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Multiple isozymes of cyclic nucleotide phosphodiesterases in the rat.

A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy.

by Brian Edward Lavan.

November 1990.

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Dedicated to my Mother and Father with all my love.

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SUMMARY

Cyclic nucleotide phosphodiesterases catalyse the hydrolysis of 3'5'-cyclic nucleotides, such as cyclic AMP and cyclic GMP, to produce 5'-derivatives. Multiple isozymes of this enzyme exist that differ in their substrate specificities, means of regulation and subcellular localisation. The soluble phosphodiesterases present in homogenates of both rat liver and rat hepatocytes have beed identified and characterised. Using a high resolution anion-exchange system (Mono Q) to rapidly separate soluble phosphodiesterases, it has been shown that five separate activities are present in both of these soluble fractions. On this basis, it was concluded that the multiplicity of phosphodiesterase species in liver is not due to the presence of multiple cell types in this organ. These soluble activities were termed PDE MQ-I to PDE MQ-V and were distinguishable from one another by a number of criteria. These included substrate specificity, kinetic properties, sensitivity to Ca^{2+}/Calmodulin or cyclic GMP, dependency on magnesium, sensitivity to a number of reference phosphodiesterase inhibitors, molecular weights and chromatographic properties.

A novel phosphodiesterase activity, PDE MQ-I, was found to be present in both the soluble fractions of rat liver and hepatocytes. This activity was found to be unable to bind to Affi-gel Blue, was markedly insensitive to all of the phosphodiesterase inhibitors tested, including the non-selective inhibitor IBMX, expressed nearly maximal cyclic nucleotide hydrolysis in the absence of added magnesium, unlike the other soluble activities, and formed a substantial proportion of the cyclic AMP phosphodiesterase activity in both rat liver and rat hepatocyte soluble fractions. It was also observed to be insensitive to Ca^{2+}/Calmodulin and to cyclic GMP.

The Ca^{2+}/Calmodulin-stimulated phosphodiesterase (PDE MQ-II) was shown to be present in both liver and hepatocytes and exhibited a marked selectivity for cyclic GMP as substrate. Calmodulin was found

to stimulate this activity only in the presence of Ca^{2+}, where up to 2.5 fold stimulation of cyclic GMP and 5 fold stimulation of cyclic AMP hydrolysis could be obtained.

The cyclic GMP-stimulated phosphodiesterase (PDE MQ-III) was once again observed in both liver and hepatocytes. In liver, the cyclic AMP hydrolysis of this activity formed the majority of the total cyclic AMP phosphodiesterase activity. In hepatocytes, this was not the case, since the IBMX insensitive activity was more predominant. At cyclic GMP concentrations up to 10 μM. cyclic AMP phosphodiesterase activity was stimulated, up to 9-fold, higher concentrations were found to be inhibitory.

The cyclic AMP-specific activity present in the rat liver soluble fraction was shown to actually consist of two activities, PDE MQ-IV and PDE MQ-V. These were shown to be highly selective for cyclic AMP as substrate and were insensitive to inhibition by micromolar levels of cyclic GMP, but sensitive to inhibition by ICI 63197 and Rolipram, which distinguishes them from the cyclic GMP-inhibited cyclic AMP-specific phosphodiesterases that have been observed in a number of tissues, including rat liver. Together these activities formed a substantial proportion of the total soluble cyclic AMP phosphodiesterase activity in both liver and hepatocytes.

Two membrane bound cyclic AMP-specific phosphodiesterases that are stimulated by hormonses such as insulin and glucagon in rat liver and hepatocytes are termed the 'dense-vesicle' and 'peripheral' plasma membrane phosphodiesterases. Using specific antisera raised to purified preparations of these phosphodiesterases the tissue distribution of immunologically related activities was assessed by quantitative immunoblotting. A species of molecular weight 63-kDa corresponding to the presumed subunit molecular weight of the native species, was identified by the antisera DV-1 (anti-'dense-vesicle' PDE) in all of the rat tissues examined (liver, fat, kidney and heart). In contrast the anitsera PM-1 (anti-'peripheral' PDE) detected a species of 52-kDa,

corresponding to the subunit molecular weight of the purified liver enzyme, in all of these tissues. It was observed that there was a differential expression of these two phosphodiesterases in these tissues. Heart and fat exhibited a predominant expression of the 'dense–vesicle' phosphodiesterase compared to liver. Expression of the phosphodiesterase activity of this species in tissue homogenates was assesssed using the inhibitor ICI 118233. This was shown to be a reversible competitive inhibitor of cyclic AMP hydrolysis and was shown to be selective for inhibition of the 'dense–vesicle' phosphodiesterase. A similar pattern of distribution of the 'dense–vesicle' PDE was obtained using this assessment of activity to that obtained by Western blotting. The 'peripheral' phosphodiesterase showed a different pattern of expression to that seen with the 'dense–vesicle' phosphodiesterase, with highest comparative levels observed in liver and the lowest in heart and white adipose tissue. The differential tissue expression of the 'peripheral' PDE was not as great as that observed for the 'dense–vesicle' phosphodiesterase.

ABBREVIATIONS.

NOTE: In some instances calmodulin has been spelt as Calmodulin. This should not be taken to imply that this represents a trade name.

Amrinone: 5-amino-3,4'-bipyridin-6(1H)-one.

ADP: Adenosine 5' diphosphate.

A-Kinase: cyclic AMP-dependent protein kinase.

ATP: Adenosine 5' triphosphate.

Cilostamide (OPC-3689): *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butyramide.

CI-914: 4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-3(2*H*)-pryidazinone.

CI-930: 4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-5-methyl-3(2*H*)-pryidazinone.

CaM: Calmodulin.

cAMP: 3'5' cyclic adenosine monophospate.

cCMP: 3'5' cyclic cytidine monophosphate.

CGI-PDE: cyclic GMP-inhibited cyclic AMP phosphodiesterase.

cGMP: 3'5' cyclic guanosine monophosphate.

CGS-PDE: cyclic GMP-stimulated cyclic nucleotide phosphodiesterase.

DE-52: DEAE-52 Cellulose.

DMSO: Dimethysulphoxide.

DPD: Rat brain *Drosophila* like phosphodiesterase.

DV-1: Antisera raised against the purified rat liver 'dense-vesicle' phosphodiesterase.

DV-PDE: 'Dense-vesicle' high affinity cyclic AMP phosphodiesterase.

ECTEOLA – cellulose: Epichlorohydrin Triethanolamine cellulose.

EDTA: ethylene diamine tetra acetic acid.

EGTA: ethyleneglycolbis (β – aminoethylether) – *N,N,N'N'* – tetra acetic acid.

Fenoximone (Enoximone or MDL 17,043): (1,3 – dihydro – 4 – methyl – 5 – [4 – methylthio) – benzoyl] – 2*H* – imidazol – 2 – one.

IBMX: 3 – isobutyl – 1 – methylxanthine.

ICI 118233: 6 – [*p* – (3 – methylureido) phenyl] – 3[2H] – pyridazinone.

ICI 58,301: 3 – acetamido – 6 – methyl – 8 – n – propyl – s – triazalo [4,3 – a] pyrazine.

ICI 63,197: 2 – amino – 6 – methyl – 5 – oxo – 4 – n – propyl – 4,5 – dihydro – s – triazalo [1,5 – a] pyrimidine.

ICI 74,917: 6 – *n* – butyl – 2,8 – dicarboxy – 4,10 – dioxo – 1,4,7,10 – tetrahydro – 1,7 – phenanthroline.

IIX: 1 – isoamyl – 3 – isobutylxanthine.

kDa: Kilodalton

8 – MeOMIX: 1 – methyl – 3 – isobutyl – 8 – methoxymethylxanthine.

Milrinone: 1,6 – dihydro – 2 – methyl – 6 – oxo – [3,4' – bipyridine] – 5 – carbonitrile.

MIX: see IBMX.

MQ: Mono Q.

MY 5445: 1 – (3 – Chloroanilo) – 4 – phenylphthalazine.

NAD: Nicotinamide adenine dinculeotide.

OPC – 3911: *N* – cyclohexyl – *N* – (2 – hydroxyethyl) – 4 – (6(1,2 – dihydro – 2 – oxoquinolyloxy))butyramide.

OPC – 13135: *N* – cyclohexyl – *N* – (2 – hydroxybutyl) – 5 – (6 – 1,2,3,4 – tetrahydro – 2 – oxoquinolyloxy) – butyramide.

PDE: phosphodiesterase.

PGI_2: Prostaglandin I_2.

PIA: N^6 – (phenylisopropyl) – adenosine.

PM – 1: Antisera raised to purified preparations of the rat liver 'peripheral plasma membrane phosphodiesterase.

PM – PDE: 'Peripheral' plasma membrane high affinity cyclic AMP – specific phosphodiesterase.

RD1, RD2 and RD3: Rat brain phosphodiesterase homologues of *Drosophila melanogaster* dunce encoded phosphdiesterase.

Ro – 15 – 2041: 7 – Bromo – 1,5 – dihydro – 3,6 – dimethyl imadazo [2,1 – b] Quinazoline 2(3H) – One.

Ro – 20 – 1724: 4 – (3 – butoxy – 4 – methoxybenzyl) – 2 – imidazolidinone.

Rolipram: 4 – (3 – cyclopentyloxy – 4 – methoxyphenyl) – 2 – pyrrolidine.

ROS: Rod outer segment.

ROS – PDE: Rod outer segment phosphodiesterase.

SD: Standard deviation.

SEM Stanard Error of the Mean.

SDS.: Sodium dodecyl sulphate.

SDS – PAGE: Sodium dodecyl sulphate – polyacrylamide gel electrophoresis.

TEMED: N,N,N',N', – tetramethylethylenediamine.

TPA: 12 – *O* – tetradecanoyl phorbol 13 – acetate.Tris:

Tris: Tris (Hydroxyaminomethan)

W – 7: *N* – (4 – amino – hexyl) – 5 – chloronapthalene – 1 – sulphonamide.

Zaprinast (M&B 22948): 2 – *O* – propoxyphenyl – 8 – azapurin – 6 – one.

CHAPTER 1.

INTRODUCTION.

INTRODUCTION

1.1: CYCLIC NUCLEOTIDES AS SECOND MESSENGERS.

Cyclic nucleotides, such as 3'5'-cyclic AMP and 3'5'-cyclic GMP, are important molecules that are capable of regulating a diverse number of biological processes in cells. Cyclic AMP was first identified by Earl Sutherland and his co-workers while investigating the mechanism of action of epinephrine and glucagon in liver glycogen breakdown. They identified a heat stable molecule that was produced by a liver particulate fraction in response to epinephrine and glucagon. This factor could activate phosphorylase when added to the supernatant fraction (Rall, et al., 1957). Later work showed that this 'factor' was 3'5'-cyclic AMP (Sutherland and Rall, 1960). These pioneering studies led on to the concept that cyclic AMP can act as a 'second messenger' for a diverse array of peptide and non-peptide hormones and neurotransmitters (for reviews see Pastan, 1972; Birnbaumer, 1990).

Increases in the intracellular concentration of this nucleotide in target cells will elicit alterations in the metabolic pathways that control the synthesis and degradation of important metabolites such as glycogen, glucose, fat and fatty acids. In addition, in some cell types, cyclic AMP has been implicated in the mechanism by which cells are stimulated to divide. Cyclic AMP thus plays a central role in the response of cells to external stimuli (for reviews see Pastan, 1972; Schacter, et al., 1988).

In 1969 Hardman and coworkers discovered 3'5'-cyclic GMP but despite intensive research efforts it's role in biological regulation remained elusive for a number of years (Hardman and Sutherland, 1969; Hardman, et al., 1971). Recent work has indicated that cyclic GMP plays a much more limited and specialised role in cell function than cyclic AMP; a role for cyclic GMP having been suggested in retinal

function, control of vessel tone and in the action of atrial natriuretic factor (ANF) (for reviews of cyclic GMP action see Goldberg and Haddox, 1977; Tremblay, et al., 1988).

A study of the systems that are responsible for the synthesis and degradation of these important molecules is thus central to an understanding of how cells respond to such diverse stimuli as hormones, neurotransmitters, growth factors and light. The structures of 3'5' cyclic AMP and 3'5' cyclic GMP are shown in Figure 1.8.

1.1.1: Synthesis of cyclic AMP: Adenylate cyclase.

Adenylate cyclase catalyses the formation of cyclic AMP from ATP at the intracellular face of the plasma membrane. Both genetic (Livingstone, et al., 1984) and biochemical (Pfeuffer, et al., 1985; Smigel, 1986; Minocherhomjee, et al., 1987) evidence indicates that there are multiple forms of this enzyme. The Calmodulin-sensitive and -insensitive bovine brain enzymes have been purified (Pfeuffer, et al., 1985; Smigel, 1986) and cDNA clones isolated (Lipkin, et al., 1989; Krupinski, et al., 1989).

The activity of adenylate cyclase can be controlled by a wide range of different hormones and neurotransmitters including adrenergic and purinergic agents and peptide hormones such as glucagon, adrenocorticotrophic hormone (ACTH) and opiates (for review see Birnbaumer, et al., 1990). Such agents can either stimulate or inhibit adenylate cyclase so that the system is under dual control (Gilman, 1984; Birnbaumer, et al., 1990).

Hormonal regulation is achieved by binding to appropriate receptors whose actions are mediated by specific guanine nucleotide regulatory proteins (G-proteins). These are heterotrimers consisting of α, β and γ subunits (Northup, et al., 1980; Manning and Gilman, 1983; Hildebrandt, et al., 1984). The individual G-protein species are defined by the type of α-subunit (Manning and Gilman, 1983; Codina, et al., 1984), since it appears that the β subunits are either identical or very similar between different G-proteins (Manning and

Gilman, 1983). There are at least three γ subunits that have been identified, one of which is specific for transducin (γ_T), the remaining two, γ_a and γ_b, appearing to be found in all populations of G-proteins (Hildebrandt, et al., 1985; Gierschik, et al., 1985; Evans, et al., 1987; Mumby, et al., 1988). The two G-protein types regulating adenylate cyclase are called G_s and G_i, which stimulate and inhibit adenylate cyclase respectively.

Stimulatory hormones bind to specific receptors located in the plasma membrane. Such activated stimulatory receptors interact with the guanine nucleotide regulatory protein G_s (in the presence of GTP) leading to dissociation of the $\alpha\beta\gamma$ trimer, producing an activated α_s subunit (containing bound GTP) and the $\beta\gamma$ dimer (Kanaho, et al., 1984; Watkins, et al., 1985; Florio and Sternweis, 1985; Florio and Sternweis, 1989). It is believed that the activated α_s subunit interacts directly with the adenylate cyclase, leading to an increase in the production of cyclic AMP (May, et al., 1985). The stimulatory signal is turned off by the intrinsic GTPase hydrolytic activity of the α_s subunit, leading to the production of an α_s subunit containing bound GDP. This action allows the reassociation of α_s with the $\beta\gamma$ dimer and the cycle can then be repeated in the presence of hormone.

Inhibitory hormones exert actions through G_i (Hildebrandt, et al., 1983), which dissociates upon activation by hormone occupied receptor to give α_i and a $\beta\gamma$ dimer (Katada, et al., 1984a; Katada, et al., 1984c). The precise mechanism of G_i action is not understood, although it may involve an inhibitory effect of $\beta\gamma$ on stimulatory input (Katada, et al., 1984b).

1.1.2: Synthesis of cyclic GMP: Guanylate cyclase.

Guanylate cyclase catalyses the formation of cyclic GMP from GTP (Hardman and Sutherland, 1969; Hardman, et al., 1971). Several forms of the enzyme exist in both the soluble and particulate fractions of most mammalian cells (Hardman and Sutherland, 1969; Schultz, et al., 1969; White, et al., 1969). Cellular guanylate cyclase activity can

be regulated by a number of different mechanisms. Which factors are operative depends on the particular cell type in question. The particulate derived forms are glycoproteins and in some cells appear to be regulated by extracellular peptides such as sperm stimulating peptides, e.g. resact and speract, and also by atrial natriuretic peptides (ANP) (Leitman, et al., 1988) and heat stable enterotoxins (STa) from *E.Coli.* (Garbers, 1989). The atrial natriuretic peptides are of particular interest, since their effects include vasodilation, natriuresis, and diuresis (Inagami, 1989). Cloning and transfection studies have indicated that the cellular receptor for ANP and guanylate cyclase activity reside in the same polypeptide (Chinkers, et al., 1989) in contrast to the adenylate cyclase system where the cyclase and receptors are distinct entities.

The soluble haem containing form, on the other hand, does not appear to be regulated by peptides, but can be stimulated by nitrovasodilators (Böhme, et al., 1984), unsaturated fatty acid oxidation products and free radicals (White, et al., 1982). The physiological significance of this mechanism of regulation is far from clear, but it is likely that the enzyme serves as a substrate for endothelial cell-derived relaxing factor (EDRF) (Furchgott and Zawadzky, 1980), a substance believed by various investigators to be nitric oxide (Garbers, 1979). Guanylate cyclase activity is present in most mammalian cells with high activities found in platelets, lung, brain, and liver (Tremblay, et al., 1988).

1.1.3: The role of cyclic AMP and cyclic GMP.

The role of cyclic AMP appears to be almost ubiquitous in that it can serve as a 'second messenger' for a wide variety of hormones and neurotransmitters that control the metabolic activity of cells. It's actions are mediated by stimulating the activity of a specific protein kinase, the cyclic AMP-dependent protein kinase (A-Kinase), leading to the phosphorylation (and consequent activation/inactivation) of a number of target enzymes, the precise nature of which will depend on

the target cell in question (Woodgett and Cohen, 1984; Belfrage, et al., 1984; Schacter, et al., 1988).

Cyclic AMP-dependent protein kinase exists as a heterodimer consisting of two regulatory subunits and two catalytic subunits (R_2C_2) (for review see Taylor, 1989). The mammalian cyclic AMP-dependent protein kinase exists in two main isozymic forms (I and II), whose regulatory subunits (RI and RII) are disimilar. These species are found in most mammalian cells (Corbin, et al., 1975). Like most protein kinases, it is tightly regulated and is maintained in an inactive form under basal conditions (sub-activatory concentrations of cyclic AMP). However, unlike other protein kinases, cyclic AMP binding to a distinct regulatory subunit (R) induces comformational changes that lead to dissociation of the holo-enzyme and it's subsequent activation.

The active, dissociated catalytic subunit (C) can phosphorylate and thus regulate the activity of a wide variety of target proteins (Schacter, et al., 1988). These include key enzymes involved in intermediary metabolism such as glycogen phosphorylase kinase (Cohen, 1982), glycogen synthetase (Woodgett and Cohen, 1984) and hormone-sensitive lipase (Belfrage, et al., 1984). This is presumed to be the mechanism by which hormones such as glucagon, β-adrenergic agonists and adrenocorticotrophic hormone (ACTH) exert their major effects on target cells.

Recent evidence has indicated that the cyclic AMP.R_2C_2 complex may also mediate biological effects. This is because it has been shown to act as a potent inhibitor of ATP-Mg(II)-dependent phosphoprotein phosphatase, the major phosphorylase phosphatase in rabbit muscle (Jurgensen, et al., 1985; Khatara, et al., 1985).

Cyclic GMP, on the other hand, appears to have a number of possible roles. However, many of these are ill defined. For example, in mammalian smooth muscle, cyclic GMP is considered to be an important regulator of vascular muscle tone (Ignarro and Kadowitz, 1985). Several smooth muscle relaxants including nitrogen-oxide

vasodilators, endothelial cell-derived relaxing factor, and atrial natriuretic peptides, stimulate cyclic GMP production in vascular smooth muscle (Schultz, et al., 1977; Rapoport and Murad, 1983; Hamet, et al., 1984b). In the mammalian retina, cyclic GMP plays a pivotal role in the transduction of the light signal by altering the conductivity of sodium ion channels in the plasma membrane of rod outer segments (Fesenko, et al., 1985).

There are a number of cyclic GMP-binding proteins in cells including a protein kinase, termed cyclic GMP-dependent protein kinase. This kinase has some sequence homology with the cyclic AMP-dependent protein kinase but has regulatory and catalytic domains as part of a contiguous polypeptide chain (Takio, et al., 1984). It's functional significance remains however to be ascertained. Other cyclic GMP-binding proteins include cyclic GMP-stimulated phosphodiesterases. These are found in many cells including bovine adrenals and liver (Martins, et al., 1982; Yamamoto, et al., 1983a). There is also a cyclic GMP-binding phosphodiesterase which has been identified in platelets and lung (Hamet and Coquil, 1978; Francis, et al., 1980).

The precise mechanism of action of cyclic GMP in eliciting responses in cells is thus unclear. However, the discovery of cyclic GMP-binding proteins other than the cyclic GMP-dependent protein kinase suggests that this cyclic nucleotide does not always act through a phosphorylation mechanism (see for example, Hartzell and Fischmeister 1986).

1.2: THE PHOSPHODIESTERASE ENZYME SYSTEM.

The initiation of an intracellular signal, such as the production of cyclic AMP, initiates a cascade of events that eventually leads to the covalent modification of a number of target enzymes and hence the cellular response. The process of signal generation is not, however, a unidirectional process. Once cyclic nucleotide levels have been raised in

the cell it is necessary to terminate their action. In addition to desensitisation of the nucleotide cyclase system, in particular the adenylate cyclase system, this can be achieved by two general mechanisms, namely the extrusion of the cyclic nucleotide from the cell or the degradation of the cyclic nucleotide. Whilst, in principle, both of these mechanisms may be operative, it seems likely that extrusion only contributes about 20% to the cyclic AMP disposal observed in most cells (Plagemann and Erbe, 1977; Brunton and Mayer, 1979). However, higher levels of extrusion of cyclic GMP have been noted in liver slices (Tjornhammer, et al., 1983).

Degradation of cyclic nucleotides is achieved in the cell by the action of the cyclic nucleotide phosphodiesterase (PDE) enzyme system. This catalyses the hydrolysis of the 3'5' phosphodiester bond of cyclic nucleotides to produce 5'-AMP derivatives (see Figure 1.1) (Butcher and Sutherland, 1962). Cyclic nucleotide phosphodiesterases have been observed in all mammalian tissues examined to date (Beavo, 1988) as well as in a number of non-mammalian systems such as the yeast *Saccharomyces cerevisiae* (Sass, et al., 1986), *Dictyostelium discoideum* (Orlow, et al., 1981; Lacombe, et al., 1986), *Mucor rouxii* (Galvagano, et al., 1979) and the fruit fly *Drosophila melanogaster* (Davis and Kauvar, 1984).

1.2.1: Multiple forms of phosphodiesterase.

Phosphodiesterases exist in multiple forms (Beavo, et al., 1970; Thompson and Appleman, 1971; Uzunov and Weiss, 1972; Russell, et al., 1973; Bergstrand and Lundquist, et al., 1976; Beavo, et al., 1982; Beavo, 1988). These differ in their physical properties, and distribution between soluble and particulate cell fractions. They also have distinct substrate specificities, kinetic characteristics, sensitivity to proteolysis and in some instances can be regulated by physiologically important molecules such as calcium, Calmodulin, cyclic GMP, hormones and the G-protein Transducin (Beavo, et al., 1982; Manning and Gilman, 1983; Yamamoto, et al., 1983a; Yamamoto, et al., 1984a;

Manganiello, et al., 1984; Houslay, 1986; Sharma and Wang, 1986c Pyne, et al., 1987a).

It has been demonstrated by immunological (Mumby, et al., 1982; Hurwitz, 1984b), pepide mapping (Takemoto, et al., 1982; Pyne et al., 1987a) and molecular cloning (Charbonneau, et al., 1986; Chen, et al., 1986; Podgorski, et al., 1986; Sass, et al., 1986; Lacombe, et al., 1986; Colicelli, et al., 1989; Davis, et al., 1989; Swinnnen, et al., 1989; Henkel-Tigges and Davis, 1990) studies that distinct isoforms exist and that they are, for the most part, distinct non-interconvertible enzymes (Erneux, et al., 1980; Keravis, et al., 1980; Hurwitz, et al., 1984a).

The reaction catalysed by cyclic nucleotide phosphodiesterase involves the breaking of a high energy phosphodiester bond in a metal ion-dependent hydrolytic process. For most enzymes, the preferred metal cofactors are divalent cations such as Mg^{2+} or Mn^{2+}, although other metals, such as Co^{2+}, Ni^{2+}, and Zn^{2+}, can support lower activity; Ca^{2+}, by itself, is virtually ineffective.

It has been suggested that there are four major classes of phosphodiesterase with different specificities for cyclic AMP and cyclic GMP and different allosteric regulators. Type I phosphodiesterases are stimulated by Ca^{2+} plus Calmodulin and in general have a higher affinity for cyclic GMP than cyclic AMP (Kincaid, et al., 1984). Type II phosphodiesterases likewise, have a higher affinity for cyclic GMP than for cyclic AMP but the activity toward cyclic AMP is markedly stimulated by low (micromolar) concentrations of cyclic GMP (Yamamoto, et al., 1983a; Pyne et al., 1986a). Type III phosphodiesterases have a higher affinity for cyclic AMP than cyclic GMP and their activity is increased in certain cells by hormones e.g. isoproteronol, glucagon, and insulin (Heyworth, et al., 1983d; Marchmont and Houslay, 1980b; Loten and Sneyd, 1970; Loten, et al., 1978). Type IV phosphodiesterases are cyclic GMP-specific enzymes, which also have an allosteric binding site for cyclic GMP. An example

of this class of enzyme is the one from retinal rod outer segments, which is activated by light via rhodopsin and the guanine nucleotide binding protein Transducin (Baehr, et al., 1979).

1.3: Ca^{2+}/CALMODULIN-STIMULATED CYCLIC NUCLEOTIDE PHOSPHODIESTERASES.

This is found in several mammalian tissues including bovine heart (Hansen and Beavo, 1982; Ho, et al., 1977), bovine brain (Sharma, et al., 1980; Sharma, et al., 1984; Kincaid, et al., 1984), rat liver (Strewler, et al., 1983), rat pancreas (Vandermeers, et al., 1983), dog thyroid (Erneux, et al., 1985b), rat testis (Purvis, et al., 1981), and bovine lung (Sharma and Wang, 1986b). The enzyme activity is also found in non-mammalian sources, for example in *Neurospora Crassa* (Tellez-Inon, et al., 1985).

The Ca^{2+}/Calmodulin-stimulated phosphodiesterase is an enzyme of relatively low abundance. Mammalian brain is the richest source of the enzyme, at a level of about 10mg/kg (Sharma, et al., 1980). Mammalian heart, although the second most abundant source of the enzyme, contains only 10-20% of the levels found in brain (LaPorte, et al., 1979).

1.3.1: Ca^{2+}/Calmodulin-stimulated phosphodiesterase isozymes.

Originally, it was thought that this was an enzyme of approximately 60-kDa subunit molecular weight, which appeared to exist as a homodimer in the native state (Morril, et al., 1979; LaPorte, et al., 1979). More recent work using improved separation procedures has shown that multiple forms exist, both in single tissues and between species.

Using rapid, immuno-affinity chromatography techniques (ACAP-1 monoclonal antibody) to purify the Ca^{2+}/Calmodulin-stimulated phosphodiesterase from different bovine tissues (brain and heart), Hansen and Beavo showed that the two enzymes isolated

differed by approximately 2-kDa in apparent molecular weight, suggesting the occurence of tissue specific forms (Hansen and Beavo, 1982). Kincaid and co-workers were, however, the first to provide evidence for the presence of distinct isozymes in a single source. They identified and purified a 59-kDa species from both bovine and ovine brain tissue that had an isoelectric point (pI) of 5.6 and also identified the presence of a fraction that had a lower pI of about 5.45. Additionally, the purified bovine and ovine 59-kDa enzymes yielded different α-chymotryptic peptide-maps, indicating that these proteins differed in primary sequence (Kincaid, et al., 1984).

Similar studies by Sharma and co-workers, also using monoclonal antibodies (A6, C1 and A2), showed the presence of two distinct isozymes in bovine brain of subunit molecular weight 60-kDa and 63-kDa (Sharma, et al., 1984). Peptide mapping of the two subunits, by partial proteolysis, demonstrated that the 60-kDa species was not derived from the 63-kDa species. Further to this, it was shown that the PDE could exist in several forms (a) a homodimer of 60-kDa (b) a heterodimer of 60-kDa and 63-kDa and (c) a homodimer of 63-kDa. In bovine brain, the homodimers were the major forms encountered (Sharma, et al., 1984).

An unusual form of the enzyme exists in bovine and rabbit lung that appears to contain Calmodulin as an integral subunit. This is analagous to the way that Calmodulin exists as the δ-subunit of phosphorylase kinase (Cohen, et al., 1978; Sharma and Wang, 1986b; Sharma and Wirch, 1979). The bovine lung enzyme is a dimeric protein of subunit molecular weight 58-kDa containing, in addition, two molecules of Calmodulin. The Calmodulin moiety cannot be dissociated from the phosphodiesterase, even in the presence of very low calcium concentrations. The enzyme is stimulated directly by calcium, indicating that binding of Calmodulin *per se*, is not sufficient to cause stimulation of the enzyme. The enzyme shows immunological cross reactivity with the bovine brain 60-kDa form based on it's ability

to bind to an immuno-affinity column (C1 monoclonal antibody) (Sharma and Wang, 1986b).

In addition, forms of the enzyme with higher subunit molecular weights have been reported in male mouse germ cells (Geremia, et al., 1984), bovine brain (Shenolikar, et al., 1985) and mouse testis (Rossi, et al., 1988). Identification of these individual forms has, however, relied upon extensive purification away from other contaminating activities. In a few instances multiple forms of the Ca^{2+}/Calmodulin-stimulated phosphodiesterase have been identified by simple ion-exchange chromatography. Using this procedure, multiple Calmodulin-stimulated forms were resolved in immature rat testis whilst two forms could be identified in rat pancreas (Purvis, et al., 1981; Vandermeers, et al., 1983). The properties of the individual isozyme forms purified to date are summarised in Table 1.1.

1.3.2: Kinetics of cyclic nucleotide hydrolysis by Ca^{2+}/Calmodulin-stimulated phosphodiesterase isozymes.

The kinetic properties of the Ca^{2+}/Calmodulin-stimulated phosphodiesterases from a variety of tissues and species have been studied in a number of laboratories. Early reports on the kinetic characteristics of these isozymes showed a marked variation in K_m and V_{max} values between different studies. For example, the K_m values for both cyclic AMP and cyclic GMP varied over two orders of magnitude among early reports. It has been suggested that such variation can be attributed to a number of factors: (a) The enzyme preparations were of different purity and may have been contaminated with different amounts of other phosphodiesterases. (b) Different assay conditions were used; agents such as imadazole or ammonium ions have been shown to increase the activity of the Calmodulin-stimulated bovine brain enzyme (Ho, et al., 1976; Nair, 1966; Cheung, 1967; Sakai, et al., 1977). Other cations such as potassium are inhibitory (Davis and Daly, 1978). (c) It was not known that different isozymes of the Ca^{2+}/Calmodulin-stimulated phosphodiesterase existed.

Kinetic characteristics of both highly purified isozymes and homogeneous preparations have now been obtained by a number of groups (Ho, et al., 1976; Sharma and Wang, 1986a; Sharma and Wang, 1986b; Hansen and Beavo, 1986; Reeves, et al., 1987; Purvis, et al., 1981). The basic kinetic characteristics are summarised in Table 1.2. On the basis of kinetic criteria, the Ca^{2+}/Calmodulin-stimulated phosphodiesterase isozymes may be divided into two general groups: a group showing high affinity for cyclic GMP but low affinity for cyclic AMP, and a group showing high affinity for both cyclic nucleotides. The first group possess K_m values for cyclic GMP and cyclic AMP in the ranges 5-20μM and 70-200μM respectively, whilst the latter group show similar K_m values for both cyclic nucleotides in the range 1-2μM. All the well characterised bovine brain and heart isozymes belong to the first group although, they represent different isoenzymic forms. Also included in this group are the purified enzymes from bovine lung and coronary artery and the partially purified enzymes from porcine brain and coronary artery and one form of the rat testis enzyme. In the second group of isozymes is the Peak I Calmodulin-stimulated rat testis PDE (identified from anion-exchange profiles of tissue extracts) and the testis-specific Calmodulin-stimulated phosphodiesterase purified from mouse testis.

Within each of these general groups is the potential for sub-groups. The four well defined bovine isozymes, all belonging to the first group, appear to be separable into two subgroups on the basis of their affinities for cyclic AMP and cyclic GMP. The 60-kDa brain, heart and lung isozymes show almost identical kinetic properties, while the 63-kDa brain isoenzyme has a higher V_{max} for cyclic GMP than for cyclic AMP.

In almost all instances Calmodulin increases both the affinity (decrease in K_m) and the V_{max} of the enzyme toward both cyclic nucleotides, although effects on the V_{max} component alone have been observed. The reported fold stimulation of cyclic nucleotide hydrolysis in the presence of saturating levels of Calmodulin varies considerably

but it is not known if this is an intrinsic property of the individual isozymes or is a consequence of different procedures used to isolate/purify the enzymes. It has been shown that limited proteolysis can reduce the extent of Ca^{2+}/Calmodulin stimulation as a result of an increase in the basal enzyme activity (Tucker, et al., 1981; Kincaid, et al., 1985). This may partly explain the different fold stimulations observed in partially purified preparations but not that of purified preparations where the subunit molecular weight can be easily assessed.

On the basis of observations made on purified preparations of bovine brain phosphodiesterase, Kincaid and his co-workers have proposed a model to explain the different fold stimulations observed. They observed that different procedures used to isolate the enzyme from bovine brain produced preparations which, although they exhibited identical subunit size, specific activity and K_m for cyclic GMP, differed in the extent of stimulation by saturating levels of Ca^{2+}/Calmodulin (Kincaid, et al., 1981b).

These highly purified preparations displayed a progressive loss in the degree of Calmodulin stimulation with an increase in basal activity. This loss of Ca^{2+}/Calmodulin responsiveness did not correlate with any detectable proteolytic degradation of the enzyme. There was however, an increase in the proportion of the phosphodiesterase that existed as a 60-kDa monomeric species and a decrease in the proportion that existed in the dimeric state. They also observed that the enzyme present in freshly prepared supernatants consistently behaved as a monomer, but on storage could be converted to a dimer. This conversion correlated with an increase in the extent of Ca^{2+}/Calmodulin stimulation. Based on these observations, it was proposed that the phosphodiesterase exists as interconvertible monomeric and oligomeric species and that these are activated to different extents by Ca^{2+}/Calmodulin (Kincaid, et al., 1981b).

Using radiation inactivation to assess the subunit structure of the enzyme under basal and Ca^{2+}/Calmodulin stimulated conditions,

Kincaid and coworkers showed that the basal activity decayed with an apparent target size of 60-kDa, whilst the stimulated activity decayed with an apparent target size of 105-kDa, giving support to their other observations (Kincaid, et al., 1981a). The degree of activation of a particular preparation of the enzyme will thus depend on the relative proportion of the two forms of the enzyme.

