cuatrecasas, P. (1986) Insulin stimulates the generation from hepatic plasma membranes of modulators derived from an inositol glycolipid. Proc.Natl. Acad.Sci.USA, 83: 5793-5797

Saltiel, A.R. and Sorbara-Cazan, L.R. (1987) Inositol glycan mimics the action of insulin on glucose utilization in rat adipocytes. Biochem.Biophys.Res.Comm., 149: 1084-1092

Saltiel, A.R.; Fox, J.A.; Sherline, P. and Cuatrecasas, P. (1986) Insulin-stimulated hydrolysis of a novel glycolipid generates modulators of cAMP phosphodiesterase. Science, 233: 967-972

Sattin, A. and Rall, T.W. (1970) The effect of adenosine and adenine nucleotides on the cyclic adenosine 3'5'-phosphate content of guinea pig cerebral cortex slices. Mol.Pharmacol., 6: 13-23

Schrader, W.P. and Bryer, P.J. (1982) Characterization of an insoluble adenosine deaminase complexing protein from human kidney. Arch.Biochem.Biophys., 215: 107-115

Schrader, W.P. and Stacy, A.R. (1977) Purification and subunit structure of adenosine deaminase from human kidney. J.Biol.Chem., 252: 6409-6415

Schrader, J.; Schutz, W. and Bardenheuer, H. (1981) Role of S-adenosylhomocysteine hydrolase in adenosine metabolism in mammalian heart. Biochem.J., 196: 65-70

Schwabe, U.; Ebert, R. and Erbler, H.C. (1973) Adenosine release from isolated fat cells and its signifi-cance for the effects of hormones on cyclic 3'5'-AMP levels and lipolysis. Naunyn-Schmeideberg's Arch.Pharmacol., 276: 133-148

Schwabe, U.; Schonhofer, P.S. and Ebert, R. (1974) Facilita-

tion by adenosine of the action of insulin on the accumulation of adensoine 3':5'-monophosphate, lipolysis and glucose oxidation in isolated fat cells. Eur.J.Biochem., 46: 537-545

Schwabe, U.; Ebert, R. and Erbler, H.C. (1975) Adenosine release from fat cells: effect on cyclic AMP levels and hormone actions. Adv.Cyclic Nucleotide Res., 5: 569-584

Setlow, B. and Lowenstein, J.M. (1967) Adenylate deaminase. II. Purification and some regulatory properties of the enzyme from calf brain. J.Biol.Chem., 242: 607-615

Sheridan, J.D. (1971) Electrical coupling between fat cells in newt body and mouse brown fat. J.Cell Biol., 50: 795-803

Shoelson, S.E.; Sivaraja, M.; Williams, K.P.; Hu, P.; Schlessinger, J. and Weiss, M.A. (1993) Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation. EMBO J., 12: 795-802

Shukla, S.D.; Coleman, R.; Finean, J.B. and Michell, R.H. (1980) Selective release of plasma-membrane enzymes from rat hepatocytes by a phosphatidyl-inositol-specific phospholipase C. Biochem.J., 187: 277-280

Singh, B.M.; Palma, M.A. and Nattrass, M. (1987) Multiple aspects of insulin resistance: Comparison of glucose and intermediary metabolite response to incremental insulin infusion in IDDM subjects of short and long duration. Diabetes, 36: 740-748

Skladanowski, A. and Newby, A.C. (1990) Partial purification and properties of an AMP-specific soluble 5'-nucleotidase from pigeon heart. Biochem.J., 268: 117-122

Skolnik, E.Y.; Batzer, A.; Li, N.; Lee, C.-H.; Lowenstein, E.; Mohammadi, M.; Margolis, B. and Schlessinger, J. (1993) The function of GRB2 in linking the insulin receptor to ras signaling pathways. Science, 260: 1953-1955

Slavin, B.G. (1985) The morphology of adipose tissue. In: A.Cryer and R.L.R.Van (Eds) New Perspectives in Adipose Tissue: structure, function and development (Butterworth & Co. Ltd.), pp.23-43

Slavin, B.G. and Ballard, K. (1978) Morphological studies on the adrenergic innervation of white adipose tissue. Anat.Rec., 191: 377-390

Smith, U.; Kuroda, M. and Simpson, I.A. (1984) Counter-regulation of insulin-stimulated glucose transport by catecholamines in the isolated rat adipose cell. J.Biol.