1.3.3: Mechanism of Calmodulin activation.

The affinity of the the bovine brain 61-kDa and 63-kDa forms for Calmodulin is about 1nM, whilst the affinity constant for the bovine heart form is about 10 fold lower at 0.1nM (Hansen and Beavo, 1986). Calmodulin activates the enzyme in the presence of calcium, presumably by binding to a domain on the enzyme, and this has been demonstrated with purified components, indicating that the activation is a direct action rather than indirect (LaPorte, et al., 1979; Hansen and Beavo, 1982; Hansen and Beavo, 1986).

Calmodulin contains four Ca^{2+} binding sites which are characteristic of the 'EF hand' structure. These sites are designated as I, II, III and IV in order of their location from the N-terminus. The crystal structure of a mammmalian Calmodulin in it's Ca^{2+} bound state has been determined (Babu, et al., 1985). The crystalline Calmodulin assumes a 'dumbbell' shape, with each lobe of the dumbbell containing a pair of 'EF hand' Ca^{2+} binding sites. The two lobes are connected by a long central helix and do not show any interactions. However, the two Ca^{2+} binding sites within each lobe appear to interact through hydrogen bonds. There appear to be two classes of Calmodulin binding sites with affinities differing by more than an order of magnitude (Seaman, 1980; Suko, et al., 1986), which corresponds with the existence of the two lobes. Sites III and IV, at the C-terminal lobe, are the high affinity sites, whilst sites I and II, at the N-terminal lobe, are the low affinity sites.

Calcium binding takes place in two distinct stages. At low levels of Ca^{2+} sites III and IV are occupied whilst at higher levels, sites I

and II become occupied (Forsen, et al., 1986) The binding of Ca^{2+} to Calmodulin is a highly cooperative process within each lobe, with no co-operativity between lobes, and this may allow for a very selective on/off switch to activate/deactivate phosphodiesterase over a very narrow range of Ca^{2+} concentrations.

The binding of calcium to Calmodulin appears to cause a conformational change since it becomes more resistant to proteoytic cleavage (Ho, et al., 1976; Liu and Cheung, 1976) and appears to be more susceptible to chemical modification (Wolff, et al., 1977). LaPorte, et al., (1980), used a number of fluorescent probes capable of sensing exposure of hydrophobic binding sites on proteins, to show that binding of Ca^{2+} to Calmodulin results in the exposure of a domain with considerable hydrophobic character. Further to this, binding of hydrophobic ligands to this domain antagonised Calmodulin-protein interactions. These workers proposed that this hydrophobic domain may serve as the interface for the Ca^{2+}-dependent binding of Calmodulin to target proteins including the Ca^{2+}/Calmodulin-stimulated phosphodiesterase (LaPorte, et al., 1980).

The binding of bovine heart and brain phosphodiesterases to Calmodulin in the presence of calcium may also cause a 'second' conformational change in the Calmodulin molecule. Hansen and Beavo, (1986) raised a monoclonal antibody (ACC-1) directed against an epitope on the carboxyl terminal of bovine Calmodulin. They showed that the ability of this antibody to bind to Calmodulin was greatly enhanced in the presence of bovine heart phosphodiesterase. However, the antibody reacted poorly with Calmodulin that was bound to heart or brain Calcineurin, skeletal muscle myosin light chain kinase or other Calmodulin binding proteins (Hansen and Beavo, 1986). This differential antibody reactivity toward Calmodulin-enzyme complexes was taken to indicate that target proteins either induce very different conformations in Calmodulin and/or interact with different geometries relative to the antibody binding site.

Calmodulin and Ca^{2+} show synergistic interactions in the activation of phosphodiesterase, so an increase in Calmodulin or Ca^{2+} concentration results in an increase in the apparent enzyme affinity towards Ca^{2+} or Calmodulin respectively (Brostrom and Wolff, 1974). The implication of this is, depending on the levels of Calmodulin, the Ca^{2+} concentrations needed for the activation of phosphodiesterase in different tissues may be quite different.

It has been proposed that at normal cellular Ca^{2+} concentrations phosphodiesterase and Calmodulin do not form a complex (Huang, et al., 1981; Cox, et al., 1981). Detailed kinetic studies of the purified bovine brain enzyme have indicated that four Ca^{2+} ions are required to bind to one Calmodulin molecule before activation can occur.

An alternative model has, however, been suggested by a number of workers (Blumenthal and Stull, 1980; Keller, et al., 1982; Greenlee, et al., 1982). In this model, Calmodulin can bind to target enzymes in the absence of calcium, albeit weakly, and with succesive binding of Ca^{2+} to Calmodulin, the affinity of Calmodulin toward the enzyme is increased. Steady state kinetic analysis of the activation of phosphodiesterase by Ca^{2+} and Calmodulin showed that the activation of the enzyme requires the binding of at least three and probably four Ca^{2+} ions to Calmodulin. Using crosslinking of I^{125}-labelled Calmodulin to a highly purified bovine heart enzyme, LaPorte, et al., (1979) were able to show that the stoichiometry of the PDE:Calmodulin complex to be $PDE_2Calmodulin_2$, i.e. one Calmodulin molecule per PDE chain. The molecular weight of the PDE:Calmodulin complex calculated from sedimentation and Stokes radius data has been shown to be consistent with this stoichiometry (Sharma, et al., 1980), although this has not been rigorously tested for all isozymes.

1.3.4: Effect of proteolysis on the Ca^{2+}/Calmodulin-stimulated phosphodiesterase.

The enzyme isolated from a number of sources is subject to proteolysis. This has been observed in both crude and purified preparations (Cheung, 1971; Tucker, et al., 1981; Kincaid, et al., 1985). For example, proteolysis of a bovine brain preparation of the Calmodulin-stimulated PDE (63-kDa subunit containing isozyme) decreased the fold stimulation of the phosphodiesterase activity in the presence of Ca^{2+}/Calmodulin from a value of 80, to a value of 1. This correlated with an increase in the basal activity to a value equal to, but not exceeding, that obtained with saturating Ca^{2+}/Calmodulin. This effect also correlated with a decrease in the molecular weight of the subunit to a molecular weight of 43-kDa. Preparations demonstrating intermediate fold activations displayed a fragment pattern with predominant polypeptides at 57-kDa, 52-kDa, 47-kDa, and 42-kDa. Proteolysis with trypsin could mimic these effects producing a fragment of molecular weight 40-kDa (Tucker et al., 1981).

Chymotrypsin treatment of a purified preparation of the bovine brain form of the enzyme also had similar effects to those observed with trypsin (Kincaid, et al., 1985). Proteolysis of the enzyme had a similar effect on the kinetic parameters to that of saturating Ca^{2+}/Calmodulin, namely, an increase in the V_{max} for cyclic GMP hydrolysis with a marginal increase in the affinity (decrease in K_m) for cyclic GMP. They also observed protection toward chymotryptic digestion in the presence of calcium and Calmodulin.

On the basis of these observations, Kincaid and co-workers have proposed a model for the structure of the Calmodulin-stimulated phosphodiesterase in bovine brain. The hypothesis is that the enzyme can exist either in an activated or inactivated state. Each state is a manifestation of different interactions between three structural domains of the enzyme: the catalytic, inhibitory and Calmodulin binding domains. Under basal conditions, the inhibitory and catalytic domains

undergo strong interactions, suggested to result in a low enzyme activity. However, the various activated forms of the enzyme result from a weakening or disruption of this interaction. The stimulation of the enzyme can thus be envisaged to involve a comformational change; binding of Ca^{2+}/Calmodulin resulting in the disruption of the interaction between the inhibitory and catalytic domains (Kincaid, et al., 1985).

The relationship between the Calmodulin-binding domain and the inhibitory domain is unclear, but the simplest model is for Calmodulin to compete with the catalytic domain for binding to the inhibitory domain. In this case, the Calmodulin-binding and inhibitory domains are believed to be the same or closely proximal. The inhibitory domain can be removed or damaged by protease action, thus relieving the inhibition of the catalytic activity (see Figure 1.2 for a diagrammatic representation). However, other workers have not observed Ca^{2+}/Calmodulin-dependent protection of the rat liver enzyme from chymotrypsin-mediated activation. This model may, therefore, only hold true for certain forms of the enzyme (Tucker, et al., 1981; Moss, et al., 1978). Furthermore, it is not known whether the action of proteases has any significance with respect to regulation of the activity.

Because of the large size and unknown three-dimensional structure of Calmodulin-regulated enzymes, the precise site(s) of interaction of Calmodulin with phosphodiesterase isozymes is at present unknown. One approach that has been taken in identifying potential Calmodulin binding sites in a Calmodulin-regulated enzyme has been to assess the ability of peptides (both synthetic and natural) to bind to Calmodulin and to compete with target enzymes for such binding (for review see Erickson-Viitanen and DeGrado, 1987). It has been suggested that this approach may identify sequences in proteins that can serve in Calmodulin binding.

Peptides such as mellitin were found to bind to Calmodulin in a 1:1 complex with affinities approaching those for many target enzymes ($K_D = 1nM$) (Comte, et al., 1983; Malencick and Anderson, 1983). Mellitin has been shown to possess an amphiphilic α-helix, a structure in which the hydrophobic residues are segregated from the hydrophilic residues along the helix, such that there results a hydrophobic patch on one face of the helix (Terwilliger and Eisenberg, 1982). Since Calmodulin also contains a hydrophobic sequence (LaPorte, et al., 1980), it has been proposed that mellitin and other peptides bind to this region of Calmodulin in an amphiphilic α-helical conformation (Erickson-Viitanen and Degrado, 1987). In addition to the important role played by hydrophobic interactions, a portion of the binding interaction appears to derive from electrostatic interactions, as all tight binding peptides carry net positive charges, whereas Calmodulin is an acidic (negatively charged) protein.

1.3.5: Methods of regulation of the Ca^{2+}/Calmodulin-stimulated phosphodiesterase.

In most tissues the amount of Calmodulin is in excess of the quantity of phosphodiesterase (Klee and Vanaman, 1982; Hansen and Beavo, 1986), such that the limiting factor for stimulation is the concentration of free calcium. The cytosolic concentrations of Calmodulin in heart and brain, have been estimated to be about 1.6μM and 22μM respectively. Thus, it would appear that a direct regulation of the enzyme activity by intracellular calcium is the most likely method of regulation. Further evidence for such a postulate is:

(i) Stimulation of phosphodiestrase requires micromolar calcium concentrations that are close to physiological concentrations of this divalent cation (Exton, 1988).

(ii) Intracellular calcium fluctuates in response to a wide variety of stimuli such as vasopressin in the liver (Combettes, et al., 1986).

(iii) the response of the phosphodiesterase to calcium and Calmodulin is rapid and comparable with the rapid hydrolysis of cyclic AMP in response to certain stimuli (Kakiuchi and Rall, 1968)

In addition to regulation by Calmodulin, some forms of the enzyme can also be regulated by phosphorylation. Using *in vitro* phosphorylation, Sharma and Wang (1985) have demonstrated that the bovine brain 60-kDa subunit is a substrate for cyclic AMP-dependent protein kinase with a maximal incorporation of 2 moles of phosphate per mole of subunit. This phosphorylation was accompanied by a 2,000% decrease in the affinity of this subunit towards Calmodulin (Sharma and Wang, 1985; Sharma and Wang, 1986c). In contrast, purified 63-kDa subunit-containing isozyme was found to be phosphorylated in a Ca^{2+} and Calmodulin-dependent manner, presumably by a Ca^{2+}/Calmodulin dependent protein kinase (Sharma and Wang, 1986a; Sharma and Wang, 1986c). Phosphorylation resulted in the incorporation of 2 moles of phosphate per mole of subunit. This also led to a decrease in the affinity of the enzyme towards Calmodulin. The decrease observed was much smaller than that noted for the 60-kDa subunit (about 50%) and was also accompanied by a small decrease in the EC_{50} for calcium stimulation of the enzyme (1.1μM for phosphorylated compared to 1.9μM for non-phosphorylated). Hashimoto and co-workers have recently extended these observations on the 63-kDa subunit of phosphodiesterase and shown that it has a regulatory phosphorylation site(s) that is phosphorylated by the auto-phosphorylated form of Ca^{2+}/Calmodulin-dependent protein kinase II. This phosphorylation can be blocked by Ca^{2+}/Calmodulin binding to the subunit (Hashimoto, et al., 1989).

The two phosphorylated species could be dephosphorylated by the calcineurin, the Ca^{2+}/Calmodulin-dependent phosphatase (Sharma and Wang, 1985; Sharma and Wang, 1986a). Dephosphorylation of the 60-kDa subunit increased the enzyme's affinity for Calmodulin (Sharma

and Wang, 1985) reversing the effect of phosphorylation. The effect of dephosphorylation of the 63-kDa subunit was not assessed however. These phosphorylation reactions have not been shown to occur *in vivo*. The data supports the notion that the isozymes are distinct species and may play different roles in controlling the levels of cyclic AMP and cyclic GMP in the brain.

1.3.6: Role of the Ca^{2+}/Calmodulin-stimulated phosphodiesterase.

The role of the enzyme in controlling cyclic nucleotide levels has been investigated in a number of tissues and cells including, astrocytoma cells (Evans, et al., 1984; Tanner, et al., 1986) and thyroid tissue (Dumont, et al., 1984; Erneux, et al., 1985b). In human astrocytoma cells, the decrease in cyclic AMP levels seen in response to muscarinic stimulation is thought to be due to Ca^{2+}-dependent increases in phosphodiesterase activity (Evans, et al., 1984). It was presumed by these workers that the phosphodiesterase responsible was one of the Ca^{2+}/Calmodulin-stimulated isozymes and that the increased intracellular calcium was stimulating this enzyme (Evans et al., 1984). Some evidence to support this notion was obtained by the use of specific Ca^{2+}/Calmodulin-dependent phosphodiesterase inhibitors to modulate the muscarinic response in these cells (Tanner, et al., 1986).

Data from Dumont and his colleagues indicate that the Ca^{2+}/Calmodulin-stimulated phosphodiesterase is an important regulator of cyclic AMP levels in thyroid tissue (Miot, et al., 1983; Miot, et al., 1984; Dumont et al., 1984: Erneux, et al., 1985b). In these studies, the potency of selective inhibitors of this enzyme, such as MIX, M&B 22948, 7-Benzyl-MIX and 8-MeOMIX, correlated well with the ability of these compouds to relieve the carbachol-induced decrease in intracellular cyclic AMP levels; this was not true of inhibitors of the cyclic AMP-specific form, such as IIX and Ro-20-1724.

1.3.7: Inhibitors of Ca^{2+}/Calmodulin–stimulated phosphodiesterase.

A large number of inhibitors of the Ca^{2+}/Calmodulin–stimulated phosphodiesterase are available. On the basis of their effects on the enzyme reaction, the inhibitors may be divided into two general categories: those counteracting Calmodulin stimulation of the enzyme and those inhibiting the enzyme reaction directly. Inhibitors in the first category may be further classified into three groups according to their mode of action. The stimulatory activity of Calmodulin is dependent on the binding of Ca^{2+}; agents that interfere with calcium binding form the first group of inhibitors. The second group of inhibitors abolish Calmodulin–stimulated phosphodiesterase activity by binding directly to Calmodulin. The third group exert their actions by binding to the phosphodiesterase to block the interaction between Calmodulin and the enzyme (for review see Vincenzi, 1982).

Numerous inhibitors which counteract Calmodulin activation of the enzyme by binding to Calmodulin have been described. They are represented by a group of highly heterogeneous compounds including proteins, peptides, amines, lipids and drugs of various chemical classes. A protein inhibitor of Calmodulin–stimulated phosphodiesterase was first described by Klee and Krinks and was later shown to be the Ca^{2+} dependent phosphatase calcineurin (Klee and Krinks, 1978). It can act as a Calmodulin–stimulated phosphodiesterase inhibitor under conditions of limiting Calmodulin since it can compete for the activator Calmodulin. Other common inhibitors, which act as Calmodulin antagonists include, N–(6–aminohexyl)–5–Chloro–1–napthalene–sulphonamide (W7) (Van Belle, 1984) and a number of W7 derivatives (such as 5–Iodo–C8 (Barton, et al., 1987)), chlorpromazine, and trifluoperazine (Van Belle, 1984). The strucutres of W7 and 5–Iodo–C8 are shown in Figure 1.7.

Using fluorescent probes such as N–Phenyl–1–napthalene (NPN), 9–anthroylcholine, 8–anilino–1–napthalenesulphonate and 2–*p*–toluidnylnapthalene–6–sulphonate, it was found that when Ca^{2+}

binds to the high affinity sites of Calmodulin it induces a conformational change which exposes hydrophobic groups. Calmodulin-stimulated enzymes or Calmodulin antagonists have been suggested to bind to this hydrophobic domain (LaPorte, et al., 1980). The use of Calmodulin antagonists to evaluate the role of the Ca^{2+}/Calmodulin-stimulated phosphodiesterase is, however, limited since they do not show specificity for the Calmodulin stimulation of but may affect all Calmodulin-stimulated processes.

The most common inhibitors which interfere with the binding of Ca^{2+} to Calmodulin are the Ca^{2+}-specific chelators such as EGTA. This agent is commonly used to demonstrate the existence of Ca^{2+}-stimulated phosphodiesterase in crude tissue extracts. Certain metal ions which bind at the Ca^{2+} binding sites of Calmodulin, but do not support activation, can act as inhibitors. A novel agent, 3-(2-benzothiazoly-4-5-dimethoxy-N[3-14-(phenylpiperidinyl) propyl] benzene sulphonamide (HT-74) that inhibits Ca^{2+} binding to Calmodulin has been described by Tanaka, et al., (1982)

Of much greater potential use are selective inhibitors that interact directly with the enzyme. These include agents such as vinpocetine (Hagiwara, et al., 1984), 7- or 8- alkyl substituted IBMX derivatives (e.g. 8-methoxymethyl-IBMX) (Wells and Miller, 1988), the IBMX derivative 2-*O*-propoxyphenyl-8-azapurin-6-one (M&B 22948) (Ruckstuhl, and Landry 1981; Broughton, et al., 1974) and HA 558 (Hidaka and Endo, 1984; Hidaka, et al., 1984). To date no specific inhibitors of the various Ca^{2+}/Calmodulin-stimulated isozymes have been reported.

1.4: CYCLIC GMP-BINDING PHOSPHODIESTERASES.

The cyclic GMP-binding cyclic nucleotide phosphodiesterases are a group of enzymes that may act as cyclic GMP "receptors". They are distinguished from other phosphodiesterases capable of hydrolysing cyclic GMP by the presence of two distinct sites for cyclic GMP. One

site functions as the catalytic site, where nucleotide hydrolysis occurs, the other site being a specific binding site for cyclic GMP which exhibits no hydrolytic activity. This is a heterogenous family of enzymes consisting of several subgroups, including the widely distributed cyclic GMP-stimulated phosphodiesterase (Beavo, et al., 1971; Erneux, et al., 1981), the retinal cyclic GMP phosphodiesterases (rod outer segment phosphodiesterases (ROS-PDE) (Miki, et al., 1975; Baehr, et al., 1979; Yamazaki, et al., 1980; Gillespie and Beavo, 1989a) and cone phosphodiesterases (Gillespie and Beavo, 1988), and the cyclic GMP-binding cyclic GMP-specific phosphodiesterase from rat and bovine lung (Hamet and Coquil, 1978; Francis, et al., 1980; Francis and Corbin, 1988) and those from rat and human platelets (Hamet and Coquil, 1978; Hamet and Coqueil, 1983; Hamet, et al., 1984a; Hamet and Tremblay, 1988).

1.4.1: Lung and platelet cyclic GMP-binding phosphodiesterases.

This activity was first observed as a distinct entity in both platelets and lung (Hamet and Coquil, 1978; Coquil, et al., 1980; Francis, et al., 1980). Later studies identified it in a number of other tissues, including rat liver (Coquil, et al., 1985; Strewler, et al., 1983). The enzyme has since been partially purified from the cytosol of rat platelets and rat lung (Hamet and Tremblay, 1988; Francis and Corbin, 1988).

1.4.2: Platelet cyclic GMP-binding phosphodiesterase.

The rat platelet enzyme displays specific, high affinity, cyclic GMP binding (K_D=353nM) and this is characteristically stimulated in the presence of the non-selective phosphodiesterase inhibitor, IBMX (K_D = 13.4nM). Cyclic GMP-binding and hydrolytic activities co-eluted during purification over a number of steps and it was presumed that the binding and hydrolytic sites were distinct entities, each associated with separate properties of the 176-kDa holoenzyme (Coquil, et al., 1980; Hamet, et al., 1984a; Hamet and Tremblay, 1988).

The rat platelet enzyme has not yet been purified to homogeneity so the actual subunit molecular weight and composition of the native enzyme is unknown. However, in studies of platelet extracts, Walseth and coworkers reported specific [^{32}P]-cGMP photoaffinity labelling of a platelet protein of molecular weight 93-kDa. This has been suggested to be the subunit molecular weight of the enzyme (Walseth, et al., 1985).

The precise role the enzyme plays in cyclic GMP metabolism is not known, but it has been shown that the phosphodiesterase activity is increased in platelets exposed to IBMX and forskolin. This effect could be mimicked with the catalytic unit of cyclic AMP-dependent protein kinase if crude material were used but not with partially purified preparations. (Tremblay, et al., 1985). This may imply that the enzyme is under the control of the cyclic AMP-dependent protein kinase in the intact cell and may represent a mechanism by which cyclic AMP levels may modulate cyclic GMP levels in platelets and lung. The need for a crude preparation may also imply the presence of a regulatory subunit that is lost during purification.

Similarly, the role of the cyclic GMP binding site is not known. However it has been suggested that since the enzyme represents the major, if not the only, cyclic GMP binding activity in platelet cytosol, it can serve as a significant buffer for intracellular cyclic GMP (Hamet and Tremblay, 1988). This will, however, be dependent on the concentration of the enzyme in the cell.

1.4.3: Lung cyclic GMP-binding phosphodiesterase.

The rat lung enzyme is similar in many respects to the platelet enzyme. Again cyclic GMP binding co-purifies with the cyclic GMP phosphodiestrase activity (Francis and Corbin, 1988) and represents a substantial proportion of the cyclic GMP binding activity in adult lung (Mumby, et al., 1982). It has an identical molecular weight on gel filtration to the platelet enzyme (176,000) and displays a subunit molecular weight of approximately 92-kDa after purification, although

other protein bands were observed by these workers in their preparations. However, a protein of molecular weight 93–kDa can be specifically labelled with [^{32}P]–cyclic GMP in photoaffinity labelling experiments (Francis and Corbin, 1988; Thomas, et al., 1988). It is presumed, therefore, that this represents the subunit molecular weight and that the enzyme exists as a homodimer under native conditions.

The phosphodiesterase activity of the cyclic GMP binding phosphodiesterase is highly specific for cyclic GMP and only hydrolyses cyclic AMP at a rate which is approximately 100 times slower than the hydrolytic rate for cyclic GMP. The purified rat lung enzyme has a K_m for cyclic GMP of about 5μM (Francis, et al., 1980). Immunological data indicate that this activity is distinct from other cyclic GMP binding phosphodiestrases, such as the cyclic GMP–stimulated phosphodiesterase and the photoreceptor enzyme (Mumby, et al., 1982; Hurwitz, et al., 1984a; Hurwitz, et al., 1984b).

As for the rat platelet enzyme the function of the cyclic GMP binding site is not known. However, it has been proposed that the allosteric site may regulate the activity of the catalytic site (Hamet and Coquil, 1978), although direct evidence for this suggestion is lacking. There does however, appear to be the capability of interaction between the two sites based on the ability of catalytic site–specific analogues to promote both [^{3}H]–cyclic GMP and [^{32}P]–cyclic GMP binding. Furthermore, cyclic GMP bound to the enzyme, causes a large shift in the elution pattern of this enzyme on DEAE chromatography (elution at higher ionic strength). This indicates a significant increase in surface electronegativity, presumably due to a conformational change.

1.4.4: Retinal rod and cone photoreceptor phosphodiesterases.

Rod outer segments (ROS) from both frog and bovine sources have been shown to contain a light and GTP–activated cyclic GMP phosphodiesterase (Miki, et al., 1973; Miki, et al., 1975; Baehr, et al., 1979). The phosphodiesterase present in rod outer segments has been well characterised by a number of groups. Highly purified preparations

of the membrane bound rod enzyme were first obtained from frog photoreceptors (Miki, et al., 1975) and later from bovine photoreceptors (Baehr, et al., 1979; Gillespie and Beavo, 1989a).

Recent work has shown that, as for other phosphodiesterase classes, isozymes of photoreceptor phosphodiesterases exist. Gillespie and co-workers have identified the presence of a soluble form of the bovine rod phosphodiesterase, which is distinct from the membrane bound form (Gillespie, et al., 1989b). A cone-specific phosphodiesterase was first observed by Hurwitz and co-workers, who found that a 94-kDa polypeptide could be immunoprecipitated with the monoclonal antibody ROS-1 (Hurwitz, et al., 1984b) from a small peak of phosphodiesterase activity (Peak I) in anion exchange separations of retinal extracts (Hurwitz, et al., 1985). The cone phosphodiesterase was purified from the soluble fraction of bovine retinas using cyclic GMP-Sepharose chromatography (Gillespie and Beavo, 1988).

Each of the isozymes has distinct properties with respect to the structure and organisation of the subunits. The membrane bound rod enzyme appears to consist of three polypeptides of molecular weights 88-kDa, 84-kDa and an inhibitory polypeptide of about 11-kDa. This small polypeptide is trypsin-labile and heat-stable and serves to keep the activity of the phosphodiesterase very low until it is released by Transducin-GTP leading to enzyme activation (Dumler and Etingof, 1976; Hurley and Stryer, 1982). The native subunit structure of the membrane associated bovine rod photoreceptor phosphodiesterase has been proposed to be $\alpha_1\beta_1\gamma_2$ (Deterre, et al., 1988). Similarly, the soluble rod phosphodiesterase has three subunits (α_{sol}, β_{sol} and γ_{sol}) which have exactly the same size as the membrane associated isozyme, as well as an additional δ-subunit of molecular weight 15-kDa (Gillespie, et al., 1989b). The cone phosphodiesterase consists of a single type of large subunit (α') of molecular weight 94-kDa and three

small subunits of molecular weights, 11-kDa, 13-kDa and 15-kDa (Gillespie and Beavo, 1988) but the precise stoichiometry is unknown.

Like the Ca^{2+}/Calmodulin-stimulated isoenzymes, the photoreceptor phosphodiesterase isoenzymes can be activated by trypsin (Miki, et al., 1975). Trypsin activates the photoreceptor phosphodiesterases by proteolysing their inhibitory subunits (Hurley and Stryer, 1982), which have a large number of basic residues (Ovchinnikov, et al., 1986).

1.4.5: Kinetics of cyclic GMP hydrolysis by retinal photoreceptor phosphodiesterases.

Trypsin-activated rod phosphodiesterase hydrolyses cyclic GMP with a K_m of 20-150μM (Gillespie and Beavo, 1988; Sitaramayya, et al., 1986; Kohnken, et al., 1981). When assayed under the same conditions, the membrane-associated rod phosphodiesterase, the soluble rod phosphodiesterase, and the cone phosphodiesterase each hydrolyse cyclic GMP with K_m values of about 20μM. The V_{max} values of both of the rod isoenzymes is about twice that of the cone isoenzymes (Gillespie and Beavo, 1988; Gillespie, et al., 1989b).

1.4.6: Cyclic GMP binding properties of retinal photoreceptor phosphodiesterases.

The frog ROS-PDE contains cyclic GMP-specific, high affinity binding sites which are discrete from the catalytic site (Yamazaki, et al., 1980; Yamazaki, et al., 1983). Scatchard analysis suggested the presence of at least two classes of cyclic GMP binding site (Miki, et al., 1975). Several lines of evidence have been used to confirm the identity of these sites as separate from the catalytic sites:

(i) Limited trypsin proteolysis rapidly damages these sites leaving the catalytic sites intact.

(ii) The non-catalytic sites show absolute specificity for cyclic GMP.

(iii) IBMX stimulates binding to the non-catalytic site and inhibits the catalytic activity of ROS-PDE. The situation is similar to the cyclic GMP-binding phosphodiesterase identified in platelets and lung (Hamet and Tremblay, 1988).

(iv) A heat stable inhibitory protein of ROS-PDE activity markedly stimulates binding of cyclic GMP to these sites (Yamazaki, et al., 1982).

The purified membrane associated bovine rod phosphodiesterase differs from the frog ROS-PDE since it contains endogenously bound cyclic GMP that occupies nearly all of the sites. Scatchard analysis of [^{3}H]-cyclic GMP binding have indicated that there are also two classes of binding site on the phosphodiesterase. The $t_{0.5}$ for the high affinity site exceeds 4h at 37°C (Gillespie and Beavo, 1989a). At 4°C, essentially no cyclic GMP dissociates, so that the phosphodiesterase isolated at this temperature retains nearly two moles of cyclic GMP bound per mole of oligomer (Gillespie and Beavo, 1989). It has been suggested that these binding sites are capable of binding the majority of the cyclic GMP in the rod outer segment. The phosphodiesterase concentration in bovine ROS has been estimated to be about 30μM (Baehr, et al., 1979; Sitaramayya, et al., 1986). It has been estimated that there is sufficient enzyme to bind nearly all of the cyclic GMP in rod outer segments.

The bovine cone phosphodiesterase also has two moles of non-catalytic binding sites per mole of oligomer. The affinity of these sites is, however, much lower than for the bovine rod isozyme (K_D=11nM) (Gillspie and Beavo, 1988).

1.4.7: Activation of photoreceptor phosphodiesterases.

Cyclic GMP hydrolysis by membrane-associated rod phosphodiesterase can be modulated by the degree of photobleaching of rhodopsin (Stryer, et al., 1981). The molecular basis for activation of this enzyme by light appears to be a mechanism analagous to hormonal coupling to adenylate cyclase. Light bleaching of rhodopsin is coupled

to the activation of the phosphodiesterase via a guanine nucleotide binding protein called Transducin. This GTP-binding protein is composed of two large polypeptides of 39-kDa (α-subunit) and 36-kDa (β-subunit) and a smaller subunit of molecular weight 10-kDa (γ-subunit) (Shinowaza, et al., 1980; Fung, et al., 1981). Light activated rhodopsin facilitates the formation of a Transducin-GTP complex, which in turn elicits the dissociation of the complex with the release of activated α subunit. This associates with the phosphodiesterase, dissociating the inhibitory subunit. The activation process is fully reversible.

This state of activation also appears to be modified by cyclic nucleotide-independent phosphorylation of rhodopsin by opsin kinase (Kuhn, et al., 1973), though the control mechanism of the kinase is not yet known. This cascade of events results in the rapid hydrolysis of cyclic GMP. It is presumed that there is a large local change in the concentration of cyclic GMP which is sensed by a cyclic GMP binding site on, or closely associated with, a cation channel (carrying primarily Na^+) in the plasma membrane (Fesenko, et al., 1985). The channel requires cyclic GMP to remain open and the resulting decrease allows closing of the channel leading to the hyperpolarisation characteristics of vertebrate photoreceptors.

The sequence of events responsible for visual transduction in cones has not been established. However, it is known that there are distinct cone opsins (Nathans, et al., 1986), transducin, (Lerea, et al., 1986), phosphodiesterase (Hurwitz, et al., 1985; Gillespie and Beavo, 1988) and cyclic GMP-sensitive channels (Cobbs, et al., 1985; Haynes and Yau, 1985). It is likely then, that a similar sequence of events occurs in cones.

1.4.8: The cyclic GMP-stimulated phosphodiesterase.

This enzyme family is termed cyclic GMP-stimulated because relatively low concentrations of cyclic GMP will increase the rate of cyclic AMP hydrolysis. It represents a potentially important point of

interaction between two second messenger systems namely cyclic AMP and cyclic GMP. In this respect it resembles the Ca^{2+}/Calmodulin stimulated-phosphodiesterase which impinges on Ca^{2+} and cyclic nucleotide metabolism. Under assay conditions that mimic the concentrations of cyclic AMP and cyclic GMP in cells, up to 50-fold increases in the rate of cyclic AMP hydrolysis can be observed (with cyclic GMP in the range 0.1 to 5.0μM). Such levels of cyclic GMP may be reached in some cells in response to atrial natriuretic factor or sodium nitroprusside (Hamet, et al., 1984b; Tremblay, et al., 1986).

The cyclic GMP-stimulated phosphodiesterase (CGS-phosphodiesterase) has been found in several mammalian tissues, including rat liver (Beavo, et al., 1971; Russel, et al., 1973; Terasaki and Appleman, 1975; Yamamoto, et al., 1983a; Pyne, et al., 1986a; Lavan, et al., 1989), thymocyte extracts (Franks, et al., 1979), human platelets (Hidaka, et al., 1976), thyroid gland (Erneux, et al., 1977) and adrenal medulla (Egrie, et al., 1977).

Beavo and co-workers (Hurwitz, et al., 1984b; Mumby, et al., 1982) have attempted to estimate the contribution of the soluble enzyme in extracts from several bovine tissues. Using non-inhibitory antibodies to immunoprecipitate the enzyme selectively, they estimated that it contributed a significant proportion of the total cyclic AMP hydrolysis in several bovine tissues, approximately 80% in adrenal tissue and liver, 60% in spleen, 40% in heart and lung and 20-30% in testis and brain, when assayed at a saturating cyclic AMP level (0.5mM).

The activity has been observed in both the soluble and particulate fractions of mammalian cells (Beavo, et al., 1971; Moss, et al., 1977; Terasaki and Appleman, 1975; Pyne, et al., 1986a). The cytosolic form of the enzyme has been purified from a number of sources including rat liver (Pyne, et al., 1986a), bovine adrenals and heart (Martins, et al., 1982), calf liver (Yamamoto, et al., 1983a) and rabbit cerebral cortex (Whalin, et al., 1988). Other cyclic GMP-stimulated phosphodiesterases isoenzyme forms which have been

reported include a particulate form of the enzyme purified from rat liver. This has been shown to possess distinct properties from the soluble form also found in rat liver including different sensitivities to certain fatty acids and different tryptic peptide maps (Pyne, et al., 1986a). The properties of the individual forms are summarised in Table 1.3.

The enzyme isolated from bovine sources (liver, adrenals and heart) appears to have a subunit molecular weight of between 102-kDa and 107-kDa (Beavo, et al., 1982; Martins, et al., 1982; Yamamoto, et al., 1983a), whilst the rat liver enzymes have smaller subunit molecular weights (66-67-kDa) (Pyne, et al., 1986a). Native molecular weight determinations indicate that all the bovine and rat forms are similar in that they exist as dimers. However, the rabbit cerebral cortex form may exist as a tetramer (Whalin, et al., 1988).

High affinity monoclonal antibodies have been raised against the bovine heart enzyme. One such antibody (CGS-2) has been used to identify the presence of a cyclic GMP-stimulated phosphodiesterase in the soluble fraction of bovine lung. This antibody did not cross react with either the Ca^{2+}/Calmodulin stimulated enzyme or a cyclic AMP-specific phosphodiesterase in bovine lung. In addition it was shown that the cyclic GMP-stimulated phosphodiesterase was immunologically distinct from the MIX-stimulated cyclic GMP-binding protein and also the cyclic GMP-dependent protein kinase present in bovine lung (Mumby, et al., 1982).

1.4.9: Kinetics of cyclic nucleotide hydrolysis for the cyclic GMP-stimulated phosphodiesterase.

Kinetic analyses of the bovine enzymes indicate that hydrolysis of both cyclic AMP and cyclic GMP displays positive co-operative behaviour (Martins, et al., 1982; Yamamoto, et al., 1983a). Hill coefficients of 1.8-1.9 and 1.25-1.5 having been reported for cyclic AMP and cyclic GMP hydrolysis respectively. All the purified forms appear to hydrolyse cyclic GMP preferentially to cyclic AMP with K_m

values of 10 – 15μM for cyclic GMP and 33 – 40μM for cyclic AMP and with V_{max} values of 120 – 200 units/mg for cyclic GMP and 120 – 170 units/mg for cyclic AMP. The two rat liver forms differ somewhat in their kinetic characteristics from the bovine forms in that, whilst they exhibit similar K_m values for cyclic GMP and cyclic AMP hydrolysis, they exhibit much lower V_{max} values for the hydrolysis of these two substrates (4 – 4.8 units/mg and 1.6 – 2.1 units/mg for cyclic AMP and cyclic GMP hydrolysis respectively). In addition, the hydrolysis of cyclic GMP of the two rat liver forms does not exhibit positively co – operative kinetics (Pyne, et al., 1986a). This has been attributed to the ability of cyclic GMP to induce a loss of positive homotropic effects for both cyclic AMP and cyclic GMP hydrolysis in these isolated forms. The enzyme would thus be expected to follow Michaelis – Menten kinetics, as both the regulatory and substrate sites would be occupied by cyclic GMP.

At micromolar concentrations of substrate and effector levels, cyclic GMP can stimulate cyclic AMP hydrolysis at least five fold for bovine adrenal and heart forms and also for the rat liver forms (Martins, et al., 1982; Pyne, et al., 1986a). A somewhat higher fold stimulation (32 fold) was obtained for the bovine liver enzyme (Yamamoto, et al., 1983a). In partially purified preparations of the soluble enzyme from rat liver, Erneux and co – workers reported between 15 and 30 fold stimulation of cyclic AMP hydrolysis (Erneux, et al., 1981). The apparent activation constant for cyclic GMP stimulation in the presence of magnesium is similar in all the purified forms (0.23 – 0.28μM for the rat liver forms and 0.5μM for the bovine liver form). Yamamoto, et al., (1983a) demonstrated that cyclic AMP could stimulate cyclic GMP hydrolysis at least five fold but only at nanomolar levels of cyclic GMP. The apparent activation constant for this process was shown to be approximately 10μM.

1.4.10: Mechanism of cyclic GMP stimulation of cyclic AMP hydrolysis.

The molecular basis for activation by cyclic GMP is ill-understood (Beavo, et al., 1982). Using a number of cyclic nucleotide analogues Erneux, et al., (1981) proposed the presence of two distinct sites (activatory and inhibitory) in a partially purified preparation of soluble rat liver enzyme. The hydrolytic site was proposed to be able to interact with both cyclic AMP and cyclic GMP whilst the activatory site shows a much greater specificity for cyclic GMP. They also observed stimulation of cyclic GMP hydrolysis by various cyclic nucleotide analogues and also cyclic AMP (at 10μM). The fold stimulation observed for cyclic AMP was however much lower, (20%), but this probably reflects the use of a much higher cyclic GMP concentration (0.3μM). Indeed, Yamamoto, et al., (1983a) showed that stimulation of cyclic AMP hydrolysis of the bovine liver form could only be observed at nanomolar levels of cyclic GMP and not at all at 0.5μM cyclic GMP.

In accordance with these observations, Scatchard plots of cyclic GMP binding to the purified enzyme from bovine adrenals displayed curvilinear concave upward-curves consistent with heterogeneity of sites (Martins, et al., 1982). Results obtained by Miot and co-workers suggested that cyclic GMP-binding activity was directly related to the allosteric cyclic GMP binding site (Miot, et al., 1985). Using a number of cyclic nucleotide analogues, Erneux and co-workers showed a perfect correlation between potencies of stimulation of cyclic AMP phosphodiesterase and displacement of cyclic GMP binding (Erneux, et al., 1985a). The cyclic GMP-stimulated phosphodiesterase thus appears to be similar to the cyclic GMP-binding phosphodiesterases in platelets and lung and also to the ROS-PDE in that it also possesses a non-catalytic cyclic GMP binding site distinct from the catalytic site.

1.4.11: Other effectors of the cyclic GMP-stimulated PDE.

Yamamoto, et al., (1984a) have also observed that another effector binding site, specific for cis double bonded fatty acids, is

present on the purified bovine liver enzyme. In general, they found that unsaturated fatty acids were inhibitory, except for myristoleic acid and palmitoleic acid, which increased the hydrolysis of cyclic AMP under basal conditions. Palmitoleic acid at 10 – 100μM could increase cyclic AMP hydrolysis by up to 50%, presumably through interaction with a hydrophobic domain, higher concentrations were inhibitory. Such stimulatory effects were evident at low cyclic AMP concentrations where homotropic effects were minimal. Indeed, immobilisation of the cytosolic rat liver enzyme upon the hydrophobic matrix, hexyl agarose reduced the ability of cyclic GMP to stimulate cyclic AMP hydrolysis, suggesting an important functional role for such a novel regulatory site. Pyne and co-workers (1986a) also observed stimulation of cyclic AMP hydrolysis by palmitoleic acid at low concentrations and inhibition at higher concentrations. This effect was only observed for the cytosolic form of the enzyme, with the particulate form being insensitive to palmitoleic acid under basal conditions. Under cyclic GMP stimulated conditions no activation was observed for either form but both forms were inhibited by high concentrations of this fatty acid (Pyne, et al., 1986a).

1.4.12: Regulation and role of the cyclic GMP-stimulated PDE.

Little is known concerning the regulation of the cyclic GMP-stimulated or it's physiological role in the control of metabolism in intact cells. In cultured rat hepatoma cells (HTC), the levels of the cyclic GMP-stimulated phosphodiesterase can be decreased by prolonged (48 – 72h) treatment with dexamethasone, with little or no effect on the 'low-K_m' cyclic AMP phosphodiesterase activity (Ross, et al., 1977).

The precise role of the enzyme in controlling cyclic nucleotide levels is unknown but it has been suggested that insulin may activate this enzyme in rat liver via the intermediancy of a 'mediator' (Pyne, et al., 1988). Beavo and co-workers demonstrated that adrenal glandular tissue contains the highest amount of cyclic GMP-stimulated

phosphodiesterase activity; in the gland itself, virtually all the cyclic GMP-stimulated phosphodiesterase activity is confined to the 1-2mm layer of the adrenal gland (Beavo, 1988). This has suggested an important role for the enzyme in zona glomerulosa function, i.e. aldostrone production and adrenal cell growth. These workers have suggested that agents which might increase cyclic GMP e.g. ANF (atrial natriuretic factor), might bring about some of their physiological effects by increasing cyclic AMP hydrolysis and inhibiting cyclic AMP mediated processes.

1.5: CYCLIC AMP-SPECIFIC PHOSPHODIESTERASES.

Initially, these were identified as activities that elute at high salt concentrations as the third peak of activity from DEAE-cellulose columns ('PDE III' or 'low K_m' cyclic AMP phosphodiesterase) and show a marked preference for cyclic AMP as substrate (when assayed at low µM substrate concentrations). In some instances they have been shown to be insensitive to Ca^{2+}/Calmodulin and insensitive or partially inhibited by cyclic GMP. (Weishaar, et al., 1985; Weishaar, et al., 1986; Grady and Thomas, 1986; Gulyassy, et al., 1975; Russel, et al., 1973; Manganiello, et al., 1984; Turnbull and Hickie, 1984). It has become apparent from more recent work that the cyclic AMP-specific class of phosphodiesterase includes a number of activities that differ primarily in their response to cyclic GMP and also to a number of pharmacological agents (Yamamoto, et al., 1984a; Pyne, et al., 1987; Whitson and Appleman, 1982; Weber and Appleman, 1982; Weishaar, et al., 1987a; Weishaar, et al., 1987b; Weishaar, et al., 1987c; Weishaar, et al., 1987d). Identification of these 'subclasses' has relied both upon extensive purification protocols and improved separation methods that allow separation from the cyclic GMP-stimulated phosphodiesterase, an activity that could serve to mask the presence of these isozymes. (Yamamoto, et al., 1984a; Reeves, et al., 1987; Weber and Appleman, 1982). In general these isozymes have been classified

into two groups, namely the cyclic GMP–inhibited phosphodiesterases and a cyclic GMP–insensitive class also referred to as the Rolipram–sensitive phosphodiesterases.

1.5.1: The cyclic GMP–inhibited phosphodiesterase (CGI–PDE).

This class of phosphodiesterase exhibits very characteristic properties including a low K_m for both cyclic AMP and cyclic GMP (usually submicromolar) but with a much greater V_{max} for cyclic AMP than for cyclic GMP (4–10 fold greater). For this reason they are deemed to be cyclic AMP–specific despite the fact that they can hydrolyse cyclic GMP. Characteristically, hydrolysis of cyclic AMP is potently inhibited by cyclic GMP with a $K_i = K_m$ indicating that the inhibition is competitive. They are potently inhibited by a number of inotropic and anti–thrombic agents but are relatively insensitive to inhibition by the phosphodiesterase inhibitors, Ro–20–1724 and Rolipram.

The apparent subcellular localisation of the phosphodiesterase varies depending on the tissue. Whereas, most of the human platelet CGI–PDE appears to be cytosolic (MacPhee, et al., 1986; Grant and Colman, 1984), the rat adipocyte and hepatocyte enzymes both appear to be membrane bound (Heyworth, et al., 1983d; Loten, et al., 1978; Pyne, et al., 1987a; Degerman, et al., 1987). The precise organelle in each of these cases has not been identified, although the rat liver enzyme appears to be located in a unique vesicle fraction termed by Houslay's group the 'dense–vesicle' (Heyworth, et al., 1983d). The subcellular localisation of the enzyme in a particular tissue also appears to depend on the species in question. In the heart, it has been shown that the enzyme from bovine, human and canine sources is membrane bound whilst in guinea pig it is soluble (Harrison, et al., 1986b; Reeves, et al., 1987; Weishaar, et al., 1987a). Such differences in subcellular distribution may imply the existence of distinct tissue and species-specific isozymes of this subclass of phosphodiesterase.

The enzyme has been identified in a large number of tissues and cells including rat liver and hepatocytes (Loten, et al., 1978; Whitson and Appleman, 1982; Pyne, et al., 1987a), adipose tissue (Saltiel and Steigerwalt 1986; Degerman, et al., 1987), human platelets (Umekawa, et al., 1984; Grant and Colman, 1984), ventricular muscle (Weishaar, et al., 1987a; Harrison, et al., 1986b; Harrison, et al., 1988) and 3T3-L1 adipocytes (Manganiello, et al., 1983).

The cyclic GMP-inhibited phosphodiesterase has been purified from a number of sources including rat liver (Whitson and Appleman, 1982; Pyne, et al., 1987a; Boyes and Loten, 1988), rat adipose tissue (Saltiel and Steigerwalt, 1986c; Degerman, et al., 1987), human platelets (Grant and Colman, 1984; Umekawa, et al., 1984) and bovine cardiac muscle (Harrison, et al., 1986; Harrison, et al., 1988). The characteristics of the purified species are summarised in Table 1.4.

1.5.2 The rat liver 'dense-vesicle' phosphodiesterase.

The rat liver enzyme (CGI-PDE or 'dense-vesicle' phosphodiesterase) is membrane bound and can be released in a catalytically active form by endogenous proteases released either by hypotonic shock (Loten, et al., 1978; Loten, et al., 1980; Pyne, et al., 1987a) or by freeze-thawing (Whitson and Appleman, 1982). The enzyme may also be solublilised by controlled chymotrypsin action under isotonic conditions (Boyes and Loten, 1988). Non-ionic detergents such as Triton X-100 (Whitson and Appleman, 1982; Loten, 1983) or anionic detergents such as Triton QS-9 (Loten, 1983) can also release the activity. Whilst the detergent solubilised form probably reflects the holo-enzyme, it has proved difficult to purify since it precipitates out of solution in the absence of detergent (Loten, 1983). Consequently, all purifications of this species from rat liver have involved a proteolytic solubilisation from a crude membrane fraction.

The enzyme is capable of hydrolysing both cyclic AMP and cyclic GMP but appears to have a preference for cyclic AMP (Whitson

and Appleman, 1982; Pyne, et al., 1987a; Boyes and Loten, 1988) and is not stimulated by Ca^{2+}/Calmodulin (Whitson and Appleman, 1982).

Kinetic analysis of cyclic nucleotide hydrolysis indicates that the enzyme has similar high affinities (low K_m) for both cyclic nucleotides (K_m cyclic AMP 0.24–0.5μM, K_m cyclic GMP 0.17–0.43μM) but a much lower V_{max} for cyclic GMP (5–200 times lower). Cyclic GMP acts as a competitive inhibitor of cyclic AMP hydrolysis with a K_i similar to it's K_m (Boyes and Loten, 1988). Whilst the K_m values determined for the high affinity component of cyclic nucleotide hydrolysis agree between the different groups, V_{max} values differ significantly. The highest V_{max} value reported was 6.2 μmol/min/mg for cyclic AMP as substrate and this corresponded to a fold purification in excess of 100,000 over the homogenate (Boyes and Loten, 1988). Lower V_{max} values were reported by Pyne, et al., (1987a) (V_{max1} 0.7 μmol/min/mg, V_{max2} 0.114 μmol/min/mg) and Whitson and Appleman (1982), (V_{max} 0.025 μmol/min/mg). In both instances, these correlated with much lower fold purification values (2,773 fold purification over the homogenate (Pyne, et al., 1986) and 178 fold purification over the homogenate (Whitson and Appleman, 1982).

The reasons for these discrepancies are unclear but it has been suggested that the preparations of Pyne, et al., (1987a) and Whitson and Appleman, (1982) were contaminated with other proteins including a low affinity phosphodiesterase. This was based on the observation that the preparations of Pyne, et al., and Whitson and Appleman both displayed non-linear kinetics for cyclic AMP hydrolysis whilst those of Boyes and Loten displayed linear Michaelis-Menten kinetics (Boyes and Loten, 1988). However, Pyne, et al., assessed purity by a number of different criteria including both non-denaturing and denaturing gels and thermal inactivation measurements and concluded that only one species of phosphodiesterase was present. These differing reports may reflect the purification of different species of enzyme or alternatively, the different solubilisation procedures employed by the three groups may result in the release of differently 'clipped' versions of the CGI

phosphodiesterase which may alter both the kinetic and chromatographic properties of the enzyme. It is well documented that limited proteolysis can alter the properties of certain phosphodiesterases including the Ca^{2+}/Calmodulin-stimulated phosphodiesterase and the cyclic GMP-stimulated phosphodiesterase (Epstein, et al., 1978; Tucker, et al., 1981; Kincaid, et al., 1985; Price, et al., 1987).

The reported subunit molecular weights also differ between different groups. Pyne, et al., (1987a) obtained a protein of molecular weight 57-kDa which formed a dimer of 112-kDa under non-denaturing conditions. An antibody (DV-1) raised to the purified protein could immunoprecipitate a larger form of the enzyme (62-kDa) from cholate extracted membranes indicating that this was the native subunit molecular weight. Whitson and Appleman, (1982) also obtained a single protein band on SDS-PAGE with a molecular weight of 67-kDa whilst the preparation of Boyes and Loten, (1988) displayed a larger subunit molecular weight of 73-kDa which also formed a dimer under non-denaturing conditions.

1.5.3: Cyclic GMP-inhibited phosphodiesterase in other tissues.

A similar activity to the rat liver 'dense-vesicle' phosphodiesterase has been identified in rat adipose tissue (Kono, et al., 1977; Makino, et al., 1982; Weber and Appleman, 1982; Ueda, et al., 1984; Osegawa, et al., 1985; Saltiel and Steigerwalt, 1986c; Degerman, et al., 1987). This species resembles the rat liver 'dense-vesicle' phosphodiesterase in a number of respects, including it's activation by insulin (Kono, et al., 1975), high affinity for cyclic AMP, association with the P-2 fraction, (Manganiello and Vaughan, 1973; Sakai, et al., 1974; Zinman and Hollenberg, 1974) and solubilisation by detergents or by hypotonic shock (Lovell-Smith, et al., 1977; Loten, et al., 1980; Makino, et al., 1980; Makino and Kono, 1980).

Saltiel, et al., (1986c) and Degerman, et al., (1987) have both purified the rat adipocyte enzyme. The former group identified a protein of 60-kDa molecular weight purified some 54 fold from a

Triton X–100 extract of a crude P–2 fraction. This appeared to have a much higher K_m for cyclic AMP (6μM) than the membrane bound form and was less sensitive to inhibition by cyclic GMP (Saltiel, et al., 1986c). The latter group purified a protein of 64–kDa molecular weight some 65,000 fold from a sonicated particulate fraction (Degerman, et al., 1987). The properties of the purified phosphodiesterase resembled the properties of the CGI–PDE isolated by Boyes, et al., (1988) from rat liver, in particular, linear Michaelis–Menten kinetics and high affinities for both cyclic AMP and cyclic GMP. In addition, the V_{max} values obtained for cyclic AMP hydrolysis are very similar between the different purified preparations (8.5 μmol/min/mg for the adipocyte enzyme compared to 6.2 μmol/min/mg for the rat liver enzyme). The actual substrate range used in determining these values was not stated by this group, so it is not possible to assess if the kinetics were linear due to only a narrow substrate concentration range being used. Linear kinetics would be observed if only low cyclic AMP concentrations were analysed. In agreement with the rat liver enzyme studies, the fat derived enzyme also appears to form a dimer under native conditions.

The CGI–PDE has also been purified from bovine heart (16,000 fold) and human platelets (2,000 fold) (Harrison, et al., 1986b; Grant and Colman, 1984). Both enzymes hydrolysed cyclic AMP and cyclic GMP with normal Michaelis–Menten kinetics. For the platelet enzyme the K_m for cyclic AMP was 0.18μM and V_{max} 3 μmol/min/mg, the K_m for cyclic GMP was 0.02μM and V_{max} was 0.3 μmol/min/mg. For the cardiac enzyme, the K_m for cyclic AMP was 0.15μM and the V_{max} was 6 μmol/min/mg. The sensitivity to inhibition of cyclic AMP hydrolysis by cyclic GMP and several cardiotonic drugs is similar for the cardiac (Harrison, et al., 1986b), platelet (Grant and Colman, 1984; MacPhee, et al., 1986) and adipocyte enzymes (Degerman, et al., 1987). The platelet enzyme may also be a dimer (140–kDa) of similar if not identical subunits (62–kDa).

Unequivocal identification of the native subunit molecular weight of the CGI-PDE in bovine heart and human platelets has been achieved using monoclonal antibodies raised to the purified bovine heart phosphodiesterase (CGI-2, 4 & 5) (Harrison, et al., 1986; Harrison, et al., 1988) and to the platelet enzyme (Grant, et al., 1988). The evidence indicates that the native subunit molecular weight of the cardiac CGI-PDE is 110-kDa in fresh extracts and can be readily degraded to 80-kDa presumably by endogenous cardiac proteases. Indeed, the purified phosphodiesterase used to raise these monoclonal antibodies contained largely proteolysed enzyme of 80-kDa, 67-kDa and 60-kDa. Using a monoclonal antibody raised to the cardiac form of the enzyme (CGI-5), MacPhee, et al., (1986) have shown that the human platelet CGI-PDE also has a subunit molecular weight of 110-kDa and that the 62-kDa form of the human platelet enzyme identified by Grant and Colman (1982) may represent a proteolytic fragment. Identical results have been obtained using a monoclonal antibody raised to the purified platelet phosphodiesterase (Grant, et al., 1988).

The activity of this particular species appears to be under hormonal control in tissues such as hepatocytes (Loten, et al., 1978; Heyworth, et al., 1983d; Heyworth, et al., 1984a; Bennelli, et al., 1986; Heyworth, et al., 1986; Gettys, et al., 1987), and adipocytes (Loten and Sneyd, 1970; Zinman and Hollenberg, 1974; Kono, et al., 1975; Weber and Appleman, 1982; Degerman, et al., 1987). In platelets, it may act as the target for a variety of pharmacological agents that inhibit platelet aggregation and hence act as anti-thrombic agents, (Cilostamide and it's derivatives (Umekawa, et al., 1984; MacPhee, et al., 1986), and Ro-15-2041 (Muggli, et al., 1985; MacPhee, et al., 1986). A number of cardiotonic agents such as Milrinone, Fenoximone and Amrinone, which act as positive inotropes, are all potent and selective inhibitors of a bovine heart cyclic GMP-inhibited phosphodiesterase (Harrison, et al., 1986a; Harrison, et al., 1986b). This ability to inhibit the CGI phosphodiesterase selectively has been

proposed to be the mechanism by which these agents exert their positive inotropic effects in the heart since there is a correlation between their ability to act as phosphodiesterase inhibitors and their ability to produce a positive inotropic response (Harrison, et al., 1986a; Weishaar, et al., 1987b).

Despite the presence of several types of phosphodiesterase in adipocytes and hepatocytes, the CGI-PDE may regulate a cyclic AMP pool important in lipolysis and glycogenolysis (Beebe, et al., 1985). Likewise, of several phosphodiesterases in cardiac tissue the CGI-PDE, which seems to be a target for several cardiotonic drugs, may serve a critical role in the regulation of a cyclic AMP pool important in myocardial contractility (Harrison, et al., 1986; Weishaar, et al., 1987b). The possibility thus exists that these 'low-K_m' cyclic AMP phosphodiesterases are isoenzymes involved in the regulation of discrete processes mediated by cyclic AMP or, perhaps of more fundamental importance, may be examples of specific phosphodiesterases regulating functional compartmentalisation of cellular cyclic nucleotide responses.

Weishaar, et al., (1987b) have suggested that functional subclasses of the cyclic AMP-specific phosphodiesterse exist in ventricular muscle and that the cyclic GMP-inhibited subclass of phosphodiesterase is important in modulating the *in vivo* positive inotropic response to agents such as Imazodan, Amrinone and several other cardiotonic agents. They also suggest that species differences that exist in the cardiotonic response to Imazodan and Amrinone can be accounted for by differences in the intracellular localisation of the Imazodan-sensitive phosphodiesterase (CGI-PDE). In species such as the dog, where the ventricular CGI-PDE is membrane bound, then this appears to correlate with strong inotropic responses to Imazodan. In other species, such as the guinea pig and the rat, where the ventricular CGI-PDE appears to be soluble, then responses to Imazodan are much weaker. These results suggest that there may indeed be distinct pools

of cyclic AMP regulating myocardial contractility and that specific subclasses of phosphodiesterase may be important in regulating them.

Further evidence for distinct functional pools of cyclic AMP has been provided by Elks and Manganiello. They reported that in 3T3-L1 cells, both the non-selective phosphodiesterase inhibitor Isobutylmethylxanthine (IBMX) and the inhibitor Ro-20-1724, which is selective for a subclass of the cyclic AMP-selective phosphodiesterase, could enhance differentiation of the 3T3-L1 fibroblasts' into adipocytes. In contrast, the inhibitor Cilostamide, which is selective for the cyclic GMP-inhibited sub-class of cyclic AMP-specific phosphodiesterase, had no effect on differentiation. On this basis, they suggested that a specific pool of cyclic AMP regulated by the Ro-20-1724-sensitive subclass of cyclic AMP-specific phosphodiesterase was important in differentiation (Elks and Manganiello, 1985). These authors have also shown that the Cilostamide-sensitive subclass of the cyclic AMP-specific phosphodiesterase (CGI-PDE) may be more important in regulating lipolysis than the Ro-20-1724-sensitive subclass (Elks and Manganiello, 1984a).

The molecular relationships between the various purified cyclic GMP-inhibited enzymes remains, however, to be established; they may be hormone responsive isozymes which possess similar substrate binding sites as well as sites for a specific class of drugs and/or endogenous effectors such as cyclic GMP. The reported differences in properties between the individual purified species may be due to either tissue specific expression of individual CGI-isozymes or alternatively species specific differences in the physical and kinetic properties of the CGI-PDE. Additionally, given the proteolytic sensitivity observed in this particular class of phosphodiesterase and the probable consequences this has on the properties of the enzyme, an unequivocal identification of the subunit molecular weight of the rat tissue forms of this enzyme is

required to allow comparison to the bovine and human forms of this enzyme.

1.5.4: <u>The Rolipram-sensitive (or cyclic GMP-insensitive) class of cyclic AMP-specific phosphodiesterase.</u>

Although also capable of specifically hydrolysing cyclic AMP, this class of phosphodiesterase appears to differ from the cyclic GMP-inhibited class in a number of respects. In general a number of pharmacological agents can distinguish this activity from other phosphodiesterases such as the CGI-PDE, namely it's sensitivity to Ro-20-1724 and Rolipram (Schering ZK-62711) and it's relative insenstivity to cardiotonic agents such as Cilostamide, Imazodan (CI-914) and Methyl-imazodan (CI-930). In addition, cyclic AMP hydrolysis is insensitive to concentrations of cyclic GMP that potently inhibit the CGI-phosphodiesterase.

This activity has been identified in a large number of tissues (Schneider, et al., 1987) including calf liver (Yamamoto, et al., 1984), canine, human and guinea pig left ventricle (Weishaar, et al., 1987a; Weishaar, et al., 1987b; Reeves, et al., 1987), rat liver, (Whitson and Appleman, 1982; Turnbull and Hickie, 1984; Pyne, et al., 1987b) and rat adipocytes (Weber and Appleman 1982).

Identification of this activity has largely relied upon the ability to separate it from the cyclic GMP-inhibited and cyclic GMP-stimulated phosphodiesterases. In calf liver, for example, chromatography of calf liver supernatant on $N^6-H_2N(CH_2)_s$-cyclic AMP agarose separated the cyclic GMP-stimulated phosphodiesterase from the 'low-K_m' cyclic AMP phosphodiesterase activity which did not bind to this matrix. Further chromatography, on Ultrogel AcA-34, separated the cyclic GMP-inhibited phosphodiesterase from the cyclic GMP-insensitive phosphodiesterase (Yamamoto, et al., 1984a). In canine ventricular muscle the CGI-PDE can be effectively separated from the non CGI-PDE activity, since the former is membrane bound whilst the latter is soluble (Weishaar, et al., 1987a). In tissues where

the two activities reside in the same compartment (both are soluble activities in rat ventricle), pharmacological evidence such as biphasic inhibition curves for DEAE-resolved 'PDE III' to drugs such as Imazodan and Ro-20-1724 has been used to suggest the presence of both the CGI-PDE and non CGI-PDE classes (Weishaar et al., 1987b).

1.5.5: Characteristics of purified forms of cyclic GMP-insensitive phosphodiesterases.

A membrane bound enzyme, which appears to belong to this subclass of cyclic AMP phosphodiesterase has been identified in rat liver plasma membranes. The enzyme behaved as a 'peripheral' protein associated exclusively with the plasma membrane (Marchmont and Houslay, 1980a; Marchmont and Houslay, 1980b; Houslay and Marchmont, 1981) and for this reason has been termed the 'peripheral' plasma membrane phosphodiesterase (PM-PDE). It can be activated by it's phosphorylation which is triggered by insulin (Marchmont and Houslay, 1980b). It is found to be exclusively associated with the plasma-membrane due to it's binding to a specific integral membrane protein (Houslay and Marchmont, 1981).

The enzyme has been extensively purified from both rat liver plasma-membranes (Marchmont, et al., 1981b) and a rat liver particulate fraction (Pyne, et al., 1986b; Houslay, et al., 1988). The purified enzyme from both procedures exhibited anomalous non-linear kinetics with a high affinity K_{m1} for cyclic AMP of 0.7-0.9μM and a V_{max1} of 1.24 μmol/min/mg. The Hill coefficient of 0.54 indicated apparent negative co-operativity. Cyclic GMP acts as a poor substrate for the enzyme with a K_m of 120μM and a V_{max} of 0.4 μmol/min/mg. The IC_{50} for cyclic GMP inhibition of cyclic AMP hydrolysis was 150μM at 0.4μM cyclic AMP. The enzyme was moderately inhibited by Ro-20-1724.

The purified enzyme had a subunit molecular weight of 52-kDa and appeared as a monomer on sucrose density gradient centrifugation,

although a dimeric species could be found on molecular sieving on Sepharose S-200. Tryptic peptide mapping showed it to be distinct from the rat brain Ca^{2+}/Calmodulin-stimulated enzyme and the bovine rod outer segment phosphodiesterase, (Takemoto, et al., 1982). Whitson and Appleman have reported that conversion of the 'dense-vesicle' phosphodiesterase (CGI-PDE) to a cyclic GMP-insensitive form, with altered chromatographic properties, can occur in an extract of rat liver membranes solubilised by freeze-thawing. This raises the possibility that the 'peripheral' plasma-membrane phosphodiesterase may be derived from the 'dense-vesicle' phosphodiesterase (Whitson and Appleman, 1982).

A soluble, cyclic GMP-insensitive, activity has been purified by Thompson, et al., (1979) from canine kidney displaying linear kinetics toward cyclic AMP and cyclic GMP with K_m values of 2.2μM and 312μM respectively. Initial data indicated that the enzyme was a monomer of molecular weight 60-kDa. However, immunological data (Sarada, 1982), indicated that the 60-kDa protein was devoid of phosphodiesterase activity and indeed later work suggests that the enzyme is capable of binding avidly to this 60-kDa protein, with up to 20μg of enzyme bound per mg of 60-kDa protein. A different purification scheme yielded approximately 60μg of protein from 27g of homogenate with a subunit molecular weight of 82-kDa on SDS-PAGE. The relative substrate affinities are identical to the previous report, whilst the V_{max} values for cyclic AMP are somewhat higher at 10-20 μmol/min/mg (Thompson, et al., 1988).

The relationship between these purified species is unclear although it would appear that there may be both soluble and membrane bound enzymes within this subgroup and that these may display distinct properties. Table 1.5 details the properties of the purified species as well as the properties of selected partially purified species. There is a diversity in the subunit molecular weights reported for the purified enzymes. It has been proposed that limited proteolysis

can generate phosphodiesterases of lower molecular weight that still retain catalytic activity (Thompson, et al., 1984).

1.6: HORMONAL REGULATION OF PDE ACTIVITY.

In addition to controlling the synthesis of 'second messengers', hormones may also modulate the degradation of important second messengers such as cyclic AMP and cyclic GMP. The activity of phosphodiesterase appears to be under hormonal control in a number of tissues including rat epididymal fat pads (Loten and Sneyd, 1970; Manganiello and Vaughan, 1973; Zinman and Hollenberg, 1974; Makino, et al., 1980; Makino and Kono, 1980; Weber and Appleman, 1982), rat liver (House, et al., 1972; Tria, et al., 1976; Loten, et al., 1978, Loten, et al., 1980; Marchmont and Houslay, 1980b; Heyworth, et al., 1983d; Keppens and De Wulf, 1984; Benelli, et al., 1986; Pyne, et al., 1987a), human platelets, (MacPhee, et al., 1987; MacPhee, et al., 1988), 1321N1 human astrocytoma cells (Tanner, et al., 1986), rat granulosa cells (Conti, et al., 1984), 3T3-L1 adipocytes (Elks, et al. 1983a; Elks, et al., 1983b), *Xenopus* Oocytes (Sadler and Maller, 1987) and human neutrophils, (Grady and Thomas, 1986). In addition, phosphodiesterase activity may be under negative control since it has been demonstrated that TPA treatment of hepatocytes inhibits total cyclic AMP phosphodiesterase activity (Irvine, et al., 1986) probably through a phosphorylation mechanism, although the precise identity of the inhibited species is unknown.

1.6.1: The anti-lipolytic and anti-glycogenolytic actions of insulin in adipocytes and hepatocytes.

The polypeptide hormones insulin and glucagon are involved in regulating circulating metabolite levels, particularly glucose levels. The anabolic hormone insulin promotes glucose uptake into target tissues such as adipose tissue and muscle, whilst the catabolic hormone glucagon will promote an increase in the blood levels of glucose and free fatty acids by increasing the breakdown of stored glycogen in the

liver and fat in adipose tissue. In addition, it can increase the *de novo* synthesis of glucose by promoting gluconeognesis in the liver. Insulin also has anti-lipolytic actions in adipose tissue (Steinberg, et al., 1975; Strålfors, et al., 1984; Jungas, et al., 1963) and anti-glycogenolytic actions in liver (Denton, et al., 1981). In this respect it counteracts the action of glucagon in liver. The precise mechanism by which this occurs is uncertain but it has been suggested that these effects of insulin are due to an ability of insulin to counteract the actions of cyclic AMP. Such an action may be achieved at several levels.

One potential mechanism that has been investigated is the ability of insulin to decrease intracellular cyclic AMP levels. In adipose tissue and liver for example, insulin has been shown to inhibit the production of cyclic AMP in response to agonists (Ray, 1970; Jungas, 1966; Butcher, et al., 1968; Hepp, 1971). Furthermore, in isolated hepatocytes, insulin can also reduce cyclic AMP levels that have been raised by previous challenge with glucagon (Blackmore, et al., 1979; Heyworth, et al., 1983d; Pilkis, et al., 1975). Since there is no hormonal regulation of cyclic AMP export from tissues such as liver (Pilkis, et al., 1975), a potential mechanism that has been focussed on is the ability of insulin to exert an effect on the activity of phosphodiesterases.

Evidence for such a mechanism of insulin action was provided by Beebe, et al., (1985). They showed that insulin could selectively antagonise the lipolytic and glycogenolytic actions of a variety of analogues of cyclic AMP and this correlated with the ability of these analogues to serve as substrates for phosphodiesterase. Insulin could not antagonise the action of poorly hydrolysed analogues, whilst the reverse was true for analogues that were good substrates for phosphodiesterase (Beebe, et al., 1985).

However, it has also been suggested that insulin may antagonise the action of cyclic AMP by affecting it's ability to activate the cyclic AMP-dependent protein kinase. This model was proposed by Gabbay

and Lardy, (1984), who showed that in hepatocytes, insulin could antagonise the glycogenolytic action of exogenously added cyclic AMP even in the presence of the phosphodiesterase inhibitors IBMX and Ro-20-1724. Under these conditions they concluded that the effect of insulin could be explained by an inhibitory action of insulin on the cyclic AMP-dependent protein kinase.