Chem., 259: 8758-8763

Smith, C.J.; Vasta, V.; Degerman, E.; Belfrage, P. and Manganiello, V.C. (1991) Hormone-sensitive cyclic GMP-inhibited cyclic AMP phosphodiesterase in rat adipocytes. J.Biol.Chem., 266: 13385-13390

Sollevi, A. and Fredholm, B.B. (1981) Role of adenosine in adipose tissue circulation. Acta Physiol.Scand., 112: 293-298

Spychala, J.; Madrid-Marina, V. and Fox, I.H. (1988) High K_m soluble 5'-nucleotidase from human placenta. J.Biol. Chem., 263: 18759-18765

Spychala, J.; Madrid-Marina, V.; Nowak, P.J. and Fox, I.H. (1989) AMP and IMP dephosphorylation by soluble high- and low-K_m 5'-nucleotidase. Am.J.Physiol., 256: E386-E391

Stanley, K.K.; Edwards, M.R. and Luzio, J.P. (1980) Subcellular distribution and movement of 5'Nase in rat cells. Biochem.J., 186: 59-69

Stochaj, U.; Flocke, K.; Mathes, W. and Mannherz, H.G. (1989) 5'-Nucleotidases of chicken gizzard and human pancreatic adenocarcinoma cells are anchored to the plasma membrane via a phosphatidylinositol-glycan. Biochem.J., 262: 33-40

Stochaj, U.; Dieckhoff, J.; Mollenhauer, J.; Cramer, M. and Mannherz, H.G. (1989a) Evidence for the direct interaction of chicken gizzard 5'-nucleotidase with laminin and fibronectin. Biochim.Biophys. Acta, 992: 385-392

Strålfors, P. and Alemany, S. (1990) Phosphorylation control by insulin in adipocytes is interfered with at a post-receptor step by phosphoinositol and glucosamine. FEBS Lett., 268: 169-172

Strålfors, P. and Honnor, R.C. (1989) Insulin-induced dephosphorylation of hormone-sensitive lipase. Eur.J.Biochem. 182: 379-385

Strassheim, D.; Milligan, G. and Houslay, M.D. (1990) Diabetes abolishes the GTP-dependent, but not the receptor-dependent inhibitory function of the inhibitory guanine-nucleotide-binding regulatory protein (G_i) on adipocyte adenylate cyclase activity. Biochem.J., 266: 521-526

Sugden, M.C.; Steare, S.E.; Watts, D.I. and Palmer, T.N. (1983) Biochem.J., 210: 937-944

Suhail, M. and Rizvi, S.I. (1989) Erythrocyte membrane acetylcholinesterase in type 1 (insulin-dependent)

diabetes mellitus. Biochem.J., 259: 897-899

Sun, X.J.; Rothenberg, P.; Kahn, C.R.; Backer, J.M.; Araki, E.; Wilden, P.A.; Cahill, C.A.; Goldstein, B.J. and White, M.F. (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature, 352: 73-77

Sun, X.J.; Miralpeix, M.; Myers, M.G. Jr.; Glasheen, E.M.; Backer, J.M.; Kahn, C.R. and White, M.F. (1992) Expression and function of IRS-1 in Insulin Signal Transmission. J.Biol.Chem., 267: 22662-22672

Sung, C.K. and Goldfine, I.D. (1992) Phosphatidylinositol-3-kinase is a non-tyrosine phosphorylated member of the insulin receptor signalling complex. Biochem.Biophys. Res.Comm., 189: 1024-1030

Suzuki, S.; Toyota, T.; Tamura, S.; Kikuchi, K.; Tsuiki, S.; Huang, L.; Villar-Palasi, C.; Larner, J. and Goto, Y. (1987) ATP-Mn^{2+} stimulates the generation of a putative mediator of insulin action. J.Biol.Chem., 262: 3199-3204

Suzuki, K.; Furukawa, Y.; Tamura, H.-O.; Ejiri, N.; Suematsu, H.; Taguchi, R.; Nakamura, S.; Suzuki, Y. and Ikezawa, H. (1993) Purification and cDNA of bovine liver 5'-nucleotidase, a GPI-anchored protein, and its expression in COS cells. J.Biochem., 113: 607-613