In isolated rat adipocytes, Wong and Loten, (1981), concluded that the anti-lipolytic action of insulin could be fully accounted for by a decrease in cyclic AMP and that there was no change in the sensitivity of the protein kinase to cyclic AMP. In addition Lonnroth and Smith (1986) and Kather and Scheurer (1987) both concluded that the anti-lipolytic effect of insulin in human adipocytes requires phosphodiesterase activation.

Taken together, these results suggest a role for phosphodiesterase activation in the anti-lipolytic and anti-glycogenolytic actions of insulin.

1.6.2: Desensitisation of cells to cyclic AMP-mediated hormones.

After chronic exposure to hormones which elevate cyclic AMP, cells appear to be able to 'densensitise', 'down-regulate', or produce a state of 'refractoriness'. Hormones such as glucagon and β-adrenergic agents appear to employ a desensitisation phenomena (Heyworth, et al., 1984b; Murphy, et al., 1987; Bouvier, et al., 1988) involving an uncoupling of receptor from the adenylate cylcase. A complimentary, negative feedback mechanism, that dampens the effect on cyclic AMP levels by promoting cyclic AMP disposal in response to elevated cyclic AMP via a phosphodiesterase activation mechanism, has been proposed to explain the transient nature of increases in cyclic AMP levels often observed in stimulated hepatocytes (Corbin, et al., 1985; Heyworth, et al., 1983d), adipocytes (Gettys, et al., 1987) and cardiomyocytes (Gettys, et al., 1987).

1.6.3: Hormonally regulated phosphodiesterases in rat liver.

There appears to be at least two hormonally regulated cyclic AMP-specific phosphodiesterases in rat liver, both of which are membrane bound. The 'peripheral' plasma membrane phosphodiesterase (PM-PDE) can be activated by insulin both *in vitro* (Tria, et al., 1976; Marchmont and Houslay, 1980) and *in vivo* (Heyworth, et al., 1983d). This activity belongs to the subclass of cyclic AMP-specific enzymes that is insensitive to cyclic GMP but sensitive to Ro-20-1724 (Pyne, et al., 1987a).

The second activity has been termed the 'dense-vesicle' enzyme (DV-PDE), since it resides in a membrane fraction that migrates on the high density shoulder of the endoplasmic reticulum when hepatocytes are fractionated on a Percoll gradient (Heyworth, et al., 1983d). Paradoxically, this activity can be activated in intact hepatocytes by both insulin and glucagon (Loten, et al., 1978; Heyworth, et al., 1983d). The enzyme can be distinguished from the 'peripheral' activity by a number of criteria, including the ability of cyclic GMP to potently inhibit cyclic AMP hydrolysis, as such, it belongs to the CGI subclass of cyclic AMP specific phosphodiesterases (Loten, et al., 1978; Pyne, et al., 1987a).

A similar activity has been identified in rat adipocytes, which is also membrane bound, and can be activated by insulin (Manganiello and Vaughan, 1973; Zinman and Hollenberg, 1974), and agents that raise cyclic AMP such as isoproteronol (Makino and Kono, 1980), adrenocorticotrophin (ACTH) and methylxanthines (Pawlson, et al., 1974). Weber and Appleman, (1982) showed that this insulin-stimulated phosphodiesterase in rat adipose tissue was cyclic AMP-specific and also belonged to the cyclic GMP-inhibited class. This species bears a significant resemblance to the insulin and glucagon stimulated 'dense-vesicle' phosphodiesterase in rat liver. Other cells including platelets (MacPhee, et al., 1988), differentiated 3T3-L1 adipocytes (Elks, et al., 1983a: Elks, et al., 1983b) and heart (Harrison,

et al., 1986b) also possess an activity that resembles the 'dense-vesicle' phopshodiesterase.

1.6.4: Insulin activation of the 'peripheral' plasma-membrane PDE.

Activation of the rat liver 'peripheral' plasma membrane cyclic AMP phosphodiesterase by insulin, in isolated plasma membranes, is of particular interest since it undergoes an insulin-mediated cyclic AMP-dependent phosphorylation (Marchmont and Houslay, 1980b), resulting in an increase in cyclic AMP hydrolysis. Phosphorylation of this species elicited a decrease in the apparent Hill coefficient for cyclic AMP from 0.62 to 0.47 such that the activated enzyme displayed greater negative cooperativity (Marchmont and Houslay, 1981). The phosphorylation of this phosphodiesterase from rat liver plasma membranes exhibited a K_a for cyclic AMP and insulin of 1.6×10^{-6} and 10^{-10} respectively (Marchmont and Houslay, 1980b). Utilisation of both the high affinity receptors for insulin and basal intracellular cyclic AMP concentrations suggests that the activation process is physiologically important and indeed it has been observed in intact hepatocytes under basal conditions (Heyworth, et al., 1983d), where intracellular concentrations of cyclic AMP are of the order of 0.3-0.5μM (Exton, et al., 1973; Smith, et al., 1978).

The mechanism of insulin-induced activation of the enzyme is unclear, but the requirement for cyclic AMP in the process indicates the involvement of the cyclic AMP-dependent protein kinase as does the ability of an A-kinase inhibitor to block both phosphorylation and activation (Marchmont and Houslay, 1980b). Marchmont and Houslay (1981) were able to purify the 'peripheral' phosphodiesterase in an active state from insulin-treated membranes. They concluded that the phosphorylation was alkali-labile and occured on serine residues. However, subsequent work, using an anti-sera raised to the 'peripheral' plasma-membrane phosphodiesterase to immunoprecipitate the enzyme after insulin challenge of intact cells, indicated that the enzyme was phosphorylated on alkali-stable tyrosine residues (Pyne, et al., 1989).

It would thus appear that the mechanism whereby insulin treatment can lead to the phosphorylation and activation of this enzyme in broken membranes does not reflect that employed in intact cells.

1.6.5: Evidence for the involvement of a G-protein in insulin's actions on phosphodiesterase activation.

In intact cells, the 'peripheral' plasma-membrane phosphodiesterase is not activated by glucagon treatment or by agents which increase intracellular cyclic AMP, such as dibutyryl cyclic AMP or IBMX (Heyworth, et al., 1983d). It can, however, be activated by cholera toxin, an agent which causes the NAD^+-dependent ribosylation and activation of the guanine nucleotide binding protein G_s. This observation has been taken to imply the involvement of a guanine nucleotide binding protein in the insulin-induced activation of the 'peripheral' plasma membrane phosphodiesterase. In support of this contention, guanine nucleotides have been shown to activate the insulin stimulated phosphodiesterase in isolated plasma membranes (Heyworth, et al., 1983c). A similar observation was made in both rat brain and adipocyte membranes where guanine nucleotides were found to stimulate membrane bound high affinity cyclic AMP-specific phosphodiesterase activity (deMazancourt and Guidicelli, 1984a; deMazancourt and Guidicelli, 1984b). This putative G protein is unlikely to be either G_s or G_i; glucagon, an activator of G_s, does not activate the enzyme (Heyworth, et al., 1983d) and the insulin induced activation is insensitive to pertussis toxin (Heyworth, et al., 1986), an agent that causes the uncoupling of receptor-mediated activation of G_i

Houslay, (1986) has proposed that a novel G-protein (G_{ins}) accounts for this particular phenomenon. G_{ins} resembles G_s in that cholera toxin elicits NAD^+-dependent ribosylation of both of these G-proteins (Heyworth, et al., 1985a). This results in stimulation of G_{ins} and activation of the 'peripheral' plasma membrane phosphodiesterase in a similar manner to toxin elicited activation of adenylate cyclase via G_s (Heyworth, et al., 1983d). Surprisingly, in

intact hepatocytes, glucagon can bring about the inactivation of G_{ins} through occupancy of a unique fraction of high affinity glucagon receptors. This results in insulin being unable to activate the 'peripheral' plasma membrane phosphodiesterase (Heyworth, et al. 1983a). The occupancy of such receptors also elicits the densensitisation of hormonally stimulated adenylate cyclase (Heyworth, et al., 1983d; Wakelam, et al., 1986; Murphy, et al., 1987).

Intriguingly, adenosine and N^6-phenylisopropyl adenosine (PIA) reverse the modification of G_{ins} elicited by glucagon, such that insulin can now trigger activation of the phosphodiesterase (Wallace, et al., 1984). Indeed, hepatocytes that are pre-treated with adenosine prior to glucagon and then insulin, express an increase in the rate of cyclic AMP degradation compared to control cells (Heyworth, et al., 1984). Phorbol esters such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA) mimic the action of adenosine and PIA in releasing glucagon's block upon the ability of insulin to activate the 'peripheral' plasma-membrane enzyme (Heyworth, et al., 1985b). As phorbol esters mimic diacylglycerol in activating C-Kinase (Nishizuka, 1983) these studies indicate that G_{ins} maybe a substrate for phosphorylation by this kinase and therefore sensitive to a multitude of other hormones that may elicit differential effects upon the ability of insulin to stimulate the plasma membrane phosphodiesterase.

1.6.6: Hormonal activation of the 'dense-vesicle' phosphodiesterase.

The 'dense-vesicle' phosphodiesterase can be activated by exposure of intact isolated hepatocytes to insulin, glucagon or agents that mimic cyclic AMP in activating the cyclic AMP-dependent protein kinase, such as dibutyryl cyclic AMP (Loten, et al., 1978; Heyworth, et al., 1983d). Activation can be observed in the proteolytically solubilised enzyme (Loten, et al., 1978) and also in the membrane bound form, isolated by Percoll fractionation (Heyworth, et al., 1983d). The mechanisms employed by glucagon and insulin to activate the 'dense-vesicle' phosphodiesterase appear to be different. Glucagon has

been proposed to mediate activation of the enzyme through an activation of the cyclic AMP-dependent protein kinase by elevated cyclic AMP levels. Insulin cannot activate the enzyme by raising cyclic AMP levels since it has been shown to decrease cyclic AMP levels in hepatocytes (Heyworth, et al., 1983d).

The glucagon mediated activation is energy dependent since depletion of intracellular ATP levels causes an impairment of the ability of glucagon to activate the enzyme (Boyes, et al., 1981; Heyworth, et al., 1984a). Such data have been taken to imply the involvement of the cyclic AMP-dependent protein kinase in the glucagon mediated activation of the 'dense-vesicle' enzyme (Loten, et al., 1978; Heyworth, et al., 1983d).

Kilgour, et al., (1989), showed that in isolated hepatocyte membranes, a purified preparation of cyclic AMP-dependent protein kinase (A-Kinase) could activate the 'dense-vesicle' phosphodiesterase provided that the membranes had been pre-treated with $MgCl_2$ to activate phosphatases. This activation corresponded with the labelling of proteins of molecular weight 57-kDa and 51-kDa corresponding to the proteolytically 'clipped' forms of the enzyme. Dephosphorylation of the enzyme, whilst necessary to enable subsequent phosphorylation by A-Kinase, does not cause any change in activity. This has led to the proposal that there are two regulatory sites on the enzyme which can be phosphorylated. One being a site for phosphorylation by A-kinase, which enhances enzyme activity, and the other a 'silent' site which prevents phosphorylation and activation by A-kinase. One suggestion is that the silent phosphorylation of this enzyme might be elicited by the 5'-AMP-stimulated kinase. This would be analagous to the situation with acetyl CoA carboxylase (Munday, et al., 1988) where phosphorylation by the 5'-AMP-stimulated kinase (Carling, et al., 1987) results as a consequence of cells becoming anoxic upon breakage leading to an increase in 5'-AMP and activation of the enzyme. The resultant effect is the phosphorylation of acetyl CoA carboxylase on a site which prevents protein kinase A from

phosphorylating and activating this enzyme. Indeed, such an effect has also been observed with HMG–CoA–reducatase (Carling, et al., 1987).

The adipocyte enzyme may also be activated by phosphorylation by cyclic AMP–dependent protein kinase. *In vitro* addition of the catalytic subunit of cyclic AMP–dependent protein kinase to a crude particulate fraction could elicit the activation of phosphodiesterase activity (Gettys, et al., 1988). However, unlike in the hepatocyte, it was not necessary to first dephosphorylate the enzyme, indicating that different control mechanisms may exist between hepatocytes and adipocytes.

The observation that the proteolytically solubilised enzyme from adipocytes and hepatocytes is not a substrate for the cyclic AMP–dependent protein kinase may indicate that the putative phosphorylation site lies on the membrane pedicle that anchors the enzyme to the membrane. Evidence for such an hypothesis exists for the adipocyte enzyme since detergent solubilised enzyme can be activated by addition of the purified catalytic subunit of A–Kinase whilst the proteolytically solubilised form can not (Gettys, et al., 1988). Phosphorylation may alter the conformation of the enzyme leading to a loss of a tonic inhibition, in an analagous manner to the Calmodulin induced release of tonic inhibition proposed to occur for the Ca^{2+}/Calmodulin–stimulated phosphodiesterase (Kincaid, et al., 1985).

The mechanism employed by insulin in activating the 'dense–vesicle' phosphodiesterase is unknown in detail but presumably differs from that of glucagon, since insulin does not raise cyclic AMP levels. It is likely that a membrane trafficking event is involved since preincubation of hepatocytes with lysosomotropic agents, such as NH_4Cl, chloroquine and methylamine and lowering of the incubation temperature to 22°C all blocked insulin's activation of the enzmye (Heyworth, et al., 1984a).

It is well established that the insulin receptor can internalise as a result of insulin binding to hepatocytes (Gordon, et al., 1980;

Denton, et al., 1981) and this may be involved in insulin 'signalling'. This process may be the 'membrane trafficking' event that appears to be required for insulin activation of the enzyme. However, results from Benelli, et al., (1986), using *in vivo* administration of insulin, indicated that insulin receptor internalisation was not required to activate a liver Golgi-associated phosphodiesterase, although the relationship between this species and the 'peripheral' and 'dense-vesicle' activities is unknown. Alternatively, it is possible that it is the trafficking of the 'dense-vesicle' enzyme itself that is responsible for it's activation by insulin and indeed insulin might initiate the translocation of the enzyme to the 'dense-vesicle' location where it is subsequently activated. The insulin mechanism may also involve a pertussis toxin substrate as pertussis toxin treatment of intact hepatocytes blocked the ability of insulin to activate the 'dense-vesicle' phosphodiesterase but interestingly, not activation of the 'peripheral' phosphodiesterase. (Heyworth, et al. 1986). Such an effect has also been observed for insulin activation of the CGI-PDE in 3T3-L1 adipocytes (Elks, et al., 1983).

1.6.7: Hormonal regulation of phosphodiesterases in other cell types.

The regulation of a cyclic GMP-inhibited phosphodiesterase (CGI-PDE) by agents that increase cyclic AMP levels may be a control mechanism common to a number of other cell types. In platelets, for example, hormones such as PGI_2 and PGE_1, which activate adenylate cyclase, are known to rapidly increase 'low-K_m' cyclic AMP phosphodiesterase activity (Alvarez, et al., 1981; Hamet, et al., 1983; Goldberg, et al., 1984). MacPhee, et al., (1988) have shown that the activation *in vivo* corresponds with phosphorylation of the 110-kDa subunit of the CGI-PDE and this effect could be mimicked *in vitro* by addition of the catalytic subunit of A-kinase, using immunoadsorbed phosphodiesterase as substrate. Similar studies using an immunoadsorbed cyclic GMP-inhibited phosphodiesterase from bovine heart showed that the 110-kDa subunit of the enzyme could be phosphorylated *in vitro* by the catalytic subunit of the cyclic AMP-

dependent protein kinase. This phosphorylation resulted in the activation of cyclic AMP phosphodiesterase activity, although the effect was small (20–50% stimulation of activity) (Reifsnyder, et al., 1987).

1.6.8: Role for a putative third insulin–sensitive PDE in rat liver.

Although insulin can activate at least two distinct membrane bound phosphodiesterases, the role of these enzymes in the mechanism whereby insulin can decrease cyclic AMP levels that have previously been raised by high (10nM) glucagon has been questioned. Using agents such as NH_4Cl or fructose to block insulin induced activation of the 'dense–vesicle' phosphodiesterase, Heyworth, et al., (1984) showed that insulin could still decrease cyclic AMP levels that had previously been raised by glucagon implying that this enzyme does not play an important role in this action of insulin. Under these conditions of prior glucagon challenge, insulin cannot activate the plasma membrane phosphodiesterase (Heyworth, et al., 1983).

It seems therefore that this effect of insulin is mediated by one or other of the multiple forms of phosphodiesterase found in the hepatocyte. Insulin's activation of such a phosphodiesterase is presumably either extremely labile or alternatively, reversible in nature. Two potential candidates are the Ca^{2+}/Calmodulin stimulated phosphodiesterase and the cyclic GMP–stimulated phosphodiesterase. A direct activation of the former would appear to be unlikely as insulin antagonises the action of hormones such as vasopressin, angiotensin and α_1–adrenergic agonists which result in an increase in the presumptive activator cytosolic calcium. The latter enzyme may be activated in a reversible manner by the purported small increase in cyclic GMP that occurs when hepatocytes are exposed to insulin (Czech, 1977). These two enzymes may however be reversibly activated by other mechanisms. Pyne, et al., (1988) have shown that the cyclic GMP–stimulated phosphodiesterase can be activated by a preparation of the putative insulin mediator (Saltiel, et al., 1986c) and it is possible that the same may be true of the Ca^{2+}/Calmodulin–stimulated phosphodiesterase.

However, the precise nature of the 'third' insulin-stimulated phosphodiesterase needs further investigation.

These observations do not however rule out a role of the 'dense-vesicle' and 'peripheral' phosphodiesterase in some of insulin's actions. For example, if hepatocytes are treated with PIA before glucagon challenge to block glucagon's ability to prevent the insulin activation of the 'peripheral' phosphodiesterase then the ability of insulin to decrease cyclic AMP levels was nearly doubled (Heyworth, et al., 1984a) implying that under these conditions the 'peripheral' phosphodiesterase plays an important role in decreasing cyclic AMP levels. Thus, it would appear that under various conditions, different phosphodiesterases may contribute to the ability of insulin to decrease cyclic AMP levels by a phosphodiesterase activation mechanism.

1.7: OTHER PHOSPHODIESTERASES.

A number of other phosphodiesterases that cannot be easily fitted into any of the well defined categories of phosphodiesterase have also been identified (and in some cases purified) by a number of workers.

1.7.1: 5'-Nucleotide phosphodiesterase.

A cyclic nucleotide phosphodiesterase that is not specific for purine or pyrimidine 3'5' cyclic phosphate has been identified and purified by Butler and colleauges (Kelly and Butler, 1975; Kelly and Butler, 1977; Landt and Butler, 1978). The enzyme has been purified from bovine intestine and shown to hydrolyse a wide range of phosphoesters. In addition to the hydrolysis of nucleic acids at the 3' end to yield 5'-nucleotides, the enzyme can also cleaves NAD, ADP, ATP and cyclic 3'5'-AMP to yield 5'-AMP (Kelly and Butler, 1975). Cyclic AMP can be hydrolysed efficiently (K_m of below 100μM, V_{max} of 7 μmol/min/mg). The reaction mechanism involves the participation

of a high energy phospho-enzyme intermediate (Landt and Butler, 1978).

1.7.2: Cytidine 3':5'-monophosphate phosphodiesterases.

A multi-functional phosphodiesterase capable of hydrolysing both purine and pyrimidine cyclic nucleotides has been identified and purified from pig liver by Helfman and colleauges. The purified enzyme appeared to behave as a monomer of molecular weight 31,000-37,000 under native conditions. It lacked absolute substrate specificity, hydrolysing both 3'5'-cyclic CMP and 3'5'-cyclic AMP to a comparable degree, and to a lesser extent 3'5'-cyclic GMP, yielding 5' nucleotides as products. The apparent K_m for cyclic CMP (182µM) was higher than the apparent K_m for cyclic AMP (25µM). The V_{max} value for cCMP (4.1 µmol/min/mg) was, however, higher than the V_{max} for cAMP (1.6 µmol/min/mg) (Helfman, et al., 1981).

Later studies showed that the enzyme could catalyse the hydrolysis of both purine and pyrimidine cyclic 2'3'-nucleotides, the product of hydrolysis being either 2'- or 3'- nucleotides depending on the substrate. Hydrolysis of 2'3'-cyclic CMP or 2'3'-cyclic UMP yielded predominantly 3'-nucleotides. In contrast, hydrolysis of 2'3'-cyclic AMP produced equal amounts of 2'- and 3'- derivatives, whilst the major product formed from 2'3'-cyclic GMP was the 2'-nucleotide (Helfman and Kuo, 1982).

In addition to this multi-functional enzyme, a phosphodiesterase capable of hydrolysing 3'5'-cyclic CMP but not 3'5'-cyclic AMP or 3'5'-cyclic GMP has been identified in Leukemia L-1210 cells (Cheng and Bloch, 1978) and in a variety of rat tissues (Kuo, et al., 1978b). Highest levels of activity were identified in liver, kidney and intestine with much lower levels observed in skeletal muscle, cerebellum, aorta and blood cells. This activity clearly differed from the multi-functional enzyme since it was selective for cyclic CMP as substrate, exhibiting a K_m of 2.4mM. In contrast, the multi-functional enzyme exhibited a K_m of 182µM for cyclic CMP hydrolysis

(Kuo, et al., 1978; Helfman, et al., 1981). These data would seem to indicate then that as for other phosphodiesterase classes there is also a multiplicity of phosphodiesterases capable of hydrolysing cyclic CMP.

1.7.3: Non-mammalian phosphodiesterases.

The phosphodiesterases in the eukaryotes, *Saccharomyces cerevisiae* (Bakers yeast), *Drosophila melanogaster* and *Dictyostelium discoideum* have been studied extensively by a number of groups. They have played an important role in recent studies on the molecular genetics of phosphodiesterase genes.

1.7.4: *Drosophila melanogaster* phosphodiesterases.

Drosophila contains two major types of cyclic AMP phosphodiesterase, a Ca^{2+}/Calmodulin-stimulated form and a cyclic AMP-specific form (Davis and Kauvar, 1984) The Ca^{2+}/Calmodulin-stimulated form is similar in properties to it's mammalian counterparts. The enzyme can hydrolyse both cyclic AMP and cyclic GMP and is activated by trypsin treatment. However, it appears to behave as a monomer of subunit molecular weight 120-kDa, which distinguishes it from the mammalian forms of this enzyme (for review see Davis and Kauvar, 1984).

The cyclic AMP-specific form has been much more extensively studied. It appears that the cyclic AMP-specific form is implicated in learning and/or memory processes in *Drosophila*. The gene which codes for the *Drosophila melanogaster* phosphodiesterase (PDE 2) is housed at the dunce (dnc) locus (Chen, et al., 1986), mutations at which, produce progeny that show defective conditioned behaviour in several different situations in which associative learning is evaluated (Dudai, et al., 1976; Tempel, et al., 1983).

The enzyme has been estimated to have a molecular mass of between 35-kDa and 70-kDa (Davis and Kauvar, 1984). cDNA clones encoding the transcripts of the ducne gene have been isolated and sequenced (Chen, et al., 1986). The amino acid sequence predicts a

protein of molecular weight 40-kDa (within the estimated values) which has a high degree of homology with the 61-kDa subunit of the bovine Ca^{2+}/Calmodulin-stimulated isoenzyme. Within this domain the degree of homology between the two sequences was 42% (Charbonneau, et al., 1986).

1.7.4.1: Use of the *Drosophila melanogaster* cDNA clone to isolate mammalian phosphodiesterase cDNA clones.

A probe corresponding to a portion of the dunce gene was used to screen a rat brain cDNA library at low stringency (Davis, et al., 1989) and isolate several indepenent cDNA clones. Sequence analysis of the longest cDNA clone (termed RD1) revealed that it contained an open reading frame which translated into a conceptual protein of molecular weight 68-kDa containing 610 amino acids. Two additional rat brain cDNA clones (RD2 and RD3) also isolated with the *Drosophila* probe appeared identical to RD1 over the majority of their lengths but showed sequence variations towards their N-termini. These clones were, however, incomplete at their C-termini so these workers were unable to assess sequence variations in this region. These workers have suggested that the three PDE clones isolated represent transcripts that arise by alternative splicing of messenger RNA from a single gene. Based on genomic blotting experiments these workers have also suggested the presence of at least one other rat gene homologous to this putative gene (Davis, et al., 1989).

An alignment of the conceptual translation sequence of RD1 with representatives of several subfamilies of phosphodiesterase (the Ca^{+}/Calmodulin-stimulated phosphodiesterase, the cyclic GMP-stimulated phosphodiesterase and the α-subunit of the retinal cyclic GMP phosphodiesterase (all from bovine sources) and the *Drospophila* cyclic AMP phosphodiesterase) revealed the presence of a conserved domain of approximately 270 amino acids. Within this region, the homology of RD1 with the *Drosophila* sequence was very high, with 75% of the residues showing absolute identity. The degree of

homology with the other phosphodiesterases ranged from 20–40% identity (Davis, et al., 1989).

Swinnen and co–workers (Swinnen, et al., 1989) also used a cDNA clone encoding the *Drosphila melanogaster* dunce phosphodiesterase as a probe to screen both rat testis and rat sertoli cell cDNA libraries. They isolated four different groups of clones, termed ratPDE1, ratPDE2, ratPDE3 and ratPDE4; the former two from a rat testis library and the latter two from the rat sertoli cell library. All four of these clones once again contained a region of approximately 270 amino acids where the amino acid sequence was highly conserved between both these individual clones and between known sequences of the other phosphodiesterases. Within this conserved region, the four ratPDE clones had very similar sequences but were not identical. However, at the amino and carboxyl termini the sequences shared little or no homology with each other (Swinnen, et al., 1989).

The molecular weights of the presumptive proteins encoded by ratPDE2, ratPDE3 and ratPDE4 were estimated to be 68–kDa, 67–kDa and 64–kDa respectively. In the case of the ratPDE1 clone the actual molecular weight could not be estimated since the clone was only partial in length but the minimum expected molecular weight was 47–kDa (Swinnen, et al., 1989).

These clones were used in Northern blot analyses to assess the cell distribution of ratPDE transcripts in the various cell types of the seminiferous tubule. RatPDE1 and ratPDE2 identified transcripts mainly in germ cells whilst ratPDE3 and ratPDE4 identified transcripts in the Sertoli cell with only faint hybridisation observed in peritubular and germ cell RNA. The distribution of the RNA transcripts corresponding to the four cDNA clones was also assessed in a number of different rat tissues including brain, heart, liver, kidney and testis. RatPDE1 identified a number of major transcripts in both kidney and testis with only minor transcripts identified in the other tissues. RatPDE2, on the other hand, identified major transcripts in all the

tissues examined except liver. All the organs tested expressed transcripts corresponding to ratPDE4 and ratPDE3 (Swinnen, et al., 1989).

Analysis of the entire nucleotide sequences showed that there was no sequence longer than 32 nucleotides in which any two of the four groups of cDNA clones had identical sequences. On the basis of this observation, and the differential expression of these transcripts in various cell types, these workers have proposed that these four clones represent transcripts from different genes rather than being derived from a single gene by alternative splicing (Swinnen, et al., 1989).

Using a different approach, involving complementation of a mutant in the yeast *Saccharomyces cerevisiae*, Colicelli and co-workers have isolated and characterised a rat brain cDNA encoding a high affinity cyclic AMP phosphodiesterase. This was found to be highly homologous to the cAMP phosphodiesterase encoded by the *dunce* locus of *Drosophila melanogaster* (Colicelli, et al., 1989). They have called the gene DPD (dunce-like phosphodiesterase). The cloned cDNA was capable of coding for a putative protein of molecular weight 60-kDa which was highly homologous to the *D.melanogaster dunce* gene product over a 252 amino acid region located in the centre of the rat DPD cDNA (over 80% identity). Using expression of the DPD-cDNA in the yeast *Saccharomyces cerevisiae*, these workers showed that the clone coded for a phosphodiesterase which had a high affinity for cyclic AMP (K_m of 3.5μM), was insensitive to Ca^{2+}/Calmodulin, did not hydrolyse cyclic GMP and was insensitive to inhibition by cyclic GMP. This cDNA was similar to, though distinct from, the three cDNAs isolated from a rat brain cDNA library by Davis, et al., (1989). Although these putative proteins shared over 93% identity in the central conserved region of the molecule this homology fell off dramatically in the flanking regions (amino and carboxyl termini) (Davis, et al., 1989; Colicelli, et al., 1989).

A comparison of the properties of the *Drosophila* dunce, RD1 and DPD cDNA products revealed that whilst all three coded for high affinity phosphodiesterases (K_m values between 2 and 4μM) the three activities were different with respect to their sensitivity to phosphodiesterase inhibitors. Whilst both RD1 and DPD phosphodiesterases are sensitive to inhibition by Rolipram and Ro-20-1724, the *Drosophila* dunce product is much less so. All three activities were not found to be sensitive to inhibition by Fenoximone and cyclic GMP; agents which exert a selective inhibitory effect on the cyclic GMP-inhibited subclass of phosphodiesterase (Henkel-Tigges and Davis, 1990).

1.7.5: *Saccharomyces cerevisiae* phosphodiesterases.

The Bakers yeast *Saccharomyces cerevisiae* contains both 'high-K_m' and 'low-K_m'-cyclic AMP phosphodiesterases. The 'high-K_m' enzyme has been purified to homogeneity and was shown to exist as a dimer of similar if not identical subunits of molecular weight 43-kDa (Londesborough and Suoranta, 1983). It is not, however, specific for cyclic AMP as substrate since it can also hydrolyse cyclic GMP (K_m values of 250μM and 160μM respectively) (Fujimoto, et al., 1974). This phosphodiesterase is unusual since it is active in the absence of added divalent metal cations (Fujimoto, et al., 1974). It has been shown that purified preparations of the enzyme contain 2.4 atoms of zinc/43,000 subunit (Londesborough and Suoranta, 1983).

The 'low-K_m' form has also been purified (Suoranta and Londesborough, 1984). This phosphodiesterase was found to be specific for cyclic AMP as substrate (K_m=0.2μM) and has a molecular weight of 61-kDa, although if protease inhibitors were omitted during purification, the active fraction contains subfragments of 45-kDa and 17-kDa. Like the 'high-K_m', enzyme this phosphodiesterase was also a zinc requiring enzyme. It was apparent however, from an analysis of their respective amino acid compositions, that these two activities were

distinct from one another (Londesborough and Suoranta, 1983; Suoranta and Londesborough, 1984).

1.7.5.1: Genes for *Saccharomyces cerevisiae* phosphodiesterase.

The structural gene for the 'low-K_m' phosphodiesterase (termed PDE2) has been isolated by Wigler and his co-workers by searching for genomic sequences which suppress the phenotypes of the *ras*2^{val19} mutation when carried on a high copy number plasmid (Sass, et al., 1986). The open reading frame contained within the PDE2 gene was found to be 526 amino acids long, predicting a protein product of 61-kDa, in excellent size agreement with the purified protein (Suoranta and Londesborough, 1984; Sass, et al., 1986). The predicted amino acid sequence is homologous to other phosphodiesterases including the *Drosophila* cAMP PDE and the bovine Ca^{2+}/Calmodulin- and cyclic GMP-stimulated phosphodiesterases (Charbonneau, et al., 1986). The gene was shown to be capable of expressing phosphodiesterase activity in yeast cells and exhibited a K_m for cyclic AMP hydrolysis of 1μM when partially purified preparations were analysed (Sass, et al., ,1986).

The PDE1 gene which codes for the 'high-K_m' enzyme has also been isolated by Wigler and co-workers using an identical strategy to that employed for the 'low-K_m' enzyme (Nikawa, et al., 1987). The clone isolated had an open reading frame of 369 amino acids, predicting a protein molecule of 42-kDa, in agreement with the estimates for the purified protein (Londesborough and Suoranta, 1983). In contrast to the 'low K_m' phosphodiesterase, the amino acid sequence of this enzyme demonstrates no homology with any known phosphodiesterase except one, the cAMP phosphodiesterase from *Dictyostelium discoideum* (see section 1.7.6.1).

1.7.6: *Dictyostelium discoideum* phosphodiesterases.

In this organism, the phosphodiesterase activity is found in both membrane bound and extracellular forms. The extracellular form of the enzyme has been purified and shown to consist of two proteins of molecular weight 48-kDa and 50-kDa both of which exhibit

phosphodiesterase activity. The enzyme appears to exist as a monomeric species, (Orlow, et al., 1981). This enzyme has a low K_m for cyclic AMP (8µM), while the K_m for cyclic GMP is 25µM (Shapiro, et al., 1983). It may be converted to a high K_m form (2mM) (Chang, 1968) upon interaction with an inhibitor protein (Kessin, et al., 1979). The enzyme is unusual in that it exhibits a high isoelectric point of between 7.5 and 9 and unlike most other phosphodiesterases is unable to bind to anion-exchange resins such as DE-52 (Orlow, et al., 1981).

A high molecular weight form of the enzyme (150,000-200,000), which has an isoelectric point of 5, can be isolated from cells. This activity appears to result from aggregation of the low molecular weight form of the enzyme with an acidic protein. The high molecular weight form of the enzyme can be converted to the low molecular weight momomer by isoelectricfocussing in the presence of 6M urea. Under these conditions the isoelectric point of the enzyme is shifted to that of the monomeric form. The two species have been shown to be immunologically indistinguishable from one another after urea treatment and to react identically with the cyclic nucleotide phosphodiesterase inhibitor protein (Orlow, et al., 1981; Franke and Kessin, et al., 1981).

1.7.6.1: Isolation of a cDNA encoding the extracellular phosphodiesterase of *Dictyostelium discoideum.*

The purified extracellular phosphodiesterase was used to a obtain partial amino acid sequence for the design of oligonucleotide probes. The cDNA isolated contained an open reading frame capable of coding for a protein of molecular weight 51-kDa (LaCombe, et al., 1986). The predicted amino acid sequence showed no homology with any of the known sequences except for the yeast PDE1 gene product which encodes the 'high-K_m' enzyme (LaCombe, et al., 1986; Nikawa, et al., 1987).

1.8: PHOSPHODIESTERASE INHIBITORS.

Inhibition of phosphodiesterase activity has been reported for many different classes of compounds. These include inhibitors that are presumed to interact directly with the phosphodiesterase itself and also compounds that inhibit the action of phosphodiesterase effector molecules such as the so called 'Calmodulin antagonists' (see Section 1.3.7). In general, inhibitors of the former type may be divided into two classes; those that show non-selective actions against the different phosphodiesterase classes/isozymes and those that exhibit selectivity for the different forms (for review see Weishaar, et al., 1985). Examples of the non-selective inhibitors include papaverine, theophylline, and 3-iosbutyl-1-methylxanthine (IBMX) (Wells, et al., 1975). The structures of these non-selective inhibitors are shown in Figure 1.3.