Switzer, B.R. and Summer, G.K. (1971) A modified fluorometric micromethod for DNA. Clin.Chim.Acta., 32: 203-206

Tanaka, Y.; Himeno, M.; Taguchi, R.; Ikezawa, H. and Kato, K. (1989) Cell.Struct.Funct., 14: 597-603

Thakkar, J.K.; Raju, M.S.; Kennington, A.S.; Foil, B. and Caro, J.F. (1990) [^{3}H]Myoinositol incorporation into phospholipids in liver microsomes from humans with and without type II diabetes. J.Biol.Chem., 265: 5475-5481

Thannhauser, S.J. and Bommes, A. (1914) Experimentelle Studies Ober den Nucleinstoffwechsel. II. Mitteilung. Stoffwechsel-versuche mit Adenosin und Guanosine. Z.Physiol.Chem., 9: 336-343

Thompson, L.F. (1991) 5'Nucleotidase - an overview of the last three years. In: R.A.Harkness *et al* (eds.) Purine and Pyrimidine Metabolism in Man, Vol.VII, Part B (Plenum Press, N.Y.), pp.145-150

Thompson, L.F.; Ruedi, J.M. and Low, M.G. (1987) Purification of 5'-nucleotidase from human placenta after release from plasma membranes by phosphatidyl-inositol-specific phospholipase C. Biochem.Biophys.Res.Comm., 145: 118-125

Thompson, L.F.; Ruedi, J.M.; Glass, A.; Low, M.G. and Lucas, A.H. (1989) Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. J.Immunol., 143: 1815-1821

Toutant, J.-P.; Roberts, W.L.; Murray, N.R. and Rosenberry, T.L. (1989) Conversion of human erythrocyte acetylcholinesterase from an amphiphilic to a hydrophilic form by phosphatidylinositol-specific phospholipase C and serum phospholipase D. Eur.J.Biochem., 180: 503-508

Towbin, H.; Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc.Natl.Acad.Sci.USA, 76: 4350-4354

Trost, T. and Stock, K. (1979) ??? In: H.P. Baer and E.I. Drummond (eds.) Physiological and Regulatory Functions of Adenosine and Adenosine Nucleotides (Raven Press, N.Y.), pp.379-393

Trotta, P.P. (1982) Identification of a membrane adenosine deaminase binding protein from human placenta. Biochemistry, 21: 4014-4023

Truong, V.L.; Collinson, A.R. and Lowenstein, J.M. (1988) 5'-Nucleotidases in rat heart. Biochem.J., 253: 117-121

Tsushima, K. (1986) Properties of cytosol 5'-nucleotidase and its role in purine nucleotide metabolism. Adv.Enzyme Regul., 25: 181-200

Ueland, P.M. (1982) Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomo-cysteine hydrolase. Pharmacol.Rev., 34: 223-253

Ullrich, A.; Bell, J.R.; Chen, E.Y.; Herrera, R.; Petruzzelli, L.M.; Dull, T.J.; Gray, A.; Coussens, L.; Liao, Y.-C.; Tsubokawa, M.; Mason, A.; Seeburg, P.H.; Grunfeld, C.; Rosen, O.M. and Ramachandran, J. (1985) Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature, 313: 756-761

Van Calker, D.; Muller, M. and Hamprecht, B. (1979) Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J.Neurochem., 33: 999-1005

Van den Berghe, G.; Van Pottelsberghe, C. and Hers, H.-G. (1977) A kinetic study of the soluble 5'-nucleoti-dase of rat liver. Biochem.J., 162: 611-616

Van den Bosch, R.; Geuze, H.J.; Du Maine, A.P.M. and Strous,

G.J. (1986) Transport and metabolism of 5'-nucleotidase in a rat hepatoma cell line. Eur.J.Biochem., 160: 49-54

Van den Bosch, R.; Du Maine, A.P.M.; Geuze, H.J.; Van der Ende, A. and Strous, G.J. (1988) Recycling of 5'-nucleotidase in a rat hepatoma cell line. EMBO J., 7: 3345-3351