A number of selective phosphodiesterase inhibitors have been described, that appear to show selectivity for different classes of phosphodiesterase. In general, the action of these inhibitors has been assessed using Peaks from anion-exchange chromatography of tissue extracts. It is now clear that there are several isozymes of each of the individual phosphodiesterase classes which may be represented in such peaks. Whilst these isoenzymes may be similar they may also show different susceptibility to inhibitors. However, the total number of distinct isozymes for each of the different classes has yet to be determined. Consequently, it has not yet proved possible to assess the precise isozyme distribution pattern between different tissues and/or species. Therefore, the actual inhibition profiles obtained will most likely represent an average effect on all of the individual types present. This fact must be borne in mind when reviewing the actions of the well known phosphodiesterase inhibitors against partially purified preparations of phosphodiesterase

1.8.1: 'PDE-I' inhibitors..

For the purposes of this introduction, the term 'PDE-I' refers to all of the Ca^{2+}/Calmodulin-stimulated isozymes as well as the

cyclic GMP-specific phosphodiesterases. Agents that exert a selective effect on this class of phosphodiesterase include HA-558, TCV-3B, (Kariya, et al. , 1982; Weishaar, et al., 1983), Zaprinast (M&B 22,948) and ICI 74,197 (Bergstrand, et al., 1977). Hidaka and coworkers have shown that HA-558 and TCV-3B exert comparable inhibitory effects on Calmodulin-stimulated and basal phosphodiesterase activities (Hidaka, et al., 1984; Hidaka and Endo, 1984). On the basis of this difference, it has been proposed that these latter agents selectively inhibit the hydrolytic site on the Calmodulin-stimulated phosphodiesterase rather than exerting any direct inhibitory effect on Calmodulin or the Calmodulin binding site on phosphodiesterase.

Bergstrand and co-workers showed that the anti-allergic agents, Zaprinast (M&B 22948) and ICI 74,197, were selective inhibitors of a partially purified preparation of the Ca^{2+}/Calmodulin-stimulated phosphodiesterase isolated from human lung by anion-exchange chromatography (Bergstrand, et al., 1978). More recently, it has become apparent that inhibitors such as Zaprinast also show inhibitory action against the Ca^{2+}/Calmodulin-insensitive cyclic GMP-selective phosphodiesterase as well as the Ca^{2+}/Calmodulin-stimulated phosphodiesterase (isolated from human platelets and bovine coronary arteries respectively). The IC_{50}s for inhibition were very similar being 1.3μM and 3.4μM respectively for the two enzymes (Quade, et al., 1984).

Hidaka and Endo (1984) showed that MY-5,445 could distinguish between these two classes of phosphodiesterase. It potently inhibited a cyclic GMP-specific phosphodiesterase from human platelets (K_i =0.5μM) whilst exerting a lesser inhibitory effect on the cyclic GMP-selective Ca^{2+}/Calmodulin phosphodiesterase from brain (K_i =9.5μM). The structures of all these inhibitors are shown in Figure 1.4.

1.8.2: 'PDE – II' inhibitors.

This is generally referred to as the cyclic GMP – stimulated phosphodiesterase (see sections 1.4.8 – 1.4.12). No agents have yet been identified that are truly selective agents for this class of enzyme. Whilst dipyridamole, AR – L 57 and Sulmazole (AR – L 115BS) all exert a somewhat greater inhibitory effect on cardiac 'PDE – II' than on 'PDE – I' or 'PDE – III', dipyridamole inhibits 'PDE – I' and 'PDE – II' to comparable degrees in platelets. (Quade, et al., 1984; Weishaar, et al., 1986). The structures of these inhibitors are shown in Figure 1.5.

1.8.3: 'PDE – III' inhibitors.

This class represents the cyclic AMP – specific phosphodiesterases and includes the cyclic GMP – inhibited and cyclic GMP – insensitive subclasses. Much attention has focussed on developing selective inhibitors of these classes of phosphodiesterase, particularly the cyclic GMP – inhibited form in cardiac muscle and platelets, since 'PDE – III' inhibitors have been shown to possess cardiotonic and anti – thrombic activities (Kariya, et al., 1982; Umekawa, et al., 1984; Muggli, et al., 1985; MacPhee, et al., 1986; Goldenberg, et al., 1987).

1.8.3.1: Positive Inotropic agents.

These were originally developed to replace the cardiac glycosides, such as digitalis, for the treatment of congestive heart failure. The rational for inotropic therapy is based on the observation that the contractility of failing myocardium can be augmented (Dyke, et al., 1975). Studies in animal models with myocardial dysfunction and in the failing human heart have suggested the existence of a residual myocardial contractile reserve. The cardiac glycosides have a low therapeutic toxic ratio and a limited inotropic potential so a number of new inotropic agents were developed in the hope that these agents would be suitable for the treatment of congestive heart failure.

Most of the available inotropic agents appear to work, at least partly, by increasing the amount of intracellular calcium available to

react with contractile proteins to generate a greater force of myocardial contraction (Scholz, 1983). Inotropic agents exist that act as β_1-adrenoreceptor agonists, β_2-adrenergic agonists and phosphodiesterase inhibitors. All these agents will increase intracellular cyclic AMP levels. It is presumed that increases in intracellular cyclic AMP levels will stimulate the cyclic AMP-dependent protein kinase and lead to the phosphorylation of proteins on the cell membrane and the intracellular sarcoplasmic reticulum (Tadu and Katz, 1982). These changes lead to an enhanced calcium flux that increases intracellular calcium, the intensity of actin and myosin interaction and thus the force of myocardial contraction.

The first non-catechol, non-glycoside compound described that could act as a positive inotrope was Amrinone (Win 40,680) (Alousi, et al., 1979). Other such agents include, Milrinone (Baim, et al., 1983), Fenoximone (also called Enoximone or MDL 17,043) (Kariya, et al., 1982), Piroximone (MDL 19,205) (Petein, et al., 1984), Imazodan (CI-914), methyl-Imazodan (CI-930), (Bristol, et al., 1984), AR-L 57 and Sulmazole (AR-L 115 BS) (Pouleur, et al., 1982). Of these Amrinone, Milrinone, Imazodan, Methyl-Imazodan, and Fenoximone have been shown to be inhibitors of cyclic AMP-specific phosphodiesterases (Kariya, et al., 1982; Bristol, et al., 1984; Weishaar, et al., 1985b; Weishaar, et al., 1987d).

1.8.3.2: Inhibitors of platelet aggregation.

Increased levels of cyclic AMP have been shown to inhibit the aggregation of platelets (Salzman, 1972). Interference with cyclic AMP disposal has been proposed as the mechanism of action of a number of reference platelet aggregation inhibitors, including dipyridamole (Salzman, 1972; Mills and Smith, 1971; Asano, et al., 1977; Pichard, et al., 1972). More recently, several selective phosphodiesterase inhibitors have been described that exert potent anti-aggregatory effects. Hidaka and co-workers have shown that Cilostamide (OPC-3,689), which is a selective inhibitor of the cyclic GMP-inhibited

subclass of phosphodiesterase (Hidaka and Endo, 1984; Degerman, et al., 1987), inhibits platelet aggregation induced by ADP, collagen or archidonic acid (Hidaka, et al., 1984). A number of derivatives of Cilostamide such as OPC-3911, OPC-13135 and OPC-13013 are all selective and potent inhibitors of the cyclic GMP-inhibited subclass of cAMP specific phosphodiesterase (Degerman, et al., 1987).

1.8.3.3: Other 'PDE-III' inhibitors.

A number of other selective inhibitors of cyclic AMP-specific phosphodiesterases have been described and these include agents such as Ro-20-1724 and Rolipram (ZK 62,771) (Schwabe, et al., 1976; Bergstrand, et al., 1977). Two phosphodiesterase inhibitors (ICI 58 301 and ICI 63 197) which possess potent anti-bronchoconstrictor activity (Davies, 1973) have also been shown to be selective inhibitors of cyclic AMP-specific phosphodiesterase (Somerville, et al., 1970; Davies, 1973). The cyclic AMP phosphodiesterase inhibitor ICI 118233 is also a selective inhibitor of the cyclic AMP-specific subclass of phosphodiesterase. The structures of a number of selective 'PDE-III' inhibitors are shown in Figure 1.6.

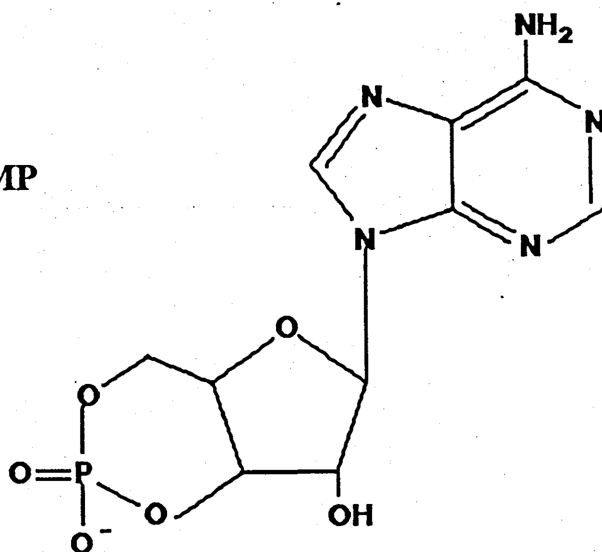
FIGURE 1.1:

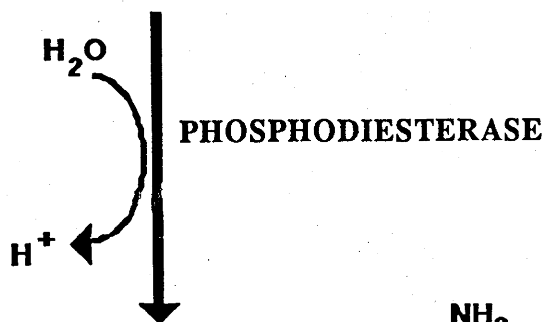
REACTION CATALYSED BY CYCLIC NUCLEOTIDE PHOSPHODIESTERSASE.

The reaction catalysed by cyclic nucleotide phosphodiesterase is depicted using 3'5'-cyclic AMP as an example. The same bond is hydrolysed when 3'5'-cyclic GMP is the substrate producing 5'-GMP as the product.

FIGURE 1.1

CYCLIC AMP





5'-AMP

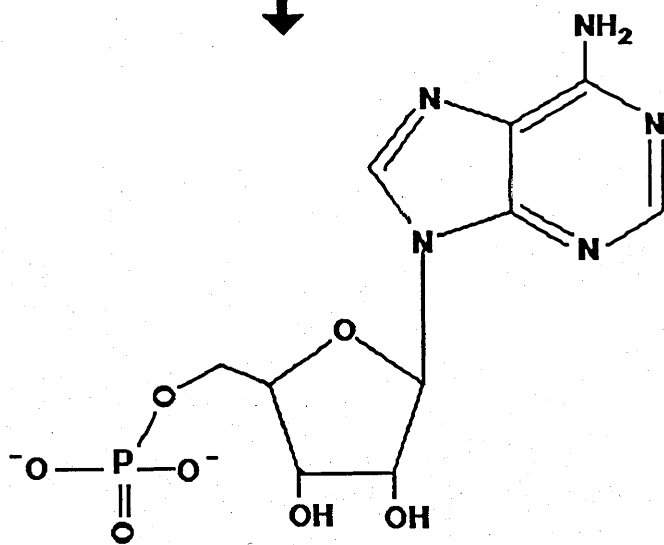
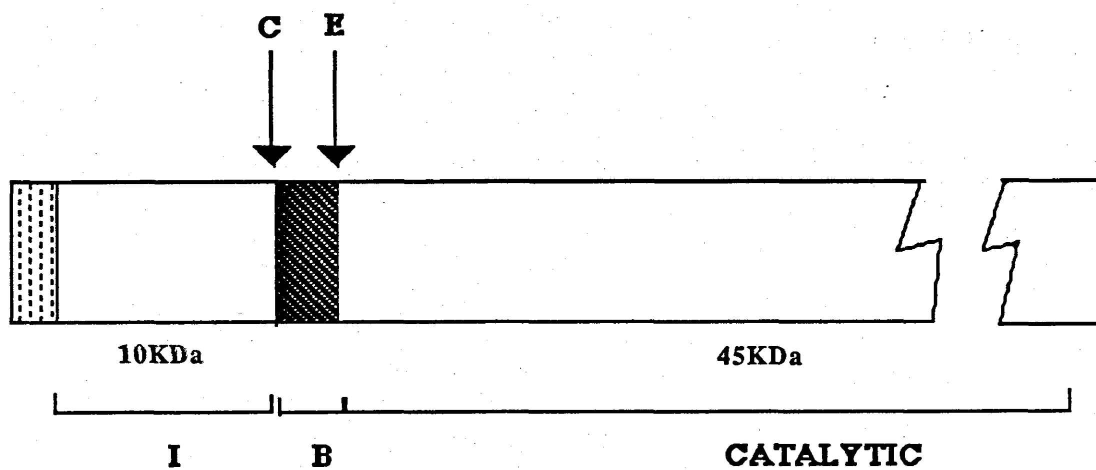


FIGURE 1.2:

POSSIBLE ARRANGEMENT OF HYPOTHETICAL DOMAINS IN BOVINE BRAIN Ca^{2+}/CALMODULIN-STIMULATED CYCLIC NUCLEOTIDE PHOSPHODIESTERASE.

This model was proposed by Kincaid, et al., (1985) on the basis of the proteolytic sensitivity of a purified preparation of bovine brain Ca^{2+}/Calmodulin-stimulated cyclic nucleotide phosphodiesterase.

FIGURE 1.2



I=Inhibitory domain

B=Calmodulin binding domain

Protease Sites:

C=Site exposed in presence of Ca2+/Calmodulin

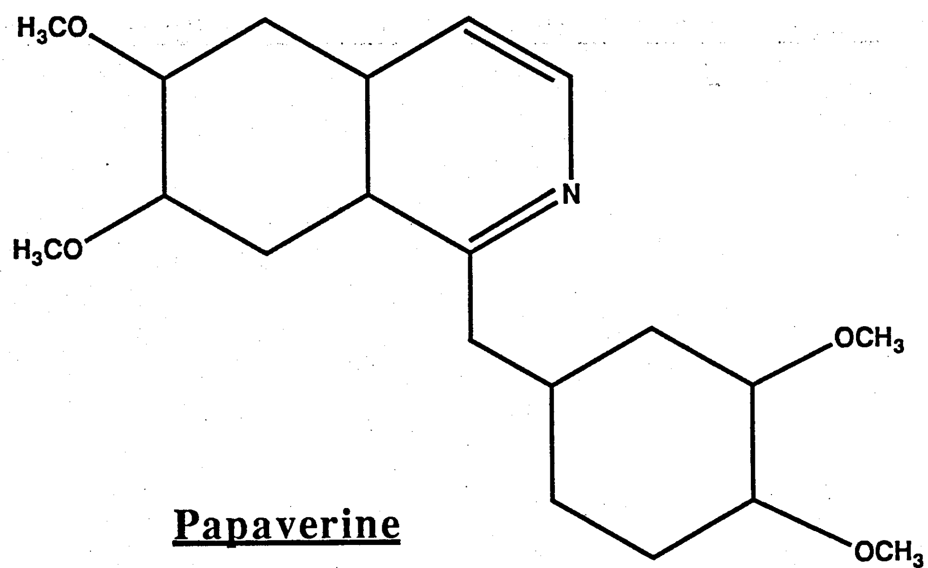
E=Site exposed in absence of Ca2+/Calmodulin

Adapated from Kincaid et.al. (1985)

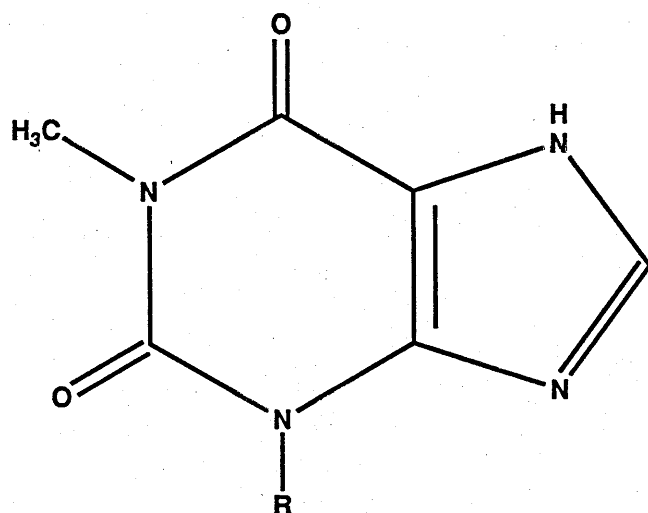
FIGURE 1.3:

STRUCTURE OF NON-SPECIFIC PHOSPHODIESTERASE INHIBITORS.

FIGURE 1.3



Papaverine



R

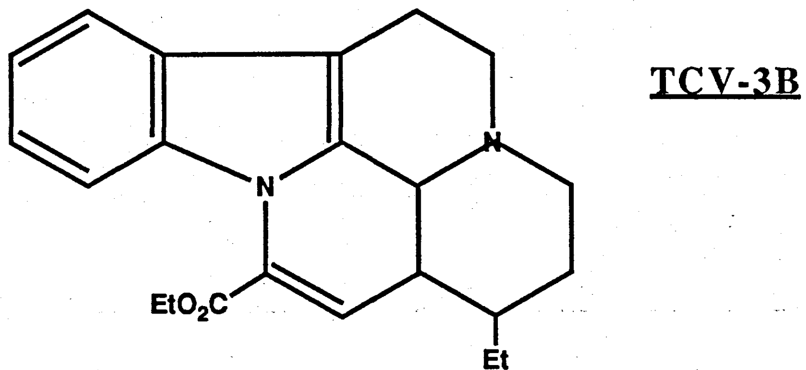
$-CH_3$ Theophylline

-iBu IBMX

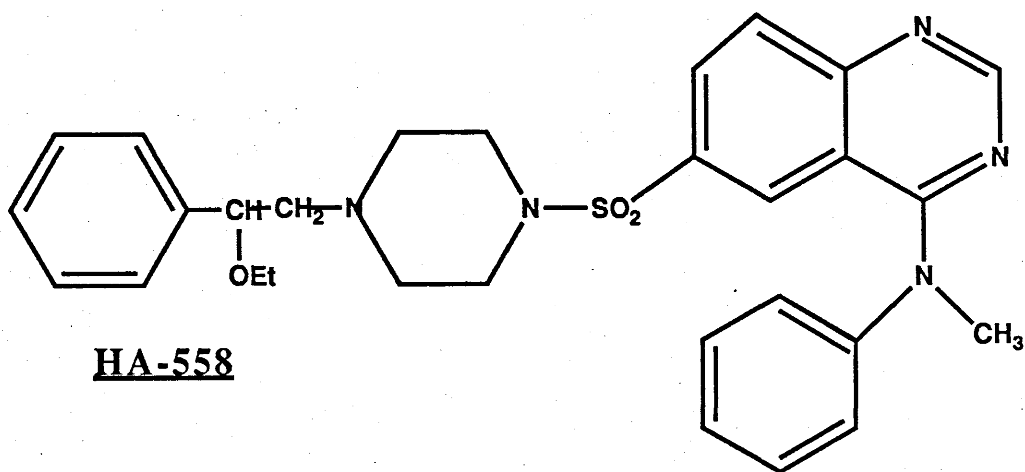
FIGURE 1.4(a):

STRUCTURE OF SELECTIVE 'PDE-I' INHIBITORS.

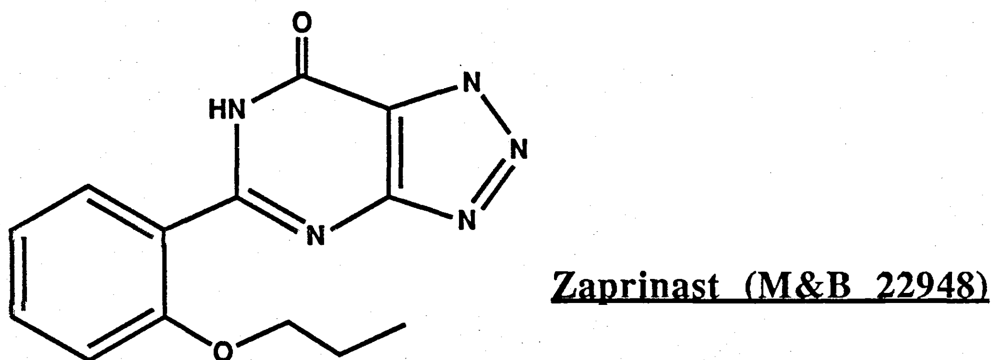
FIGURE 1.4(a)



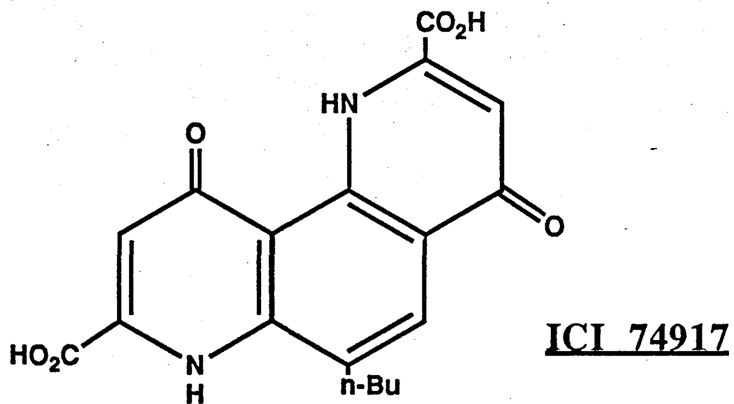
TCV-3B



HA-558



Zaprinast (M&B 22948)

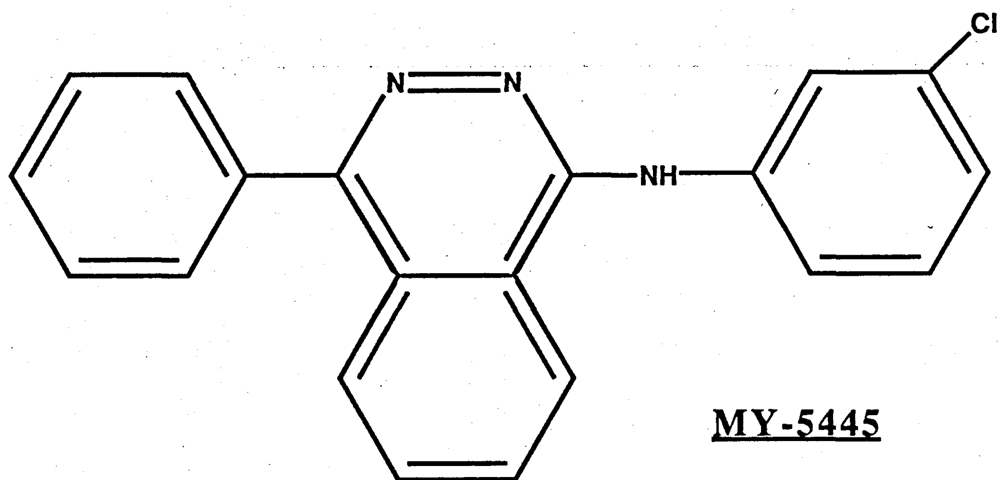


ICI 74917

FIGURE 1.4(b):

STRUCTURE OF SELECTIVE 'PDE-I' INHIBITORS.

FIGURE 1.4(b)

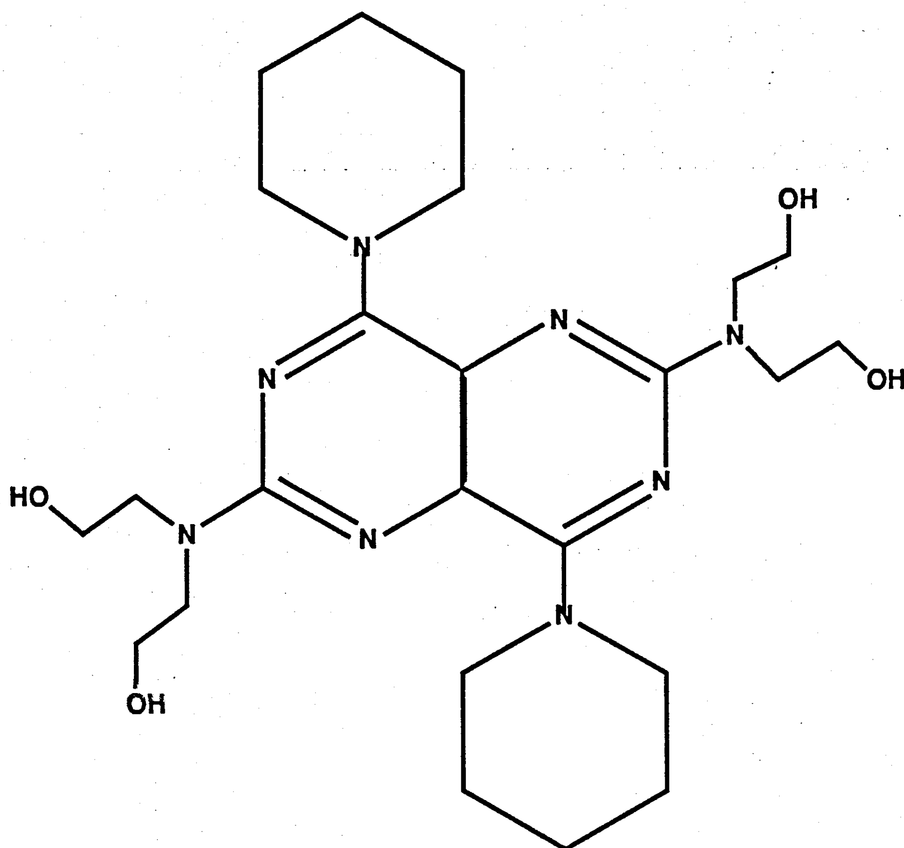


MY-5445

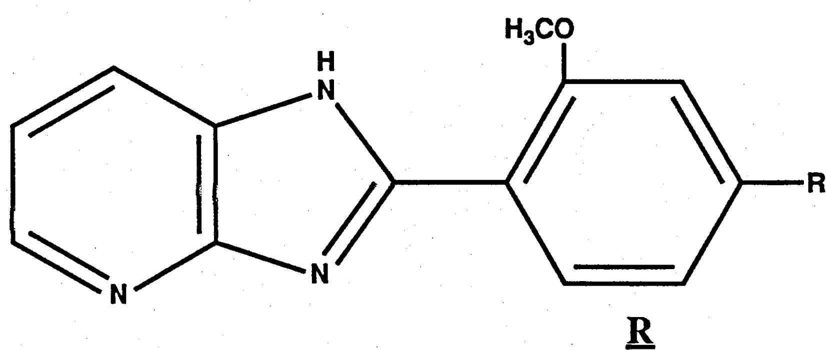
FIGURE 1.5:

STRUCTURE OF SELECTIVE 'PDE-II' INHIBITORS.

FIGURE 1.5



Dipyridamole



R

$-OCH_3$ AR-L 57

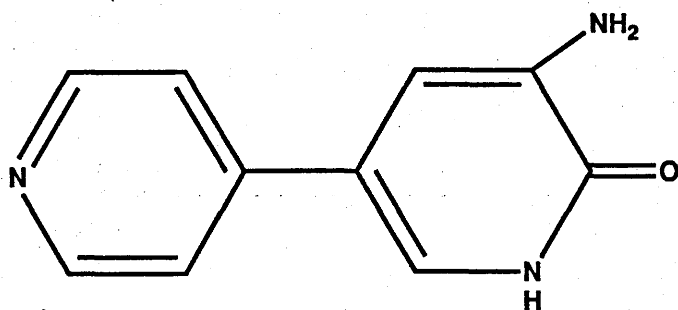
$-SOCH_3$ Sulmazole

FIGURE 1.6(a):

STRUCTURE OF SELECTIVE 'PDE-III' INHIBITORS.

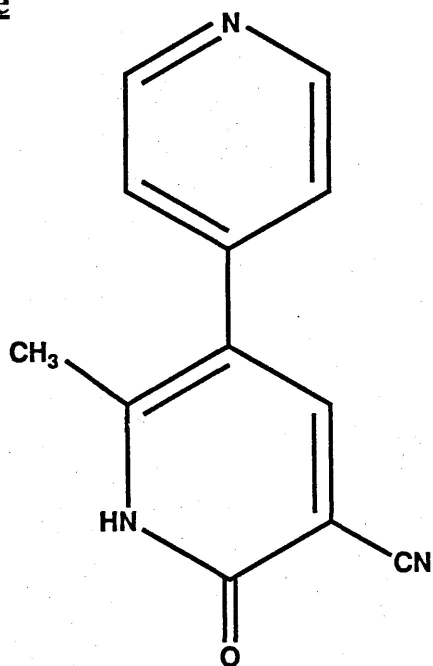
These agents are all cardiotonic agents that exert inotropic effects in the heart.

FIGURE 1.6(a)



Amrinone

Milrinone



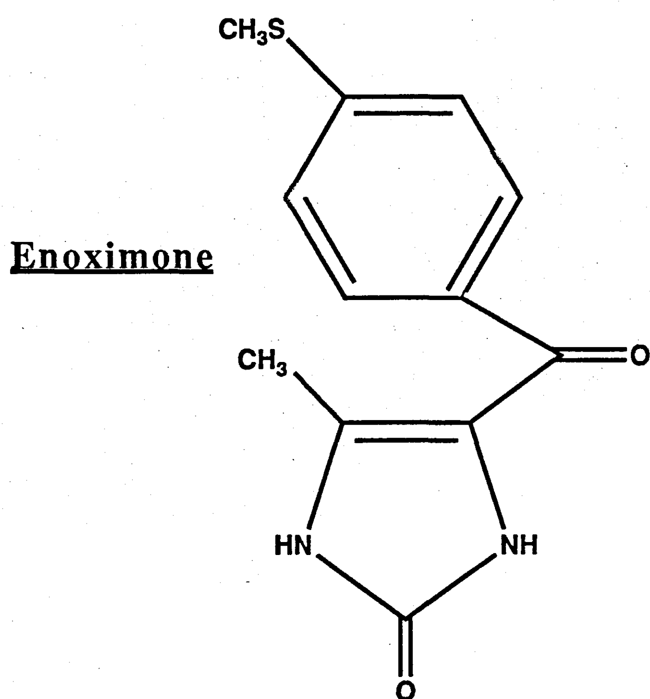
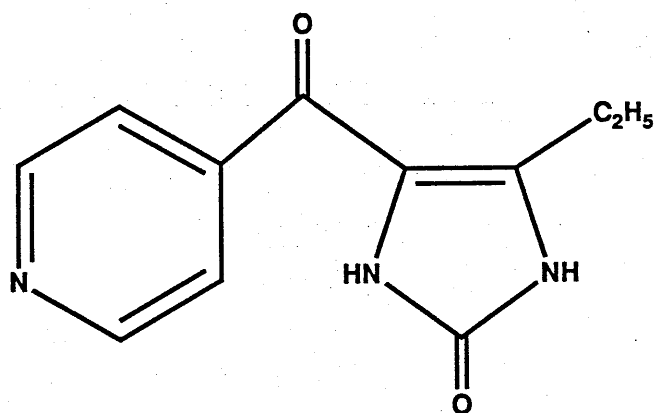


FIGURE 1.6(b):

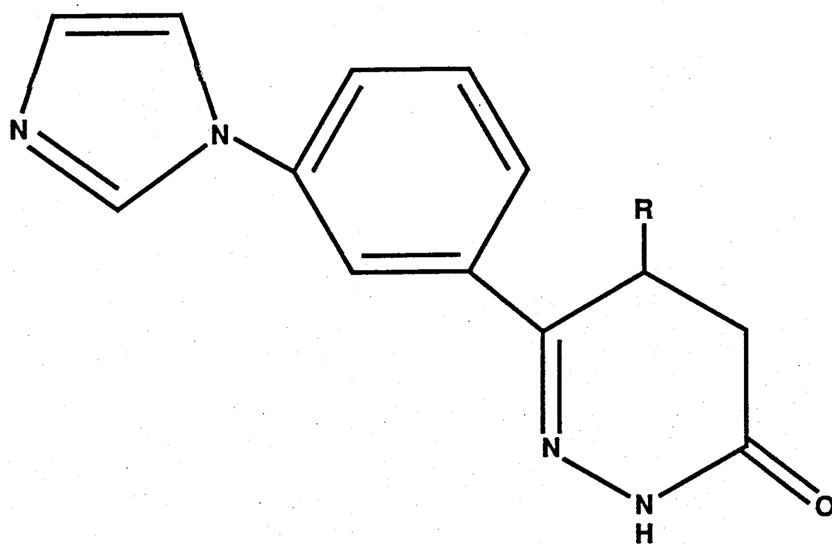
STRUCTURE OF SELECTIVE 'PDE-III' INHIBITORS.

These agents are all cardiotonic agents that exert inotropic effects in the heart.

FIGURE 1.6(b)



Piroximone (MDL 19205)



R

-H Imazodan (CI-914)

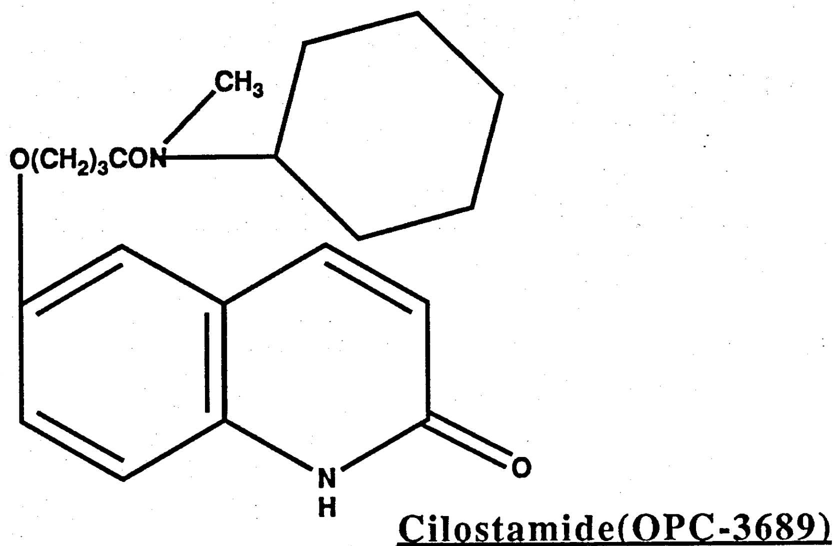
-CH_3 methyl-Imazodan(CI-930)

FIGURE 1.6(c):

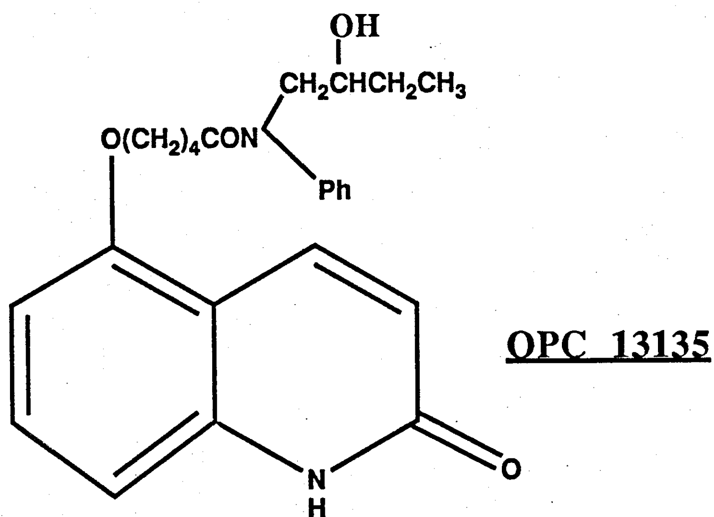
STRUCTURE OF SELECTIVE 'PDE-III' INHIBITORS.

These agents exert anti-aggregatory effects in platelets

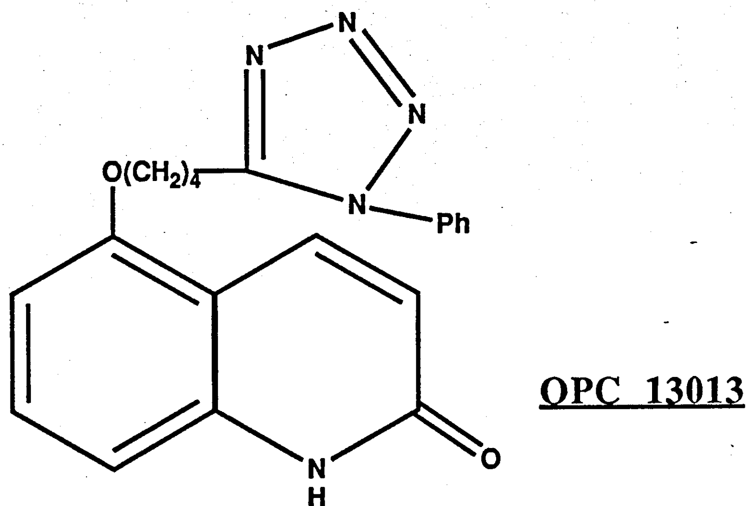
FIGURE 1.6(c)



Cilostamide(OPC-3689)



OPC 13135



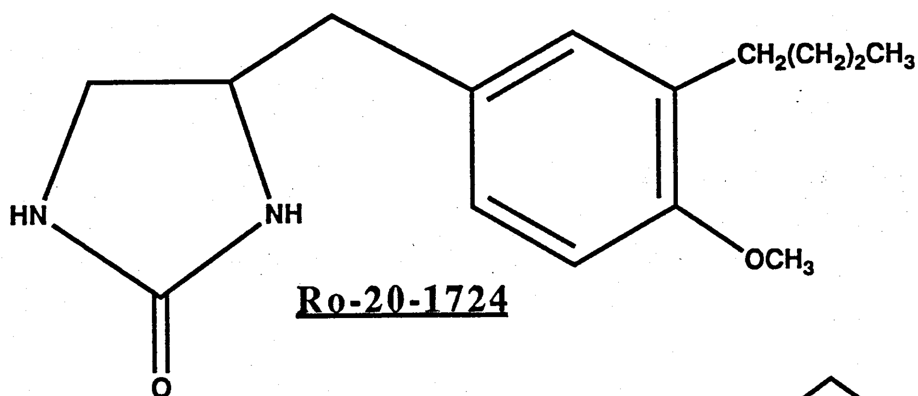
OPC 13013

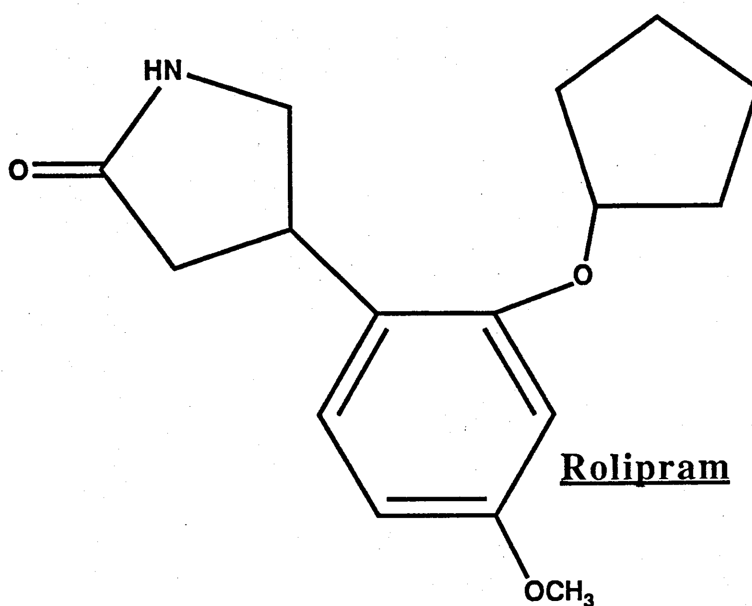
FIGURE 1.6(d):

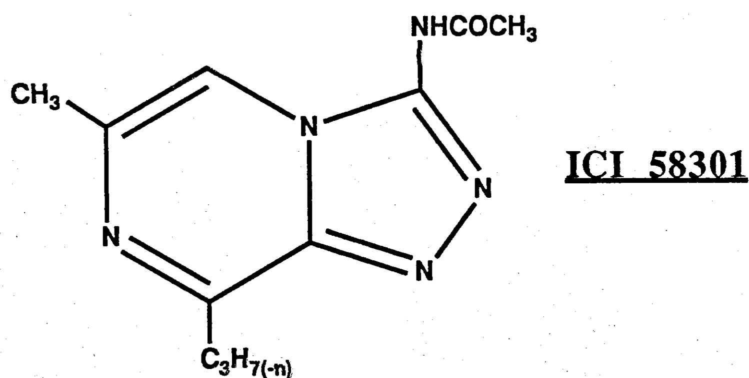
STRUCTURE OF SELECTIVE 'PDE-III' INHIBITORS.

These agents are selective for the cyclic GMP-insensitive (Rolipram sensitive) subclass of phosphodiesterase

FIGURE 1.6(d)







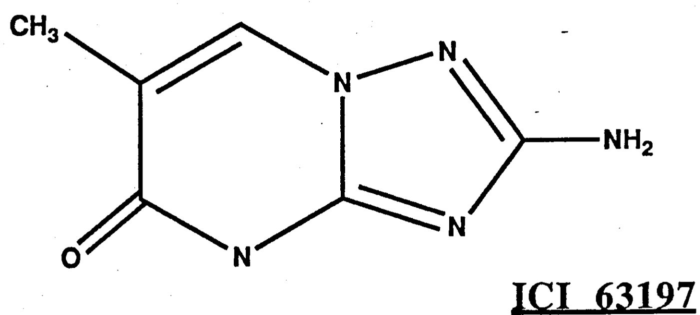
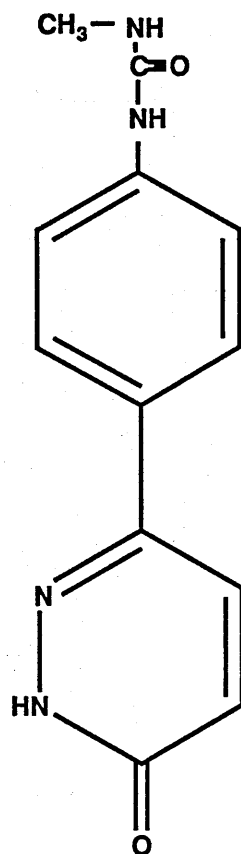


FIGURE 1.6(e):

STRUCTURE OF SELECTIVE 'PDE-III' INHIBITORS.

FIGURE 1.6(e)

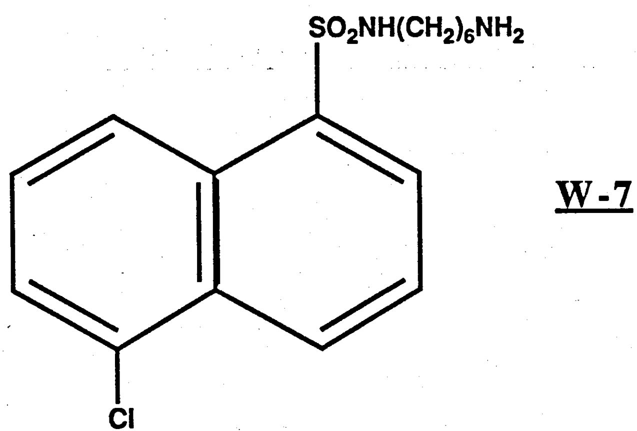


ICI 118233

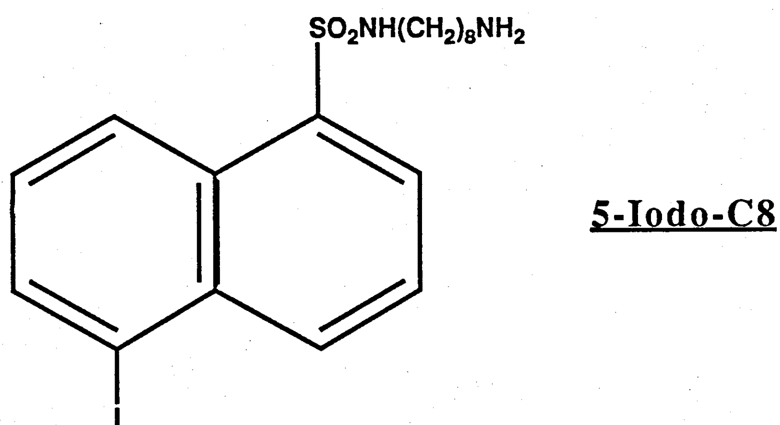
FIGURE 1.7:

STRUCTURE OF CALMODULIN ANTAGONISTS.

FIGURE 1.7



W-7

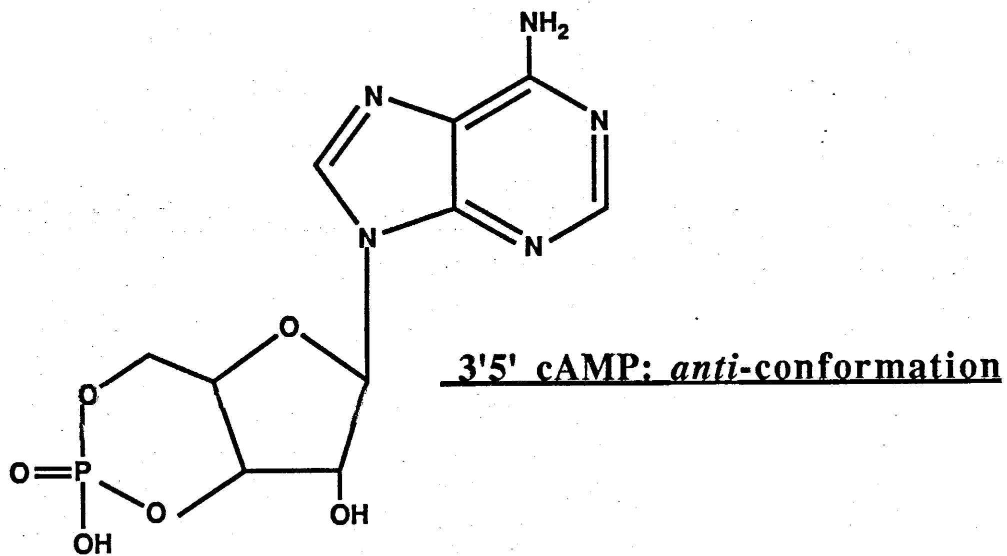


5-Iodo-C8

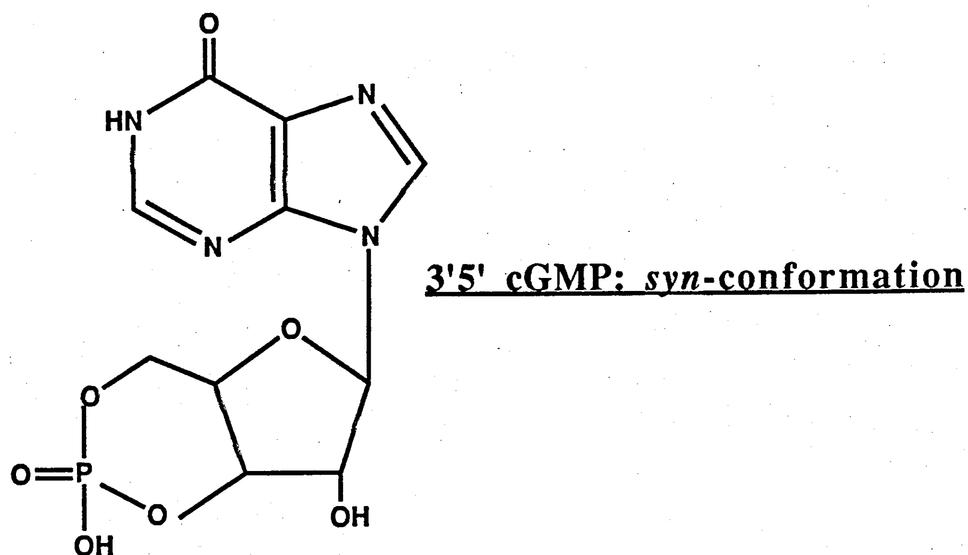
FIGURE 1.8:

STUCTURE OF PURINE AND PYRIMIDINE 3'5'-CYCLIC NUCLEOTIDES.

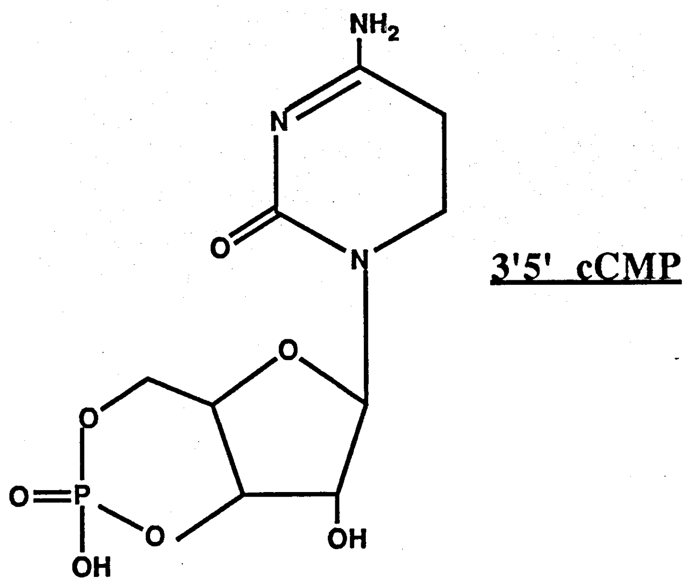
FIGURE 1.8



3'5' cAMP: *anti*-conformation



3'5' cGMP: *syn*-conformation



3'5' cCMP

TABLE 1.1:

ISOZYMES OF THE Ca^{2+}/CALMODULIN-STIMULATED PHOSPHODIESTERASE.

References for Table 1.1

1. Morril, et al., (1979).
2. Sharma, et al., (1980).
3. Hansen and Beavo, (1982).
4. Sharma, et al., (1984).
5. Hansen and Beavo, (1986).
6. Sharma, et al., (1985).
7. Sharma and Wang, (1986a).
8. Shenolikar, et al., (1985).
9. LaPorte, et al., (1979).
10. Sharma and Wang, (1986b)
11. Rossi, et al., (1988)

	Molecular mass (kDa)			
Source	Subunit	Native	Max.fold stim^{n}	Reference
Bovine brain				
	59	126	2	(1)
	58	124	15	(2)
	61	N.D.	N.D.	(3)
	61	120	4	(4,5,6)
	63	126	5	(4,5,7)
	74	150	2.5	(8)
Bovine heart				
	57	120	10	(9)
	59	N.D.	10-15	(3)
Bovine lung				
	58	130	7	(10)
Mouse Testis				
	68	180	6	(11)

TABLE 1.2:

KINETIC CHARACTERISTICS OF Ca^{2+}/CALMODULIN-STIMULATED PHOSPHODIESTERASE.

References for Table 1.2.

1. Morril, et al., (1979).
2. Sharma, et al., (1986a).
3. Sharma and Wang, (1986b).
4. Shenolikar, et al., (1985).
5. Ho, et al., (1976).
6. Rossi, et al., (1988).
7. Purvis, et al., (1981).
8. Weishaar, et al., (1986).
9. Vandermeers, et al., (1983).

Source	Purity	K_m (µM) cAMP	cGMP	V_{max} (µmol/min/mg) cAMP	cGMP	Ca^{2+}/CaM Effect	Ref
Bovine brain							
59-kDa	(Pure)	150	9	-	-	V_{max}	(1)
63-kDa	(Pure)	66-70	6-7	1.7	8.0	V_{max},K_m	(2)
61-kDa	(Pure)	68	17	6	17	V_{max}, K_m	(3)
74-kDa	(Pure)	13.3	4.8	0.12	1.09	V_{max}, K_m	(4)
Bovine heart							
	(Partial)	215,39	9	-	-	-	(5)
Bovine lung							
	(Pure)	88	15	7.15	8.5	V_{max}, K_m	(3)
Mouse Testis							
	(Pure)	2,20	2,20	8.8	6.3	V_{max}, K_m	(6)
Rat Testis							
I	(Partial)	1	1	-	-	-	(7)
II	(Partial)	3	3	-	-	-	(7)
III	(Partial)	-	1.5	-	-	-	(7)
Guinea Pig Left Ventricle							
	(Partial)	0.8, 43	0.9, 53	-	-	-	(8)
Bovine Coronay artery							
	(Partial)	2.5, 140	3, 17	-	-	- -	(8)
Rat Pancreas							
I	Pure	1,4	0.7, 10	-	-	-	(9)
II	Pure	5, 25	1.5,8.0	-	-	-	(9)

TABLE 1.3:

ISOZYMES OF THE CYCLIC GMP-STIMULATED PHOSPHODIESTERASE

References for Table 1.3

1. Beavo, et al., (1982).
2. Martins, et al., (1982).
3. Yamamoto, et al., (1983a).
4. Whalin, et al., (1988).
5. Pyne, et al., (1986a).

Property	Bovine Cardiac	Calf liver	Rabbit liver	Rat liver (soluble)
References	1,2	3	4	5
Molecular Mass (kDa)				
(subunit)	102-105	105	105	66-67
(native)	204-230	200	400	134
Apparent Km (μM)				
cAMP	36	33	28	40
cGMP	11	15	16	25
Vmax (μmol/min/mg)				
cAMP	120	170	160	4.8
cGMP	120	200	160	1.6
Hill Coefficient				
cAMP	1.9	1.5-1.8	-	1.6
cGMP	1.3	1.2-1.6	-	1.0
Ka for cGMP (μM)	-	0.5	0.35	0.28

TABLE 1.4:

<u>ISOZYMES OF THE CYCLIC AMP-SPECIFIC PHOSPHODIESTERASES</u>

(a) CYCLIC GMP -INHIBITED PHOSPHODIESTERASES.

References for Table 1.4(a)

1. Degerman, <u>et al.,</u> (1987).
2. MacPhee, <u>et al.</u>, (1986).
3. Grant, <u>et al.</u>, (1988).
4. MacPhee, <u>et al.</u>, (1988).
5. Grant and Colman, (1984).
6. MacPhee, <u>et al.</u>, (1987).
7. Harrison, <u>et al.</u>, (1986b).
8. Boyes and Loten, (1987a)
9. Pyne, <u>et al.</u>, (1987a).

Reference	Rat Adipose (1)	Bovine/human platelet (2,3,4,5,6)	Bovine heart (7)	Rat liver (8)	Rat liver (9)
Property					
Km (μM)					
cAMP	0.4	0.2	0.2	0.2	0.3, 28
cGMP	0.3	0.02	0.1	0.2	10
Vmax(μmol/min/mg)					
cAMP	8.5	3.0	6.0	6.2	0.11, 0.63
cGMP	2.0	0.3	0.6	2.1	0.004
Molecular weight (kDa)					
Native Enzyme (rapid isolation)	135	105	110		63
Purified (native)	110	N.D.	140		112
Subunits (SDS-PAGE)	66/62	83/60	110/61		57
Inhibitors IC_{50}			(K_i)		
Cilostamide	0.04	0.04	0.01	-	-
Milrinone	0.6	0.5	0.3	-	1
CI-930	0.4	-	-	-	-
Ro-20-1724	190	220	62	-	50
cGMP	0.2	0.1	0.1	(K_i) 0.2	2

TABLE 1.4:

ISOZYMES OF THE CYCLIC AMP-SPECIFIC PHOSPHO-DIESTERASES

(b) CYCLIC GMP -INSENSITIVE PHOSPHODIESTERASES.

References for Table 1.4(b)

1. Thompson, et al..(1979).
2. Thompson, et al..(1988).
3. Marchmont, et al..(1981b).
4. Conti and Swinnen, (1990)
5. Demazancourt and Guidicelli, (1988).
5. Yamamoto, et al..(1984a).
6. Weishaar, et al..(1986).

Reference	Dog Kidney (1,2)	Rat Liver (3)	Rat Sertoli (4)	Rat Brain (5)	Calf liver (6)	Canine Heart (7)
Property						
Km (μM)						
cAMP	2.2	0.7	1.6	1.0	2.0*	1.1*
cGMP	312	120	>50	-	16	-
Vmax(μmol/min/mg)						
cAMP	10	0.93	5-6	-	N.A.	N.A.
Molecular weight (kDa)						
Native Enzyme	71-74	52	67	61	N.A.	N.A.
Subunits (SDS-PAGE)	82	52	135	53	-	-
Inhibitors IC_{50}						
cGMP	600	180	>50	-	1000	1500
Ro-20-1724	2.2	7.2	2.2	4.0	2.2	18
Rolipram	-	-	0.9	1.0	-	1.0
Cilostamide	-	-	-	59	>100	3.10
IBMX	11	-	10	24	7.5	-

CHAPTER 2.

MATERIALS AND METHODS.

MATERIALS.

All chemicals used in this study were either from:

Sigma Chemical Company, (Poole, Dorset, U.K.): ω-Aminopentyl agarose, Butyl agarose, Benzamidine hydrochoride, Chloramine T, Coomassie Blue R-250, Cyanogen bromide activated sepharose-6B, DEAE-sepharose CL-6B, Dowex-1-Chloride, Ethylene glycol, Snake venom (*Ophiophagus Hannah*), Isobutylmethylxanthine, N,N,N',N',-tetramethylethylenediamine (TEMED), Phenyl-methylsulphonylfluoride (PMSF), Polyoxyethylene sorbitan monolaurate (Tween-20), Triton-X-100, Cyclic adenosine 3'5' monophosphate (cyclic AMP), Cyclic guanosine 3'5' monophosphate (cyclic GMP), Cyclic 3'5' cytidine monophosphate (cyclic CMP), dipyridamole, Antipain, Calmodulin agarose, ethylene diamine, 6-chloroguanine, Thimerosol, *o*-dianisidine, Pepsin, Sephacryl S-200, Sephacryl S-300.

Boehringer (UK) Ltd., (Lewes, East Sussex, U.K.): Ammonium persulphate, Ammonium Acetate, Bromophenol Blue, Boric Acid, Calcium Chloride, Sodium Deoxycholate, Magnesium Chloride, Acrylamide, N,N'-methylenebisacrylamide, Magnesium Chloride, Potassium dihydrogen orthophosphate, Dipotassium hydrogen orthophosphate, Sodium Chloride, Sodium deoxycholate, Sodium Dodecyl Sulphate (SDS), Tris (hydroxymethylaminomethan) (Tris), Acrylamide.

Whatman Ltd. (Maidstone, Kent, U.K.): Diethylaminoethyl cellulose (DE-52), 3MM filter paper.

Pharmacia Ltd., (Uppsala, Sweden): FPLC system and accessories.

May & Baker Ltd., (Dagenham, Essex, U.K.): Acetic acid, Butan-1-ol, Isopropanol.

Biorad Ltd., (Richmond, California, USA): Affi-gel blue agarose, Biorad protein assay.

Kodak Ltd., (Liverpool, U.K.): Kodak XR 320 film.

Amersham International, (Amersham Bucks U.K.): $[^{125}I]$-iodine, $[^{3}H]$-cyclic 3'5' guanosine monophosphate, $[^{3}H]$-cyclic 3'5'-adenosine monophosphate.

New England Nuclear (NEN), (Du Pont Ltd, Stevenage, Herts. U.K.): ^{125}I-Protein-A.

Schleicher and Schull, (Dassel, Germany): Nitrocellulose.

Millipore (Watford, Hertfordshire, U.K.): 0.22μm single use filters.

Amrinone and Milrinone were gifts from Sterling-Winthrop Ltd. (see Bristol, et al., 1984) ICI 63197 and ICI 118233 were kind gifts from ICI Pharmaceuticals, Macclesfield, Cheshire, U.K. (see Davies, et al., 1973). Ro-20-1724 was a gift from Roche Products Ltd., Welwyn Garden City Hertfordshire U.K. M&B 22948 and Zaprinast-agarose were kind gifts from Pfizer Central Research, Sandwich, Kent, U.K.

Horseradish-peroxidase linked antibody was donated free by the Scottish Antibody Production Unit (Law Hospital, Carluke, Lanarkshire, Scotland, U.K.)

METHODS

2.1: PREPARATION OF DOWEX-1-CHLORIDE.

This was prepared using a modification of the method of Pichard and Cheung (1976). 500g of Dowex-1-Chloride (200-400 mesh, Chloride form) was placed in 5 litres of 1M NaOH and gently stirred for 15 minutes. The resin was then allowed to settle and the NaOH solution poured off and replaced with distilled water. This process of washing and settling was repeated until the pH of the water was approximately 7. The resin was then suspended in 5 litres of 1M HCl and once again gently stirred for 15 minutes. The washing procedure was repeated until the pH of the distilled water was between 3 and 4. The resin was then made up to a 1:1 suspension in distilled water and stored at 4°C.

2.2: CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ASSAY.

A two step radioassay was employed to assess cyclic AMP and cyclic GMP phosphodiesterase activity. The assay was based upon that described by Thompson and Appleman (1971). The principle of the assay is that [^{3}H]-labelled cyclic nucleotide ([^{3}H]-cNMP: on the 8 position of the adenine or guanine ring) is incubated with the sample to allow hydrolysis of the 3'5' phosphodiester bond producing a [^{3}H] 5'-NMP derivative. The [^{3}H] 5'-NMP derivative is then converted to the corresponding [^{3}H] nucleoside ([^{3}H]-N) by incubating with a source of 5'-nucleotidase activity. Routinely this was provided by the snake venom isolated from *Ophiophagus hannah*. The conditions are such that complete conversion is obtained in 10 minutes at 30°C. The final step involves the separation of unhydrolysed [^{3}H]-cNMP from [^{3}H]-N. This is achieved by batch binding of the mixture to Dowex-1-Chloride. This removes any

unhydrolysed $[^3H]$-cNMP from the nucleoside ($[^3H]$-N) because the latter is uncharged.

Rutten et al., (1973) demonstrated that tissue preparations containing adenosine deaminase can lead to significant underestimations of the rate of cyclic AMP hydrolysis in the assay, because this enzyme's activity converts adenosine, produced from cyclic AMP degradation, into inosine, which is free to bind to Dowex-1-Chloride. To prevent such adsorption, ethanol was used in the Dowex batch binding step.

2.2.1: Phosphodiesterase assay stock solutions.

(a) 10mM cyclic AMP or cyclic GMP in 20mM Tris-HCl pH 7.4 10mM $MgCl_2$: The stock solutions were kept stored at -20^oC and were usually used within two months.

(b) 20mM Tris-HCl pH 7.4.

(c) Snake Venom (*Ophiophagus hannah*): This was prepared by dissolving snake venom in distilled deionised water at a concentration of 10mg/ml. Aliquots (1ml) were stored at -20^oC and were usually used within six months.

(d) Dowex-1-Chloride: This was prepared as described in section 2.1 and was stored at 4^oC as a 1:1 suspension in water.

2.2.2: Phosphodiesterase assay solutions.

(a) $[^3H]$-cyclic AMP and $[^3H]$-cyclic GMP: The stock solution of cyclic AMP or cyclic GMP was diluted in 20mM Tris-HCl, 10mM $MgCl_2$ to give the desired concentration (usually 2μM). To 10 mls of this solution was added 30μCi (30μl) of $[^3H]$-cyclic AMP or $[^3H]$-cyclic GMP.

(b) Snake Venom: This was diluted in water to give a final concentration of 1mg/ml.

(c) Dowex-1-Chloride: Before use in the assay, ethanol (absolute) was added to give final proportions of Dowex:water:ethanol of 1:1:1.

2.2.3: Routine assay of phosphodiesterase activity.

The final volume of the initial reaction mixture was always 100µl consisting of 50µl of radiolabelled substrate in 20mM Tris-HCl pH 7.4, 10mM $MgCl_2$ mixed with 25µl of sample and 25µl of Tris-HCl pH 7.4. The final concentration of cyclic nucleotide was usually 1µM.

2.2.4: Kinetic parameters of isolated phosphodiesterases.

For determination of kinetic parameters, a slighlty altered protocol was adopted. Radiolabelled substrate was prepared by adding 30µCi of stock [^{3}H]-substrate to 5mls of 20mM Tris-HCl pH 7·4, 10mM $MgCl_2$. Reaction tubes contained 25µl of this radiolabelled substrate (about 150,000 cpm); cold substrate (at the appropriate concentration) was added separately in a volume of 25µl in 20mM Tris-HCl pH 7.4, 10mM $MgCl_2$ along with 25µl of sample and 25µl of 20mM Tris-HCl pH 7.4.

2.2.5: Magnesium dependency of phosphodiesterase activity.

For determining the effect of magnesium on phophodiesterase activity, [^{3}H]-substrate was prepared in 20mM Tris-HCl pH 7.4 only and 50µl added to the assay. Sample was added in 25µl and the appropriate concentration of $MgCl_2$ added in 25µl of Tris-HCl pH 7.4.

2.2.6: Phosphodiesterase assay: Hydrolytic step.

The reaction mixture was vortexed and incubated at 30°C for a time interval, usually 10 minutes, to attain the conversion of cNMP to 5'-NMP, and terminated by boiling for 2 minutes.

2.2.7: Phosphodiesterase assay: Conversion step.

After the reaction mixture was cooled to 4°C, 25µg (25µl) of snake venom was added and the cocktail vortex mixed and incubated at 30°C for 10 minutes to attain the complete conversion of 5'-NMP to N. After this stage the tubes were once more placed on ice and 400µls of Dowex-1-Chloride slurry (1:1:1, Dowex:water:ethanol) was added and left on ice for at least 15 minutes with occasional vortexing.

Samples were then centrifuged in a Beckman microcentrifuge at full speed for 3 minutes to sediment the resin. A 150µl sample of the supernatant was then removed and counted in 4ml of Ecoscint scintillation fluid in a Beckman scintillation counter.

In all experiments, great care was taken to ensure that the rates of hydrolysis of cyclic nucleotides were linear with respect to time and routinely less than 10% of the substrate was hydrolysed. Blank tubes were prepared in all experiments containing a boiled (5 minutes) sample. This was especially important if the samples contained any salt, because it has been demonstrated that increases in ionic strength have the effect of reducing the binding of cyclic nucleotide to the Dowex resin and hence increasing the apparent amount of nucleoside in the supernatant leading to an overestimation of the degree of hydrolysis. Such effects are apparent at NaCl concentrations as low as 0.1M.

2.3: CALCULATION OF PHOSPHODIESTERASE ACTIVITY.

A simple calculation was used to calculate the rates of cyclic nucleotide hydrolysis. The values obtained for the apppropriate blanks were subtracted from the cpm obtained with the sample and substituted in the following equation:

150/391.67 x pmoles of cNMP/time(mins) x cpm/total cpm.

where cpm represents the blank corrected value and pmoles of cNMP repesents the total picomoles of cyclic AMP/cyclic GMP in the assay. The total cpm value was approximately 150,000 cpm/tube but was determined in each experiment to allow for day to day variation in the counting efficiency of the scintillation counter

This calculation gives the rate of hydrolysis in pmoles of cyclic nucleotide hydrolysed/min. To obtain total activities in a given volume of solution this figure was then multiplied by

total vol(mls)/volume assayed(mls).

Specific activities were then calculated by multiplying by

1/protein in assay(mg).

2.4: ISOLATION OF RAT LIVER HIGH SPEED SUPERNATANT.

A liver was excised from one male Sprague-Dawley rat (250-275g), diced using a pair of scissors and rinsed several times in ice cold homogenising Buffer 'A' (50mM Tris-HCl pH 7.5, 0.25M Sucrose, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 20μM leupeptin, 0.1mM EGTA, and 0.1mM dithiothreiotol). PMSF was added immediately before use from a stock solution of 35 mg/ml in ethanol. The liver was then homogenised using 5 up/down strokes on a rotating pestle (setting 5) at approximately 1:4 (v/v). Great care was taken to ensure that the homogenate was kept cool during this procedure. The resultant homogenate was then passed through two layers of muslin into a beaker on ice. The final volume of homogenate was generally between 40 and 50 mls for one rat liver. All subsequent operations were carried out at 4°C.

The homogenate was centrifuged for 10 mins. at 706g_{av} (Beckman JA-20 rotor) to obtain a low speed pellet (P1) and supernatant (S1). The pellet was discarded and the supernatant was recentrifuged (Beckman Ti-60 rotor) at 163,570g_{av} for 60 mins. to obtain the high speed supernatant (S2) and pellet (P2). The supernatant was carefully decanted to avoid any fat deposits and the pellet discarded. An identical protocol was used for the preparation of high speed supernatants from other tissues such as kidney, brain, and adipocytes.

2.5: ISOLATION OF A HIGH SPEED SUPERNATANT FROM RAT HEPATOCYTES.

Hepatocytes were prepared by a method used routinely in the laboratory (Smith, et al., 1978; Elliot, et al., 1976; Heyworth, et al., 1983d). Essentially, hepatocytes were prepared by collagenase digestion in the presence of 0.01% (w/v) Trypsin inhibitor. Isolated cells were washed three times in Ca^{2+}-free Krebs buffer by repeated resuspension and pelleting at 600rpm (MSE Centaur) for 2 minutes (three 80ml washes per 10ml of packed cells). Washed cells were used without further incubation. Cell viability was assessed by the ability of cells to exclude the dye Trypan Blue and was always greater than 90% in all preparations used. It was noted on some occasions that despite the ability of cells to exclude Trypan Blue, very poor yields of phosphodiesterase activity were obtained and that significant quantities of phosphodiesterase activity could be detected in the washes from the cells indicating that Trypan Blue was not a reliable indicator of cell viability with respect to maintenance of phosphodiesterase content. Consequently, the cellular ATP levels were also checked (see Section 2.6); only cell preparations containing a cellular ATP content in the range of 9 – 10 nmol/mg dry weight were used as valid.