Varela, I.; Alvarez, J.F.; Clemente, R.; Ruiz-Albusac, J.M. and Mato, J.M. (1990) Asymmetric distribution of the phosphatidylinositol-linked phospho-oligo-saccharide that mimics insulin action in the plasma membrane. Eur. J.Biochem., 188: 213-218

Vernon, R.G. and Finley, E. (1985) Regulation of lipolysis during pregnancy and lactation in sheep: response to noradrenaline and adenosine. Biochem.J., 230: 651-656

Vernon, R.G., Finley, E. and Taylor, E. (1983) Adenosine and the control of lipolysis in rat adipocytes during pregnancy and lactation. Biochem.J., 216: 121-128

Wada, I.; Himeno, M.; Furuno, K. and Kato, K. (1986) Biosynthesis and intracellular transport of rat liver 5'-nucleotidase. J.Biol.Chem., 261: 2222-2227

Wada, I.; Eto, S.; Himeno, M. and Kato, K. (1987) 5'-Nucleotidase in rat liver lysosomes. J.Biochem., 101: 1077-1085

White, M.F. and Kahn, C.R. (1989) The cascade of autophosphorylation in the β-subunit of the insulin receptor. J.Cell Biochem., 39: 429-441

White, M.F.; Maron, R. and Kahn, C.R. (1985) Insulin rapidly stimulates tyrosine phosphorylation of an M_r 185,000 protein in intact cells. Nature, 318: 183-186

White, M.F.; Stegmann, E.W.; Dull, T.J.; Ullrich, A. and Kahn, C.R. (1987) Characterization of an endogenous substrate of the insulin receptor in cultured cells. J.Biol.Chem., 262: 9769-9777

White, M.F.; Shoelson, S.E.; Keutmann, H. and Kahn, C.R. (1988) A cascade of tyrosine autophosphorylation in the β-subunit activates the insulin receptor. J.Biol.Chem., 263: 2969-2980

Widnell, C.C. (1974) Purification of rat liver 5'-nucleotidase as a complex with sphingomyelin. Methods Enzymol., 32(B): 368-374

Widnell, C.C.; Schneider, Y.-J.; Pierre, B.; Baudhuin, P. and Trouet, A. (1982) Evidence for a continual exchange of 5'-nucleotidase between the cell surface and cytoplas-

mic membranes in clutured rat fibroblasts. Cell, 28: 61-70

Wong, E.H.-A.; Smith, J.A. and Jarett, L. (1984) Effect of adenosine on insulin activation of rat adipocyte pyruvate dehydrogenase. FEBS Lett., 175: 68-71

Wong, E.H.-A.; Ooi, S.O.; Loten, E.G. and Sneyd, J.G.T. (1985) The action of adenosine in relation to that of insulin on the low-K_m cyclic AMP phospho-diesterase in rat adipocytes. Biochem.J., 227: 815-821

Worku, Y. and Newby, A.C. (1983) The mechanism of adenosine production in rat polymorphonuclear leucocytes. Biochem.J., 214: 325-330

Worku, Y.; Luzio, J.P. and Newby, A.C. (1984) Identification of histidyl and cysteinyl residues essential for catalysis by 5'-nucleotidase. FEBS Lett., 167: 235-240

Yamazaki, Y.; Truong, V.L. and Lownstein, J.M. (1991) 5'-Nucleotidase I from rabbit heart. Biochemistry, 30: 1503-1509

Zachowski, A.; Evans, W.H. and Paraf, A. (1981) Immunological evidence that plasma-membrane 5'-nucleotidase is a transmembrane protein. Biochim.Biophys.Acta, 644: 121-126

Zekri, M.; Harb, J.; Bernard, S. and Meflah, K. (1988) Purification of bovine liver cytosolic 5'-nucleotidase. Eur.J.Biochem., 172: 93-99

Zekri, M.; Harb, J.; Bernard, S.; Poirier, G.; Devaux, C. and Meflah, K. (1989) Differences in the release of 5'-nucleotidase and alkaline phosphatase from plasma membrane of several cell types by PI-PLC. Comp.Biochem.Physiol., 93B: 673-679

Zimmermann, H. (1992) 5'-Nucleotidase: molecular structure and functional aspects. Biochem.J., 285: 345-365