Cells were homogenised in ice cold Buffer 'A' (see section 2.4) at 1:4 (v/v) using fifteen up/down strokes in a tight fitting pestle. The final volume of homogenate was approximately 40 mls. A high speed supernatant was then prepared exactly as for a rat liver supernatant.

2.6: MEASURING HEPATOCYTE ATP CONTENT.

Hepatocyte ATP content was determined by a firefly luciferase assay.

2.6.1: Extraction of intracellular ATP.

The stock solutions used were;

(a) 20% (w/v) Perchloric acid (PCA)

(b) 0.5M Triethanolamine, 2M Potassium Hydroxide (TEA/KOH).

(c) Univeral indicator (diluted 1/10 in water).

Cells were pelleted at 600rpm (MSE Centaur) for two minutes and the supernatant discarded. The cell pellet was resuspended in 0.5ml of Krebs–Heinslet and 50µl of 20% PCA were added with mixing. The mixture was neutralised with approximately 100µl of TEA/KOH. Neutralisation was checked by adding about 2µl of this extract to 1ml of 1/10 diluted universal indicator. The extract was centrifuged to pellet precipitated protein and the supernatant saved and frozen at −20°C as 100µl aliquots.

2.6.2: ATP Measurement: Luciferase Assay.

The stock solutions used were;

(a) 80mM $MgSO_4$

(b) 10mM KH_2PO_4 pH 7.4

(c) 100mM Na_2HASO_4

These were mixed at a 1:1:1 ratio just prior to use.

(d) ATP standard solutions in KH_2PO_4 (30, 20, 10, 5 and 1µM)

(e) Luciferin/Luciferase reagent (Sigma FLE–50=dried lantern extract). This was resuspended in 5ml of water and hand homogenised and stored in the dark at 4°C.

(f) Glass vials were washed with NaOH and then HCl to reduce photoemission.

3mls of 1:1:1 buffer were added to one glass vial and 5µl of sample added and left for 15 minutes at 4°C. In the absence of flourescent light, 50µl of Luciferin/Luciferase was added and the sample counted immediately on an LKB Scintillation counter using a wide open channel (5–1024).

2.7: PROTEIN DETERMINATIONS.

Protein concentrations were determined using a Bio-Rad assay kit (Bradford, 1976; Spector, 1978) for soluble proteins and a modified Lowry assay for membrane proteins (Peterson, 1977).

2.8: SUBCELLULAR FRACTIONATION OF HEPATOCYTES.

Hepatocytes were prepared and homogenised as described for the preparation of high speed supernatants (Section 2.4) and were similarly fractionated to produce low speed P1 and S1 fractions. The resultant P1 fraction was washed to release any trapped soluble activity by resuspending in ice cold Buffer 'A' using 5 up/down strokes in a tight fitting pestle. This was centrifuged to produce a further S1 and P1 fraction. This washing procedure was repeated once more to yield another S1 and P1 fraction. This P1 was the 'washed P1 fraction'. The three S1 fractions were then spun at 163,570*gav* (Beckman Ti-60 rotor) for 60 minutes at 4°C to yield separate S2 and P2 fractions.

2.9: DE-52 SEPARATION OF RAT LIVER SOLUBLE PDEs.

This was performed using chromatography on the anion-exchange resin DE-52.

2.9.1: Equilibration of the anion-exchange matrix (DE-52).

All operations were carried out at 4°C. Approximately 10g of DE-52 was equilibrated in 50mM Tris-HCl pH 7.5 by repeated resuspension and settling in the equilibration buffer. After approximately 2 litres of buffer had been passed onto the matrix (4 x 500ml changes) the matrix was poured into a column (final dimensions 3.0cm x 1.4cm). The column was then washed with a further 20 bed volumes (approximately 100mls) of running buffer, Buffer 'B', (50mM Tris-HCl pH 7.5, 5mM benzamidine hydrochloride, 0.2mM PMSF, 2μM leupeptin, 0.1mM EGTA, and 0.1mM dithiothreitol). The column was

considered to be equilibrated when the pH of the eluant from the column was exactly 7.5.

2.9.2: Separation of soluble cyclic nucleotide PDE activties.

All operations were carried out at 4°C. The column was loaded with the high speed supernantant prepared from rat liver homogenates using a peristaltic pump set at 1.5ml/min and was then washed with approximately 100 ml of Buffer 'B' at 1ml/min. Under these conditions >95% of the phosphodiesterase activity (assessed at 1µM cyclic AMP or cyclic GMP) was bound to the column. Phosphodiesterase activity was subsequently eluted at 1ml/min using a 400ml linear salt gradient between 0 and 0.5M NaCl contained in Buffer 'B'. 4ml fractions were collected and aliquots (25µl) assayed for cyclic AMP and cyclic GMP phosphodiesterase activity.

2.10: ASSAY OF CALMODULIN FROM COLUMN FRACTIONS.

Fractions of column eluates from ion-exchange chromatography steps were boiled for 5 minutes in order to destroy any endogenous phosphodiesterase activity, whilst retaining heat stable Calmodulin. Aliquots (10µl) were assayed for their ability to stimulate a Calmodulin deficient preparation of Calmodulin-stimulated phosphodiesterase (see Section 2.17). The assay was performed in the presence of 1µM cyclic GMP as substrate and 100µM calcium. This concentration of calcium was found to be saturating for the buffer system used. The final concentration of EGTA in the assay was always 35µM.

2.11: DE-52 PEAK I CHROMATOGRAPHY ON AFFI-GEL BLUE.

Pooled fractions from Peak I of the DE-52 chromatography step were made up to 5mM $MgCl_2$ and loaded at 0.5ml/min onto a column of Affi-gel blue (1.8 x 1.1cm) which had previously been equilibrated in Buffer 'C', (50mM Tris-HCl pH 7.5, 0.15M NaCl, 5mM $MgCl_2$, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2µM leupeptin,

0.1mM EGTA and 0.1mM dithiothreitol). Total unbound protein was collected and the column was washed in 8 bed volumes of Buffer 'C', which was collected as a pooled fraction. All operations were carried out at 4°C.

2.12: MONO Q ANION-EXCHANGE CHROMATOGRAPHY.

This was performed using the Pharmacia Fast Performance Liquid Chromatography system (FPLC).

2.12.1: Mono Q column (HR 5/5) equilibration and maintenance.

All buffers and solutions were filtered through a 0.2µm filter before use, to remove any particles that could clog the resin. The Mono Q column was stored in 25% ethanol and was equilibrated before use using the programme shown in Table 2.1. The composition of Buffer 'A' was 50mM Tris-HCl pH 7.5, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2µM leupeptin, 0.1mM EGTA and 0.1mM dithiothreiotol. Buffer 'B' contained in addition 0.5M NaCl. The column was kept immersed in an ice jacket for the duration of the equilibration and the experiments. At the end of an experimental run the column was washed in 25ml of 1.0M NaCl in distilled deionised water and stored in 25% ethanol. After approximately 10 runs the column was cleaned to remove any material bound to the matrix. The protocol adopted was as follows:

The column was left overnight in a solution of 1 mg/ml pepsin, 0.1M acetic acid and 0.5M NaCl at a temperature of 37°C. This procedure removed any bound protein material. The column was rinsed thoroughly in water and then cleaned using a chemical 'scrubbing' protocol as follows:

The column was inverted and 500µl of 2M NaCl injected onto the column and rinsed thoroughly in distilled water.

500μl of 2M NaOH was then injected onto the column and rinsed with deionised water.

Finally, the column was injected with 500μl of 75% acetic acid and washed overnight in 500ml of deionised water and then stored in 25% ethanol.

2.12.2: Separation of soluble cyclic nucleotide phosphodiesterases.

High speed supernatants from rat liver (20ml containing approximately 120 mg of protein) or hepatocytes (30ml containing approximately 80 mg of protein) were loaded at 1ml/min onto the Mono Q column and the column washed with approximately 1 bed volume of Buffer 'A' (see Section 2.12.1). Under these conditions greater than 95% of the cyclic AMP and cyclic GMP phosphodiesterase activity (assessed at 1μM substrate) bound to the column. Phosphodiesterase activity was then eluted at 1ml/min using a 95ml gradient of NaCl (0-0.5M) in Buffer 'A', as is indicated in Table 3.2. Fractions of 1ml were collected on ice and 25μl aliqouts assayed for phosphodiesterase activity. A similar protocol was adopted for the separation of soluble phosphodiesterase activities from other rat tissues including adipose tissue, kidney and brain.

2.13: BINDING OF PDE MQ-I/PDE MQ-II TO AFFI-GEL BLUE.

Pooled fractions from each of the first two peaks of the Mono Q column step (PDE MQ-I and PDE MQ-II) were each made up to 5mM $MgCl_2$ and individually incubated with portions of Affi-gel Blue. Affi-gel blue was equilibrated by resuspending 100μl portions of the matrix in 1ml of Buffer 'A', (50mM Tris-HCl pH 7.5, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2μM leupeptin, 0.1mM EGTA and 0.1mM dithiothreitol) containing 5mM $MgCl_2$ and 0.1M NaCl, mixing briefly (5mins) and pelleted as below. This was repeated five times. Binding was performed by mixing 0.75ml of each peak with 100μl (packed volume) of Affi-gel Blue matrix for 30 minutes on a

rotary mixer at 4°C. The gel was pelleted using a refrigerated microcentrifuge (Hettich) at full speed (14,000g*av*) for 1 minute and the supernatant containing unbound material removed. The pelleted gel was washed by adding 1ml of Buffer 'A' (50mM Tris–HCl pH 7.5, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2μM leupeptin, 0.1mM EGTA and 0.1mM dithiothreitol) containing 5mM $MgCl_2$ and 0.1M NaCl, mixing for a further 10 minutes and pelleted as before. This was repeated once more and the two washes pooled to give a combined wash (2ml). Phosphodiesterase activity in both the unbound and the wash fractions was determined. Total unbound activity was determined by summing the activity from the binding step with the activity retrieved by washing.

2.14: EFFECT OF AGENTS ON RESOLVED PDE ACTIVITIES.

Fractions were carefully pooled from the mid–point of peaks to minimise any cross contamination from neighbouring activities. For Mono Q resolved activities generally three of four fractions (3 or 4 mls) were chosen for study. For DE–52 peaks approximately 3 fractions were chosen (12mls). Activities were diluted where necessary in Buffer 'A' (50mM Tris–HCl pH 7.5, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2μM leupeptin, 0.1mM EGTA and 0.1mM dithiothreitol). In all instances the volume assayed was 25μl. Agents were diluted to the required concentration in 20mM Tris–HCl pH 7.4 and effects compared with tubes that received 20mM Tris alone. The final concentration of EGTA in the assay was always 25μM.

2.15: DETERMINATION OF IC_{50} VALUES FOR INHIBITORS ON RESOLVED PHOSPHODIESTERASE ACTIVITIES.

Phosphodiesterase inhibitors were dissolved in DMSO at a stock concentration of 25mM and subsequently diluted in 20mM Tris–HCl pH 7.4 to provide a range of concentrations. In order to correct for

the effect of DMSO on resolved phosphodiesterase activities dose response curves were constructed to DMSO to allow for appropriate corrections to be made. It was assumed that the effect of DMSO and the inhibitory agents were additive. IC_{50} values were determined at 1μM substrate concentration using either cyclic AMP or cyclic GMP and a minimum of three concentrations for insensitive forms and five to seven for sensitive forms.

2.16: DETERMINATION OF KINETIC PARAMETERS OF PDEs.

Peak I from the Mono Q step was subjected to a further chromatographic step to minimise any possible residual contamination from Peak II. Pooled fractions from the ion-exchange step were made up to 5mM $MgCl_2$ and applied at 40 ml/hr to a column of Affi-gel Blue (2.2cm x 0.76cm) previously equilibrated in Buffer 'C' (50mM Tris-HCl pH 7.5, 0.15M NaCl, 5mM $MgCl_2$, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2μM leupeptin, 0.1mM EGTA and 0.1mM dithiothreitol). Total unbound activity was collected and the column washed with four 2ml washes of Buffer 'C'. Washes containing the majority of the phosphodiesterase activity were pooled with the unbound activity and used in the kinetic determinations. Peaks II-V, from the Mono Q step, were used without any further purification. Phosphodiesterase activity was assessed using the modified protocol detailed in Section 2.2.4. The final Mg^{2+} concentration in the kinetic determinations was 7.5mM. All assays were done in triplicate and great care was taken to ensure that the rate of hydrolysis was linear with respect to time and that no more than 10% of the substrate was hydrolysed.

2.17: GEL FILTRATION OF RESOLVED PHOSPHODIESTERASES.

The molecular weights of PDE MQ-1 and MQ-II were determined using gel filtration either on Sephacryl S-200 (PDE MQ-1)

or Sephacryl S-300 (PDE MQ-II). Samples of pooled PDE MQ-I and PDE MQ-II were applied to S-200 (66cm x 1.6cm) and S-300 (83cm x 1.6cm) columns respectively. These columns had been previously equilibrated in 50mM Tris-HCl pH 7.4, 0.1M NaCl and 0.1mM EGTA. Columns were run at 8 ml/hr at 4°C in this buffer and fractions were collected and assayed for phosphodiesterase activity. Columns were calibrated using proteins of known molecular weight, namely; cytochrome c (12,400), carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (66,000), alcohol dehydrogenase (150,000), catalase (232,000) and ferritin (440,000). Samples of protein (1-2mg) were applied to each column and detected either by absorbance at 280nm (410nm for ferritin) or by Bradford analysis with detection at 595nm. The inner and void volumes were determined using ϵ-DNP-Lysine and Blue-Dextran respectively. These were detected at both 610nm and 410nm respectively.

2.18: STANDARD CALMODULIN ASSAY.

Calmodulin activity was determined using a Calmodulin-deficient beef heart phosphodiesterase.

2.18.1: Bovine Heart Calmodulin-deficient phosphodiesterase.

500mU of enzyme was resuspended in 2mls of 20mM Tris-HCl pH 7.4, 50% (v/v) glycerol and aliquoted into 50μl aliqouts each containing 12.5mU of enzyme activity. These were stored at -20°C until further use.

2.18.2: Sample preparation.

Column fractions were boiled for 5 minutes to destroy any associated phosphodiesterase activity and spun at maximum speed in a microfuge to pellet any denatured protein. They were then diluted 1/300 in 20mM Tris-HCl pH 7.4 prior to assay.

2.18.3: Calmodulin assay.

For a standard assay, one aliquot (50μl/12.5mU) of enzyme was diluted in 0.575mls of 20mM Tris-HCl pH 7.4, 5mM $MgCl_2$ to give a final concentration of 20 mU/ml. The assay cocktail consisted of 30μl of 20mM Tris-HCl pH 7.4, 50μl of enzyme (1mU), 10μl of 1.5mM $CaCl_2$, 50μl of 1μM cyclic GMP (containing approximately 200,000 cpm of [^{3}H]-cyclic GMP) and 10μl of sample. The final volume of the assay was 150μl. The final concentration of cyclic GMP was 0.33μM, whilst the total added $CaCl_2$ concentration was 100μM. Where EGTA was present in the sample the concentration in the assay was 0.44μM. Phosphodiesterase activity was determined using the standard two step assay detailed in Section 2.2.

2.19: PURIFICATION OF CALMODULIN FROM PIG BRAIN.

2.19.1: Preparation of pig brain extract.

3 pig brains (285g) were excised from freshy killed pigs which had received a lethal dose of barbiturates. Brains were transported immediately to the laboratory on ice and were stored at -80°C overnight. This was accomplished within three hours of death. Brains were defrosted using a microwave set on 'defrost'. All subsequent operations were carried out at 4°C. The material was sliced using a scalpel blade and homogenised in 4% TCA at a final concentration of 25% (w/v) using a Waring blender.

The resulting homogenate was centrifuged at 9,470g*av* (6 x 250ml rotor) for 30 minutes in a Sorvall RC5B centrifuge at 4°C. The supernatant was discarded and the pellet resuspended in 400mls of Tris-Ca^{2+} buffer (50mM Tris-HCl, pH 7.5, 5mM $CaCl_2$, 0.1mM PMSF) using a polytron. The pH of the suspension was adjusted to 7.5 using 3M Tris base (between 10 and 20 mls were usually required). The suspension was stirred for 20 minutes on ice to allow renaturation of precipitated Calmodulin to occur. The extract was then heated to

65°C for 10 minutes and allowed to cool to 4°C before readjusting the pH of the solution to 7.5.

The extract was then spun at 48,250g*av* for 1 hour in either Sorvall SS-34 or Beckman JA-21 rotors. The supernatant was decanted and respun at the same speed for a further 30 minutes. The supernatant was once again decanted and the milky white pellet discarded. This extract (approximately 300mls) was used as the source for the purification of Calmodulin.

2.19.2: Phenyl-Sepharose Chromatography.

The supernatant was applied to a 9ml column of Phenyl-Sepharose CL-4B (3.2cm x 1.9cm) stopped with glass wool at a rate of 2ml/min. The eluate was collected and reapplied to ensure maximal binding to the resin. The column was washed with Tris-Ca^{2+} buffer at approximately 1.5 ml/min followed by approximately 100mls of Tris-Ca^{2+} buffer containing 0.5M NaCl until no protein was found to be eluting as determined by Bradford assay (see Section 2.6). Calmodulin was eluted from the column at a slow rate overnight (approximately 0.2 ml/min) using Tris-EDTA buffer (50mM Tris-HCl, pH 7.5, 2mM EGTA, 0.1mM PMSF) and one hundred 1ml fractions were collected. The protein content of the fractions was determined using the Bradford assay.

2.19.3: Dialysis of partially purified Calmodulin.

Fractions containing Calmodulin activity (about 20mls) were pooled and dialysed against 15mM ammonium acetate pH 8.0 in dialysis tubing which had been pre-soaked in 1% BSA solution to prevent adsorption of Calmodulin to the tubing. Dialysis took place at 4°C against 5 litres of buffer with two 5 litre changes over a 48hr period.

2.19.4: Mono Q anion-exchange chromatography.

The dialysed Calmodulin sample was diluted to 50mls in 50mM Tris-HCl pH 7.4, 1mM EDTA. The sample was applied at 2 ml/min to a Mono Q column previously equilibrated in 25mM Tris-HCl pH

7.4, 1mM EDTA. The column was washed at 1 ml/min in 25mM Tris-HCl pH 7.4, 1mM EDTA until the A_{265} was equal to zero. Calmodulin was eluted at 1 ml/min using a linear salt gradient between 0 and 1M NaCl contained in 25mM Tris-HCl pH 7.4, 1mM EDTA. A major peak of activity which coincided with protein was routinely observed eluting at approximately 0.35M NaCl.

2.19.5: Analysis of Calmodulin purity.

The purity of the Calmodulin preparation was determined by SDS-PAGE using a 3% stacking gel and a 15% resolving gel (see Section 2.20). Calmodulin samples were applied in Laemmli sample buffer containing in addition 1mM EGTA or 10mM $CaCl_2$. Gels were fixed and stained in Coomassie Brilliant Blue and destained as in section 2.21. The preparations routinely displayed a significant increase in mobility in the presence of calcium, a well defined property of Calmodulin. See Figs 2.1-2.3 for a typical purification. Such preparations were judged to be homogenous. Protein content of the preparations was determined by the method of Bradford (1976).

2.19.6: Dialysis of purified Calmodulin.

The purified Calmodulin preparation (5-10mg in 7mls) was dialysed against 3 x 5 litre changes of 0.1M Sodium Borate pH 8.2, 1mM $MgCl_2$, 0.02mM $CaCl_2$ at 4°C for 72 hrs. Such an extensive dialysis was necessary to remove the Tris buffer which will interfere with the coupling of the Calmodulin to the Cyanogen bromide-activated Sepharose since it contains amino groups.

2.20: PREPARATION OF CALMODULIN-SEPHAROSE.

Briefly, Calmodulin purified from porcine brain was coupled to Cyanogen bromide-activated Sepharose by the method of Klee and Krinks (1978).

2.20.1: Preparation of Cyanogen bromide – activated Sepharose.

2g of Cyanogen bromide activated – Sepharose – 4B were swelled to 7.5mls on a scintered glass funnel by washing with 200mls of 1mM HCl under a vacuum. The swollen gel was then prewashed in 40mls of Coupling buffer (0.1M Sodium Borate, pH 8.2, 1mM $MgCl_2$, 0.02mM $CaCl_2$).

2.20.2: Coupling step.

The dialysed Calmodulin preparation from the previous step was diluted 1:1 in coupling buffer and mixed with the gel slurry overnight at 4°C on a rotary mixer. Uncoupled Calmodulin was removed (and retained for analysis) and the gel washed with 200mls of Coupling buffer.

2.20.3: Blocking step.

The gel slurry was then resuspended in 0.5M ethanolamine pH 8.0 and mixed at 4°C for 2 hours on a rotary mixer. The Calmodulin – Sepharose was then washed in 0.02M Tris – HCl, pH 8.0, 1mM $MgCl_2$. It was stored at 4°C in this buffer in the presence of 0.02% azide.

2.21: NON DENATURING POLYACRYLAMIDE GELS.

Electrophoresis was performed in a discontinuous gel system, stacking occuring at pH 6.67 and resolution occuring at pH 8.83.

2.21.1: The Resolving gel.

Tube gels (15cm x 0.45cm) which had been thoroughly cleaned and soaked in chromic acid were used on all occasions. The following buffer solutions (filtered through 0.2μm millipore filter) were used in preparation of the resolving gels:

(a) Resolving gel acryalamide stock: (40%T, 2%C), (10.4% w/v Acrylamide, 0.266% w/v N,N,methylenebisacrylamide).

(b) Resolving gel buffer stock: (1.5M Tris – HCl, pH 8.83).

(c) Potassium Persulphate/Riboflavin/solution: (0.06% w/v potassium persulphate, 0.002% w/v riboflavin). This was prepared fresh just before use.

Resolving gels were prepared at a total monomer concentration of 10% acrylamide with 2% crosslinks. A maximum of twelve gels could be run at any one time.

For twelve 10% polyacrylamide gels, 6ml resolving gel acrylamide stock, 6ml resolving gel buffer stock, 6ml freshly made potassium persulphate/riboflavin solution and 6ml of distilled water were mixed in a brown bottle on ice. The gel mix was deaerated by stirring on ice for 5 minutes while attached to a vacuum pump. Triton X-100 (20% v/v) (240μl) and TEMED solution (10% v/v) (240μl) were added in order to initiate polymerisation. Gel mix (1.6ml) was added to the glass tubes to form gels of approximately 6cm in length. The top of the gels were overlayed with distilled water and photopolymerised by illuminating with fluorescent light at 4°C for 30 minutes. After polymerisation was complete, the gel surface was rinsed 2-3 times with distilled water, residual water removed by aspiration and the tubes dried carefully.

2.21.2: <u>The Stacking gel.</u>

Stock buffers used for the prepartion of stacking gels were filtered as before and consisted of:

(a) Stacking gel acrylamide stock: (8%C, 20%T), (6.4% w/v acrylamide, 1.6% w/v N,N,methylenebisacrylamide).

(b) Stacking gel buffer stock: (0.244M Tris-HCl pH 6.67).

(c) Potassium Persulphate/Riboflavin/solution: (0.06% w/v potassium persulphate, 0.002% w/v riboflavin).

Stacking gels were prepared at a total monomer concentration of 3.5% acrylamide with 20% bisacrylamide crosslinks. It was essential

that the volume of the stacking gel (usually 700µl) was at least twice that of the sample volume loaded onto the gel.

For twelve gels, 6ml of stacking gel acrylamide stock, 3ml of stacking gel buffer stock and 3ml of potassium persulphate/ribolfavin solution were mixed in a brown bottle on ice. The gel mix was deaerated before adding 120µl each of Triton X-100 (20% v/v) and TEMED solution (10% v/v) to initiate polymerisation. The gel mix (700µl) was poured into the tubes, overlaid with distilled water and photopolymerised as described for the resolving gel.

2.21.3: <u>Anode and cathode buffers.</u>

(a) The final composition of the anode buffer was, 0.0625M Tris-HCl pH 7.51.

(b) The final compostion of the cathode buffer was, 0.0426M Tris-HCl pH 8.90, 0.0461M glycine, 0.2% (v/v) Triton X-100.

2.21.4: <u>Sample application to gels.</u>

Samples were made up to 270µl with cathode buffer and a further 30µl of cathode buffer containing 2% (v/v) Triton X-100 and Amaranth dye (sufficient for the pink dye to be visible when loaded on the gel) added. After removal of the parafilm from the ends to the glass tubes and rinsing of the gel surface 2-3 times with distilled water, 300µl of sample was loaded onto the gel and overlaid with cathode buffer containing 0.2% (v/v) Triton X-100 to the top of the tube.

2.21.5: <u>Electrophoresis running conditions.</u>

Electrophoresis was perfomed at 4°C using an LKB power pack and a twelve tube gel apparatus. Anode and cathode buffers were cooled to 4°C overnight. Running conditions were as follows; voltage was fixed at 100V whilst the dye boundary moved through the stacking gel (running time approximately 4 hours) and 160-180V while the boundary moved through the resolving gel. Throughout electrophoresis a current in excess of 0.8mA per gel was avoided if possible.

2.21.6: Gel slicing and elution of phosphodiesterase activity.

Electrophoresis was considered to be complete when the dye boundary reached the end of the resolving gel. Gels were extruded from tubes by using a needle and syringe to force distilled water up the inner rim of the tube until the gel was free. The white stacking gel was discarded and the gel sliced into 1–2mm slices using a scalpel blade. Gel slices were incubated in 100μl of elution buffer containing 20mM Tris–HCl pH 7.5, 0.1mM EGTA and 2mM β–mercaptoethanol and shaken overnight before samples were taken for assay of phosphodiesterase activity.

2.22: SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS–PAGE).

2.22.1: Stock solutions.

The following stock solutions were used in all SDS–PAGE gels:

(a) Buffer 1 (1.5M Tris–HCl pH 8.8, 0.4% (w/v) SDS)

(b) Buffer 2 (0.5M Tris–HCl pH 6.8, 0.4% (w/v) SDS)

(c) Acrylamide/Bisacrylamide (30% (w/v) Acrylamide, 0.8% (w/v) N,N,methylenebisacrylamide).

(d) Electrophoresis Buffer (0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS).

(e) Ammonium Persulphate solution (10% (w/v) ammonium persulphate in water).

(f) Laemmli sample buffer (30% (w/v) urea, 5% (w/v) SDS, 50mM Tris–HCl pH 8.0, 6% (w/v) dithiothreitol (added fresh before use) and a trace amount of Bromophenol blue).

2.22.2: Preparation of the lower (resolving) gel.

For one 10% gel the following were mixed with stirring, 10.4mls of water, 7.5mls of Buffer 1, 10mls of Acrylamide:Bisacrylamide, 2mls of

50% (v/v) glycerol, 112.5μls of ammonium persulphate. Polymerisation was initiated by the addition of 10μls of TEMED. The solution was poured between the plates and gently overlayed with water to form an interface.

For one 15% resolving gel, the following volumes were mixed; 4.4mls of water, 7.5mls of Buffer 1, 15mls of Acrylamide:Bisacrylamide, 3mls of 50% (v/v) glycerol, 7μls of TEMED, and 87.5μls of ammonium persulphate solution.

2.22.3: Preparation of the upper (stacking) gel.

For one 5% stacking gel the following were mixed with stirring, 8.725mls of water, 3.75mls of Buffer 2, 2.525mls of Acrylamide:Bisacrylamide, 0.3mls of ammonium persulphate. Polymerisation was initiated by the addition of 10μl of TEMED. The gel was poured around a comb (usually 10 spaces).

3% stacking gels were used in some instances, consisting of: 9.75mls of water, 3.75mls of Buffer 2, 1.5mls of Acrylamide:Bisacrylamide, 150μls of ammonium persulphate and 8μls of TEMED.

2.22.4: Sample preparation and application to gels.

Samples containing 0.1μg–100μg of protein were mixed with 50–100μl of Laemmli sample buffer and boiled for 5 minutes in a boiling water bath. Pre-stained molecular weight markers were used in all preparations and contained standard proteins of molecular weights 210-kDa, 97-kDa, 68-kDa, 45-kDa, 25-kDa, 18-kDa and 14-kDa.

Boiled samples were applied to gels using a hamilton syringe, care being taken to avoid spillage of the material into adjacent wells.

2.22.5: Precipitation of samples for SDS-PAGE.

For samples that were in a very dilute solution, a precipitation protocol was adopted involving the use of trichloroacetic acid and sodium deoxycholate. The following stock solutions were used;

(a) 2% (w/v) sodium deoxycholate

(b) 24% (w/v) trichloroacetic acid (TCA)

(c) Neutralising solution (1.0M Tris-base)

25µl of sodium deoxycholate was added to the sample and the volume made up to 3mls with water. 1ml of TCA was then added and precipitation allowed to occur at room temperature for 2 minutes. The cloudy suspension was spun at setting 7 in a Beckman TJ-8 centrifuge for 20 minutes. The supernatant was aspirated off and the tubes inverted over tissue to remove as much of the TCA as possible. 20µl of Tris-Base was added to neutralise the pellet. The pellet was solubilised in Laemmli sample buffer as in Section 2.22.4. In cases where the Bromophenol blue indicator was yellow then further aliquots of Tris-Base were added until a blue colour was obtained.

2.22.6: Electrophoresis conditions.

Electrophoresis was performed on an LKB two gel apparatus. Gels were normally run at a constant current of 60mA and a voltage of 300V for approximately 2½ hours or until the Bromophenol Blue tracking dye reached the end of the gel. Gels were cooled where possible to prevent excessive heating and consequent distortion of the resolved bands.

2.23: COOMASSIE BLUE STAINING OF SDS-PAGE GELS.

SDS-PAGE gels were fixed and stained in a solution of 0.25% Coomassie Blue (Brilliant Blue R250 form) in 5:1:5, water:acetic acid:methanol, for at least one hour at room temperature. Destaining was performed in the same solution lacking the Coomassie Blue R250.

2.24: SILVER STAINING OF SDS-PAGE GELS.

The method adopted was based on that of Wray, et al., (1981)

2.24.1: <u>Silver staining solutions.</u>

All solutions were prepared using distilled and deionised water. To stain one gel the following solutions were prepared:

(a) 50% (v/v) Methanol (Bulk), 200mls

(b) Solution 'A': 20% (w/v) silver nitrate, 4mls

(c) 0.3% (w/v) sodium hydroxide, 100mls

(d) Solution 'B': 21mls of 0.3% (w/v) sodium hydroxide solution, 1.4mls of 14.8M ammonium hydroxide.

(e) 1M Citric acid.

(f) Developer: 0.12 mls of 1M Citric acid and 0.25mls of 38% formaldehyde solution. These were mixed and the volume increased to 500mls with distilled deionised water. This solution was prepared fresh just prior to use.

2.24.2: <u>Silver staining proceedure.</u>

SDS-PAGE gels to be silver stained were fixed in 50% methanol overnight (3 x 200ml changes) to fix the proteins in the gel and allow for the removal of glycine. Since glycerol and urea produce high backgrounds these were removed by transferring the methanol fixed gel to water and allowing it to swell. The water was changed several times to allow for the diffusion of urea and glycerol out of the gel. The use of acetic acid in the fixing procedure was avoided since this has been shown to inhibit the silver staining reaction.

4mls of solution 'A' was added dropwise to solution 'B' with constant stirring and the volume increased to 100mls with water. This solution was used within 5 minutes The gel was placed in this mixture in a clean container and left for 15 minutes on an orbital shaker.

The gel was then washed in deionised water for 1 hour at 40°C (4 x 200ml washes). The gel was then incubated in fresh developer until the appearence of protein bands. In some instances the appearance of bands was enhanced by the addition of further aliquots

of formaldehyde to the developer solution (0.25mls per 500mls). Gels were removed and washed extensively in water and then placed in 50% (v/v) methanol to stop the reaction.

2.25: DESTAINING SILVER STAINED SDS-PAGE GELS.

2.25.1: Destaining solutions.

To destain one SDS-PAGE gel the following reagents were prepared;

(a) Reagent 'A': A 150ml solution containing 4.9% (w/v) sodium chloride and 4.9% (w/v) Copper (II) Sulphate. Concentrated ammonium hydroxide was added until the precipitate that formed was completely dissolved to give a deep blue solution.

(b) Reagent 'B': A 200 ml solution containing 43.85% (w/v) sodium thiosulphate.

2.25.2: Destaining method.

Reagents 'A' and 'B' were mixed in equal volumes just prior to use. The gel was covered in this solution and gently shaken in a fume cupboard. The gel usually destained within 5-10 minutes. The gel was then soaked in water on a shaker to remove reagents from the gel. This was usually performed overnight at room temperature.

2.26: AUTO-RADIOGRAPHY OF DRIED GELS.

Gels were dried down onto Whatman 3MM paper under a vacuum at 80°C for two hours. Gels were exposed to X-ray film using Kodak autoradiography cassettes fitted with intensifying screens at -80°C.

2.27: IODINATION OF CALMODULIN-STIMULATED PDE.

Samples of purified Calmodulin-stimulated phosphodiesterase were prepared by the method described in Chapter 4. Iodination was performed using the Chloramine T method. This selectively catalyses the iodination of tyrosine residues (see Roholt and Pressman, (1972) for a general method).

2.27.1: Iodination reagents.

The following reagents were prepared:

(a) Chloramine T solution: 0.04% Chloramine T in distilled water. This was prepared just before use.

(b) 1M Tris-HCl pH 7.5

(c) 0.1M Tris-HCl pH 7.0

(d) Stopping Buffer (9.8mls of 0.1M Tris-HCl pH 7.0 and 200μl of β-mercaptoethanol).

(e) 10% (w/v) SDS solution.

(f) 50% (v/v) glycerol containing a trace of Bromophenol Blue.

2.27.2: Iodination reaction.

The sample was dialysed extensively against 50mM Tris-HCl pH 7.5, 0.2mM PMSF, 5mM Benzamidine, 2μM leupeptin, 0.1mM EGTA at 4°C to remove any traces of dithiothreitol before freeze drying. All subsequent operations were carried out at room temperature. 22μl of 1.0M Tris-HCl pH 7.0 was added to the lyophilised sample followed by 8μl of 10% (w/v) SDS. The sample was thoroughly vortexed mixed to ensure complete resuspension of the freeze dried powder. Approximately 200μCi of ^{125}I was then added followed by 10μl of Chloramine T solution to initiate the iodination reaction. After 2 minutes the reaction was stopped by adding 40μl of stopping buffer. 15μl of 50% glycerol containing Bromophenol blue was then added and the sample boiled for 1 minute prior to SDS-PAGE on 10% gels with a 5% stacking gel. If the samples were to be subsequently analysed by

one dimensional peptide mapping the gels were fixed in 50% (v/v) methanol and dried down under a vacuum at 80°C for two hours.

2.28: ONE DIMENSIONAL PEPTIDE MAPPING.

This was performed by the method of Cleveland, et al., (1977). Gel chips were excised from gels that had been fixed in 50% (v/v) methanol. It was important to ensure that gels were not fixed in acid in order to prevent acid hydrolysis of the peptide bond of the protein during drying down at 80°C. The gel chips were rehydrated in 40μl of 0.125M Tris-HCl pH 6.7, 0.1% SDS, 10mM dithiothreitol overnight at room temperature. The rehydrated sample was then removed from the backing paper, sliced into very small slices. and placed in the well of an SDS-PAGE gel consisting of a 5% stacking gel with a 15% resolving gel (section 2.20). The sample was overlayed with 40μls of 0.125M Tris-HCl pH 6.7, 10% (v/v) Glycerol and 0.1% (w/v) Bromophenol Blue in the presence or absence of 200ng of TPCK treated trypsin.

Gels were electrophoresed at a constant current of 60mA and a voltage of 300V. After the Bromophenol Blue dye front was about to enter the separating gel the current was switched off for 30 minutes to allow trypsinisation to occur. Thereafter, electrophoresis was allowed to continue at the above current and voltage. The gel was fixed in a 5:1:5 mixture of water:acetic acid:methanol and dried at 80°C for two hours prior to autoradiography. Exposed autoradiographs were then scanned using a Biorad model 620 gel scanner operated by an Olivetti M21 computer.

2.29: ISOLATION OF AN IgG FRACTION FROM RABBIT BLOOD.

Immunised rabbits were bled by puncturing of a vein in the ear. Routinely 20-30mls of blood were collected at any one time. The blood was allowed to clot for 2 hours at 4°C. The clot was removed manually with gentle squeezing to remove trapped serum. The resultant

serum was then spun at 15,000g for 15 minutes at 4°C to remove blood cells and any traces of blood clot. The supernatant was retained for further purification which was either performed immediately or at a later date after storage at −20°C.

The supernatant was mixed with 6g wet weight of DEAE−Cellulose which had previously been equilibrated in equlibration buffer (10mM KH_2PO_4/K_2HPO_4 pH 7.4) at 4°C. Incubation was continued for 30 minutes with occasional stirring. Unbound protein containing the IgG fraction was separated from the DEAE−Cellulose by filtration using a Buchner flask attached to a vacuum pump. An equal volume of equilibration buffer was then added and sufficient ammonium sulphate added to bring the solution to 45% saturation. The solution was stirred on ice for 15 minutes prior to spinning at 15,000g for 15 minutes to pellet the precipitated IgG fraction. The pellet was resuspended in approximately 5mls of equilibration buffer and dialysed overnight against two 2 litre changes of equilibration buffer. The IgG fraction was stored as 50μl aliquots at −80°C. The purity of the preparation was determined by SDS−PAGE electrophoresis and always demonstrated significant enrichment of protein bands corresponding to the heavy and light chains of IgG.

2.30: WESTERN BLOTTING.

2.30.1: Western Blotting: Solutions.

(a) Transfer buffer: (0.025M Tris−HCl, 0.192M Glycine, 20% (v/v) methanol).

(b) Tris−buffered saline (TBS): (20mM Tris−HCl pH 7.5, 0.5M NaCl).

(c) Tris−Tween buffered saline (TTBS): (20mM Tris−HCl pH 7.5, 0.5M NaCl, 0.05% (v/v) Tween−20).

(d) Blocking buffer: 3% (w/v) gelatin in TBS.

2.30.2: Western Blotting: Primary antibody.

The antibody was dissolved at a suitable dilution (usually 1/200 or 1/100) in 50 mls of TBS containing 1% (w/v) gelatin. The antibody was then stored in this buffer at 4°C. At this temperature the gelatin solidifies, so before use the buffer was placed in a 37°C water bath to melt. After use the antibody was made up to 0.05% (w/v) Thimerosol, an anti-bacterial agent, to act as a preservative. This addition was repeated after every use of the antibody up to a maximum of five times or when signals from the blot became noticeably weaker.

2.30.3: Western Blotting: Secondary antibody.

Horseradish peroxidase linked goat anti-rabbt IgG was dissolved at a suitable dilution (usually 1/200) in TBS containing 1% (w/v) gelatin. This solution was treated in the same way as the primary antibody.

2.30.4: Western Blotting: Method.

After separation of proteins on a suitable SDS-PAGE gel the proteins were transferred to nitrocellulose paper in an LKB Transphor apparatus at maximum current (2A) for 90 minutes at room temperature using a cooling apparatus. Once transfer had been achieved (assessed by the presence of prestained molecular weight markers) the nitrocellulose was blocked in 100mls of blocking buffer for 90 minutes at 37°C. The blocking buffer was then removed and the blot washed thoroughly in distilled water until all the unbound gelatin had been removed. The blot was then placed in the primary antibody solution for 12-18hrs at 37°C. The antibody solution was subsequently removed and retained if necessary and the blot then rinsed in TBS followed by two 10 minute washes in TTBS and one further 10 minute wash in TBS. The second antibody solution was then placed on the blot and incubated for 2-3 hours at 37°C. The antibody solution was then removed and retained and the blot washed as above in TTBS and TBS. Bands were detected by adding 40 ml of 10mM Tris-HCl pH 7.4 followed by 1ml of 1% (w/v) *o*-dianisidine and finally 10μl of 30%

(v/v) hydrogen peroxide to initiate colour development. Bands usually became detectable within 1–2 minutes. The blot was then rinsed well in water and dried between filter paper and stored away from light.

2.30.5: Use of ^{125}I–Protein A in Western Blotting.

In instances where quantification of immuno–stained bands was required Iodinated (^{125}I)–Protein A was used in place of horseradish peroxidase linked goat anti–rabbit IgG. The antibody solution, containing a suitable amount of radioactivity (2–5 µCi/blot), was prepared in 1% (w/v) gelatin in TBS, was used exactly as for the peroxidase linked goat anti–rabbit IgG but was not retained for further use.

2.31: PREPARATION OF A RAT LIVER HYPOTONIC EXTRACT.

Routinely this was prepared from 5 rat livers by a slight modification of the method detailed in Pyne, et al., (1987a). In some instances a hypotonic extract was prepared from one rat liver. In such cases the volumes used were approximately one fifth of the ones detailed below.

5 male Sprague–Dawley rats (250–275g) were sacrificed and the livers excised chopped, and rinsed in TES Buffer (10mM Tris–HCl pH 7.4, 1mM EDTA, 0.25M Sucrose) on ice. All subsequent operations were carried out at 4°C. Livers were rapidly homogenised in 8 volumes of TES Buffer by placing in Waring blender at maximum speed for 1 minute. This procedure was found to be much faster than hand homogenisation of the diced livers, which could take up to 40 minutes to complete for 5 rat livers. The homogenate (550mls) was filtered through cheese cloth and the filtrate centrifuged for 10 minutes at 1000rpm in an MSE Minor Centrifuge. The supernatant (450mls) was removed and recentrifuged at 30,000*gav*, for 20 minutes in a Beckman JA–20 rotor. The pellet was gently resuspended in 300mls of TE Buffer (10mM Tris–HCl pH 7.4, 1mM EDTA) using a hand

homogeniser and left at 4°C for 40 minutes. The hypotonically solubilised extract was then centrifuged at 48,000g*av* for 30 minutes. The supernatant (285mls) was removed and made up to 10mM $MgCl_2$ and recentrifuged at 48,000g*av*. This extra step was included because it was routinely observed that addition of $MgCl_2$ caused precipitation of some protein. When assayed for phosphodiesterase activity the $MgCl_2$ precipitated pellet contained less than 10 pmol/min of cyclic AMP PDE activity assessed at 0.1μM cyclic AMP (0.14% of total). The supernatant (hypotonic extract) from this procedure was then used as the source of the enzyme.

2.32: PRODUCTION OF DV-1 SEPHAROSE AFFINITY RESIN.

Polyclonal antisera raised to the purified 'dense-vesicle' phosphodiesterase (DV1) by Pyne, et al., (1987a) was coupled to Cyanogen bromide-activated Sepharose 4B using the following method;

0.5g of Cyanogen bromide-activated Sepharose was swelled by washing in 1mM HCl (100mls) on a glass fibre disc. The swelled gel was washed in approximately 150mls of Coupling Buffer (0.1M $NaHCO_3$ pH 8.5, 0.5M NaCl). This produced a gel of approximately 1.75mls.

The gel was coupled to the antisera DV-1 (0.4mls containing 0.6mg of protein) in the presence of 3.1mls of Coupling Buffer. The mixture was applied to a rotary mixer and coupling allowed to occur at room temperature for 2½ hours.

The coupled gel was washed in Coupling Buffer (150mls) on a scintered glass filter. Unreacted sites were blocked by incubating in Blocking Buffer (0.1M $NaHCO_3$ pH 8.0, 0.5M NaCl, 0.1M ethanolamine) for 2½ hours at room temperature. The gel was washed with 200mls of coupling buffer followed by 200mls of 0.1M Sodium acetate, 0.5M NaCl pH 4.0 and then a further 200mls of coupling buffer. The gel was stored at 4°C in 3.5mls of coupling buffer final pH 8.5 until use.

The gel was equilibrated in TE Buffer (10mM Tris-HCl pH 7.4, 1mM EDTA) prior to use by withdrawing samples from the stroage buffer and resuspending in TE Buffer (0.5ml per 3.33μl of DV-1 sepharose matrix). The gel was pelleted at maximum speed in a microfuge (30 seconds) and the washing procedure repeated four more times.

FIGURE 2.1:

ELUTION OF CALMODULIN ACTIVUTY FROM PHENYL SEPHAROSE.

Calmodulin activity was eluted from Phenyl-Sepharose as indicated in Section 2.29.2. Calmodulin activity was assayed by the ability of aliquots of column fractions to stimulate the activity of a crude preparation of Calmodulin deficient beeg heart phosphodiesterase (Sigma) see Section 2.18. Protein was determined by the method of Bradford (Section 2.7).

(□) Protein (A_{595})

(■) Calmodulin activity expressed as fold stimulation of phosphodiesterase activity.

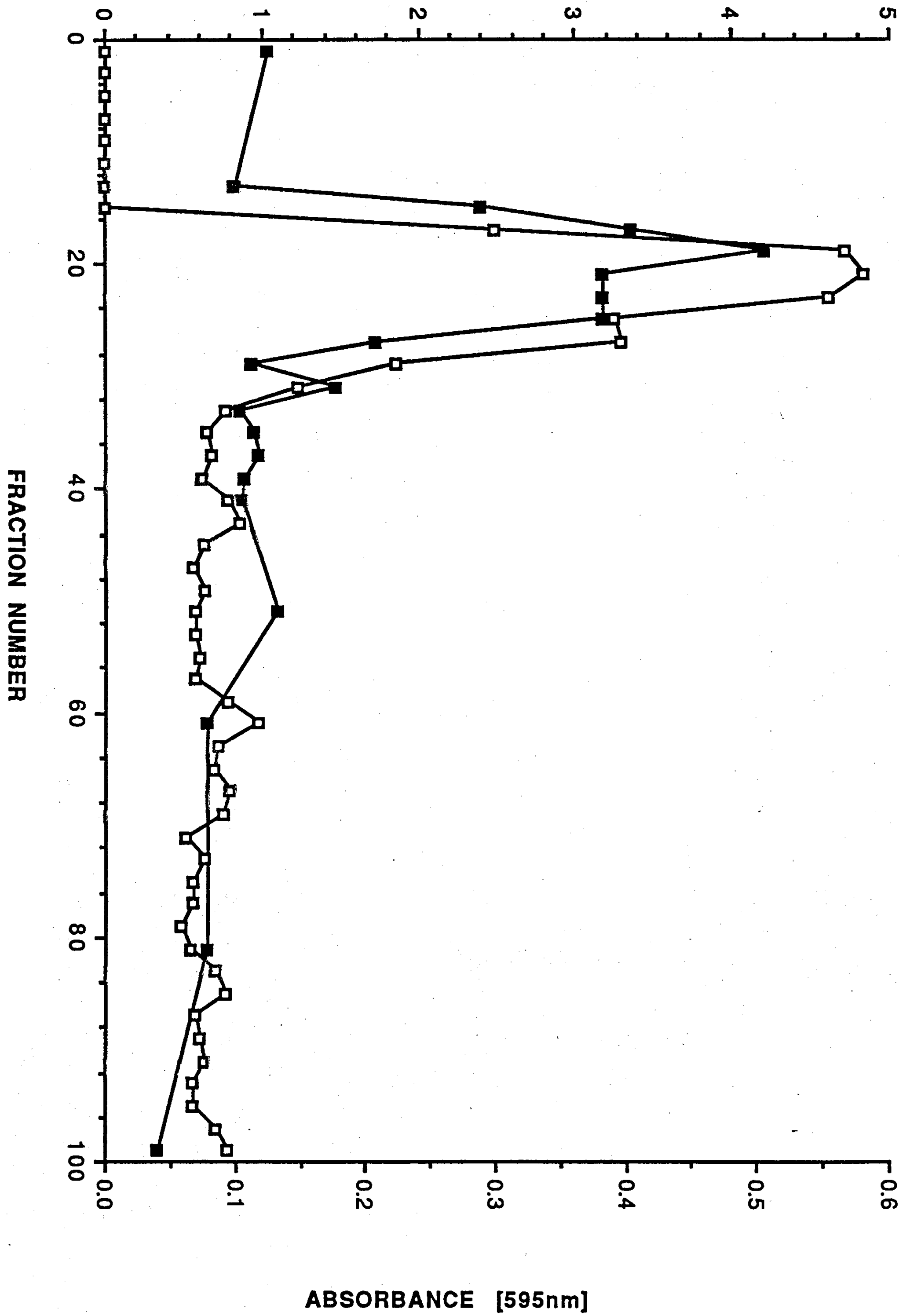


FIGURE 2.1:

FIGURE 2.2:

ELUTION OF CALMODULIN PROTEIN FROM MONO Q ANION-EXCHANGE.

Calmodulin was eluted from the Mono Q anion-exchange resin using a sodium choride gradient between 0 and 1.0M NaCl and 95 x 1 ml fractions collected. Aliquots were assayed for protein by the method of Bradford (Section 2.7).

FIGURE 2.2

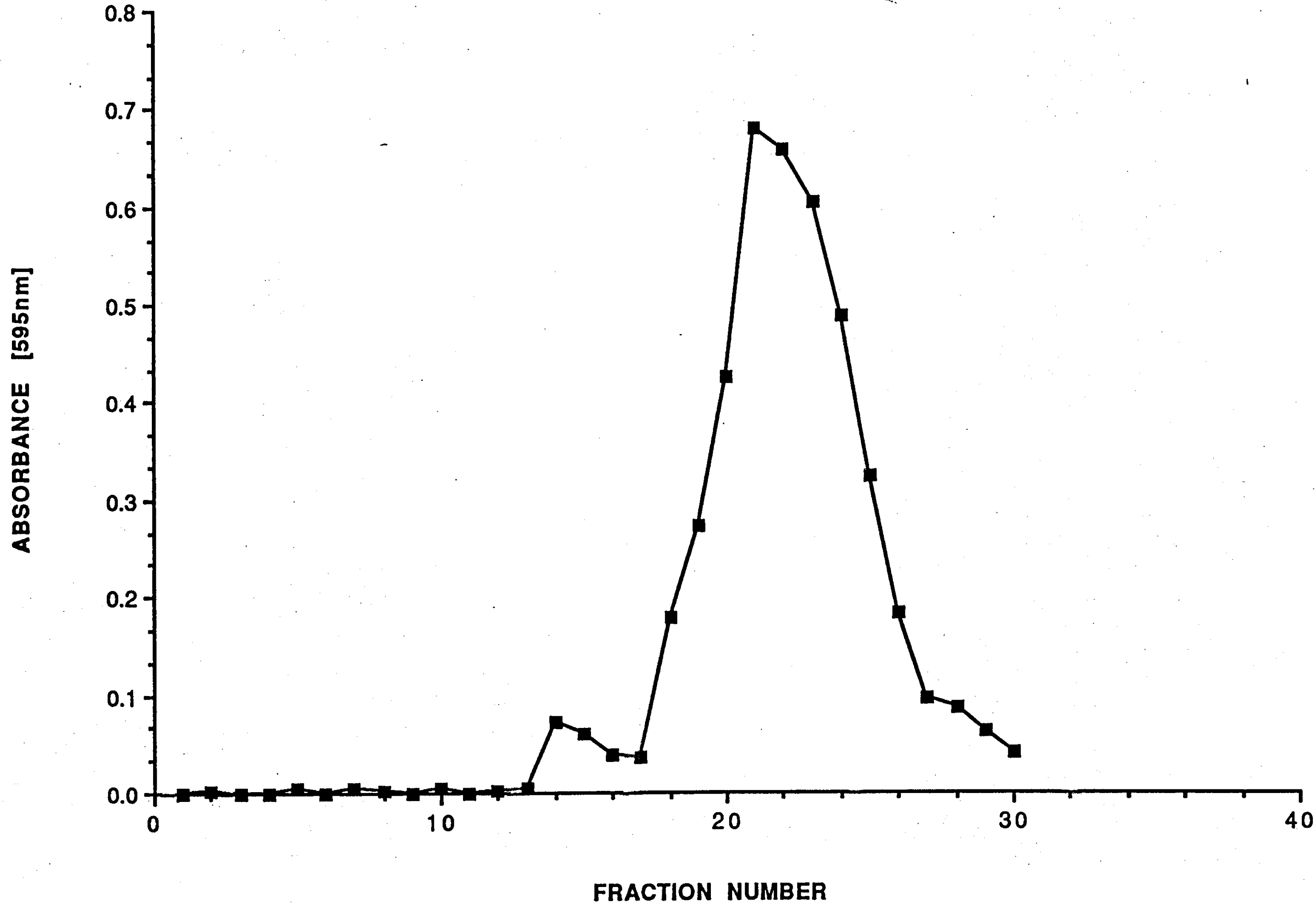
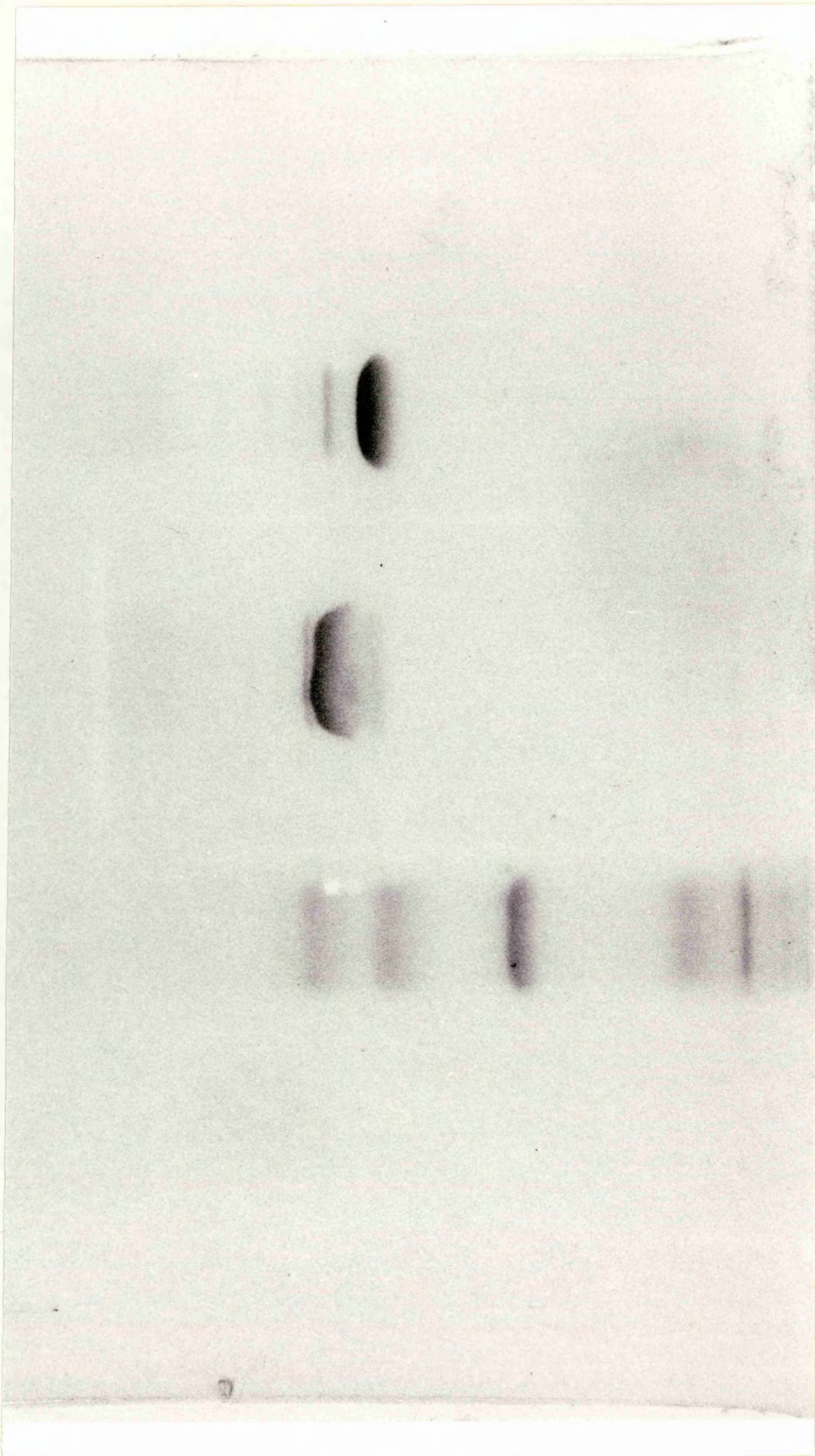


FIGURE 2.3:

SDS-PAGE GEL OF PURIFIED PORCINE BRAIN CALMODULIN.

The purified calmodulin preparation was electrophoresed on a 15% SDS-PAGE gel (Section 2.22) in the either the presence of 10 mM $CaCl_2$ or 1 mM EGTA. The gel shows the increase in mobility observed in the presence of $CaCl_2$.



CHAPTER 3.

RAT LIVER AND RAT HEPATOCYTE SOLUBLE PHOSPHODIESTERASES.

Multiple forms of cyclic nucleotide phosphodiesterase have been demonstrated in a wide range of tissues, including heart (Weishaar,et al.,.1987a; Weishaar, et al., 1987b), adipose tissue (Weber and Appleman, 1982), liver (Heyworth, et al., 1983d) and brain (Thompson and Appleman, 1971). Various techniques have been employed in the separation of phosphodiesterase isozymes, with anion exchange resins such as DE-52 or DEAE-Sephacel being the most commonly employed techniques (Russel, et al., 1973; Thompson and Appleman, 1971; Reeves, et al., 1987; Turnbull and Hickie, 1984; Weishaar, et al., 1987b; Hidaka and Asano, 1976; Grady and Thomas, 1986, Lugnier, et al., 1986; Erneux, et al., 1980).

These resolved isozymes are usually analysed as pooled fractions of peaks of phosphodiesterase activity obtained after salt elution from the columns. Such resolved peaks of cyclic nucleotide phosphodiesterase activity have been characterised on the basis of substrate specificity, sensitivity to agents such as calcium and calmodulin, kinetic parameters and response to various phosphodiesterase inhibitors (for reviews see Beavo, et al., 1982; Beavo, 1988). Studies of this nature have provided strong evidence for the existence of functionally distinct enzyme forms and this, in turn, raises the possibility that each form may have a unique role in the regulation of intracellular cyclic AMP levels.

Rat liver contains cyclic nucleotide phosphodiesterase activity in both the soluble and the particulate fractions. Using a Percoll fractionation procedure it has been shown that phosphodiesterase activity is associated with all membrane fractions except the lysosomes and the nucleus (Heyworth, et al., 1983d). The activities in the plasma membrane fraction (Marchmont and Houslay, 1980a), the mitochondrial fraction (Cercek and Houslay, 1983a), and the endoplamic reticulum fraction (Cercek and Houslay, 1983b; Wilson and Houslay, 1983) have been identified and characterised.

A number of these particulate phosphodiesterase isozymes have been studied in

some detail. These include two cyclic AMP-specific forms: the 'peripheral' plasma membrane phosphodiesterase (Marchmont, et al., 1981b; Pyne, et al., 1986b; Pyne, et al., 1989) and the 'dense-vesicle' phosphodiesterase (Pyne, et al., 1987a; Loten, et al., 1978; Boyes and Loten, 1988, Whitson and Applman, 1982). Both of these two latter enzymes have been shown to be stimulated by hormones.

In comparison to such analyses the soluble fraction of rat liver has received very little attention. A crude separation of the phosphodiesterase activity of rat liver-derived soluble fractions has been perfomed using DEAE-chomatography, which identified three peaks of phosphodiesterase activity (termed PDE I, PDE II and PDE III) based upon their order of elution (Russell, et al., 1973)). However, studies on phosphodiesterases in cardiac (Weishaar, et al., 1987a; Weishaar, et al., 1987b; Weishaar, et al., 1987c; Reeves, et al., 1987) and adrenal tissue (Beavo, et al., 1982) have implied that the complement of enzyms in the soluble may be more complex than originally surmised from crude separations performed on DEAE-cellulose chromatography. Furthermore, earlier investigators have used conditions such as hypotonic extraction and sonication (see for example Moss, et al., 1978; Turnbull and Hickie, 1984; Strada, et al., 1981).which have been shown to release membrane bound phosphodiesterases into the soluble fraction (Loten, et al., 1978, Loten, et al., 1980; Pyne, et al., 1987a; Whitson and Appleman, 1982). Also protease inhibitors, which are essential to prevent modification of phosphodiesterases (Wilson and Houslay, 1983; Strada, et al., 1981; Tucker, et al., 1981; Kincaid, et al., 1985; Price, et al., 1987) were not added. With this in mind it was decided to undertake to resolve and characterise the cyclic nucleotide phosphodiesterases from the soluble fraction of rat liver prepared under isotonic conditions and in the presence of protease inhibitors.

Further to this, it was also decided to analyse the phosphodiesterases present in the soluble fraction derived from hepatocytes. Whilst most studies of this nature are conducted using soluble prepations prepared from whole tissue/organ

homogenates there is always the concern that multiplicity observed is due to the presence of multiple cell types rather than a true reflection of the number and type of phosphodiesterases present. With this in mind it was also decided to analyse the soluble phosphodiesterase isozymes present in soluble fractions-derived from hepatocytes.

3.1: CHARACTERISTICS OF RAT LIVER SUPERNATANT.

A supernatant prepared from one rat liver homogenate under isotonic conditions and in the presence of the protease inhibitors, Benzamidine, Leupeptin and PMSF (see Section 2.4), contained both cyclic AMP and cyclic GMP phosphodiesterase activity. At 1 μM substrate, these were present in approximately equal amounts since the ratio of cAMP:cGMP hydrolysis was 0.95 +/- 0.05 (n=3, +/- SEM). At this substrate concentration, the total cyclic AMP and cyclic GMP phosphodiesterase activities in one rat liver were 12,968 pmol/min +/- 2,128 pmol/min (n=3, +/- SEM) and 13,804 pmol/min +/- 2,381 pmol/min (n=3, +/- SEM) respectively. The average protein content of the supernatant prepared from one rat liver under these conditions was 259 mg +/- 27 mg (n=3, +/- SEM).

The supernatant was further characterised by analysing the response of both the cyclic AMP and cyclic GMP phosphodiesterase activities to Ca^{2+} and cyclic GMP to assess for the presence of the Ca^{2+}/calmodulin-stimulated and the cyclic GMP-stimulated/inhibited phosphodiesterases respectively.

Figure 3.1 shows that addition of increasing calcium concentrations stimulated the total cyclic AMP and cyclic GMP phosphodiesterase activities of the rat liver supernatant. The maximal responses produced were, however, small in both cases. Cyclic GMP PDE activity was stimulated some 26.9% +/- 2% (n=5, +/- SEM) and cyclic AMP PDE activity 19.3% +/- 6.35% (n=3, +/- SEM). Stimulation occured in a dose dependent manner with maximal stimulation occuring between 10 μM and 50 μM added Ca^{2+}. The actual free calcium concentration was not calculated. Addition of calmodulin alone, at a saturating concentration (2 μg/ml) did not stimulate total cyclic GMP or cyclic AMP PDE activity (less than 1% stimulation) indicating the presence of endogenous calmodulin in the rat liver supernatant. This was later confirmed by assaying for endogenous calmodulin activity (see Section 3.3). The addition of

Ca^{2+} to a maximal level (100 µM), in the presence of 2 µg/ml of calmodulin, did not produce any greater stimulation of cyclic GMP PDE activity than that observed with Ca^{2+} alone. Under these conditions, the cyclic GMP phosphodiesterase activity was stimulated 25% +/− 4.2% (n=3, +/− SEM) and cyclic AMP phosphodiesterase activity some 15.7% +/− 2.8% (n=3, +/− SEM). This was taken to imply that the supernatant contained saturating levels of calmodulin.

Since the rat liver supernatant was prepared in the presence of 0.1 mM EGTA, in order to chelate endogenious Ca^{2+}, it was necessary to assess that this level of EGTA was sufficient for this purpose. Figure 3.2 shows that addition of further EGTA up to 500 µM, did not decrease either cyclic GMP or cyclic AMP PDE activity (activities were 99.7% +/− 1.7% (n=3, +/− SEM) and 96.5% +/− 4.6% (n=3, +/− SEM) of control cyclic GMP and cyclic AMP PDE activities respectively).

Rat liver contains a soluble Ca^{2+}−activated neutral protease (CANP) (Takai, et al., 1977: Demartino, 1981) that is activated in the presence of micromolar levels of calcium. It is well documented that phosphodiesterase activity can be activated by limited proteolysis (Cheung, 1971; Tucker, et al., 1981; Kincaid, et al., 1985) so the calcium effect may be mediated by activation of the CANP. The data shown in Figure 3.3 indicates that the Ca^{2+}−mediated activation of cyclic GMP PDE activity could be reversed by an addition of EGTA, indicating that involvement of the CANP was unlikely since it's action would be irreversible. Reversibility of the Ca^{2+} activation of soluble cyclic AMP PDE activity was not performed because of the smaller level of stimulation observed.

These data indicated then, that there was a Ca^{2+}−stimulated cyclic nucleotide phosphodiesterase activity present in the supernatant extract. In addition, the cyclic GMP−stimulated cyclic AMP PDE was shown to be present by the observation that cyclic AMP hydrolysis, at

1 μM substrate, could be stimulated by 2 μM cyclic GMP (stimulated levels were 273% +/- 31% (n=3, +/- SEM) of control values).

3.2: DE-52 SEPARATION OF RAT LIVER SOLUBLE PHOSPHODIESTERASE ACTIVITY.

In order to assess the number and 'type' of phosphodiesterases present in the rat liver supernatant, it was decided to attempt separation of the individual isozymes by anion-exchange chromatography, a technique that has been used extensively by a number of other groups to identify phosphodiesterase isozymes (Russel, et al., 1973; Moss, et al., 1978; Turnbull and Hickie, 1984; Reeves, et al., 1987).

Fractionation of total rat liver supernatant phosphodiesterase activity, by anion-exchange chromatography on DE-52, resulted in the resolution of three peaks of phosphodiesterase activity, when assessed at 1 μM cyclic AMP and cyclic GMP, as shown in Figure 3.4. These are referred to as DE-52 Peaks I to III inclusive.

Peak I eluted at approximately 0.07 M NaCl and hydrolysed both cyclic AMP and cyclic GMP equally well at 1 μM substrate; the cAMP:cGMP hydrolysis ratio for the Peak being 1.07 +/- 0.09 (n=7, +/- SEM).

Peak II eluted at approximately 0.16 M NaCl and also hydrolysed both substrates, with some selectivity for cyclic GMP, since the ratio of cAMP:cGMP hydrolysis for this activity was 0.35 +/- 0.12 (n=3, +/- SEM).

Peak III partially co-eluted with Peak II but appeared to be more selective for cyclic AMP as substrate since the cAMP:cGMP hydrolysis ratio was 4.16 +/- 1.74 (n=3, +/- SEM). The actual yields of activity for the individual Peaks were not calculated due to the partial co-elution of activities.

The total yield of cyclic AMP and cyclic GMP PDE activities from the column were 84% +/- 1% (n=6, +/- SEM) and 92% +/- 2% (n=6, +/- SEM). Extending the gradient to 0.8M NaCl did not elute any further activity.

3.3: RESPONSE OF DE-52 FRACTIONATED RAT LIVER SOLUBLE PHOSPHODIESTERASES TO EFFECTORS.

In order to characterise the individual Peaks more fully, the responses of both the cyclic AMP and cyclic GMP PDE components to saturating levels of Ca^{2+}/calmodulin and also the response of the cyclic AMP PDE component to 2 μM cyclic GMP were investigated. The responses of the individual Peaks are summarised in Table 3.1.

Peak I contained a Ca^{2+}/calmodulin-stimulated phosphodiesterase since the cyclic AMP and cyclic GMP PDE activities were both stimulated by these agents. The response for the cyclic GMP PDE component of the activity was however much larger than the response of the cyclic AMP PDE component (110% stimulation compared with 32% stimulation of control activity for cyclic AMP and cyclic GMP respectively). Ca^{2+} alone did not significantly activate either this Peak of activity or any of the other two. This implied that calmodulin was dissociated from the phosphodiesterase under these conditions. Assays of calmodulin activity were performed and it was shown that calmodulin eluted as a single peak of activity from the DE-52 column at a NaCl concentration of approximately 0.3 M NaCl (Figure 3.4) and did not co-elute with phosphodiesterase activity.

Peak II clearly contained the cyclic GMP-stimulated phosphodiesterase, since cyclic AMP hydrolysis was stimulated some 6.9 fold by 2 μM cyclic GMP. Both the cyclic AMP and cyclic GMP activities in this Peak were stimulated to some extent by Ca^{2+}/calmodulin (25% and 19% stimulation respectively).

Peak III contained a cyclic AMP PDE activity that was unresponsive to cyclic GMP since no significant stimulation, or inhibition, of cyclic AMP hydrolysis was observed in the presence of this nucleotide.

These observations showed the presence of a Ca^{2+}/calmodulin-stimulated PDE activity, a cyclic GMP-stimulated PDE activity and a cyclic GMP-insensitive cyclic AMP-specific PDE activity in the soluble fraction of rat liver.

Although Peak I clearly contained the Ca^{2+}/calmodulin-stimulated phosphodiesterase activity, it was apparent that it could hydrolyse both cyclic AMP and cyclic GMP with nearly equal efficiency and that the fold stimulation of cyclic AMP hydrolysis was much less than for cyclic GMP hydrolysis. Evidence from a number of workers has indicated that most of the forms of this enzyme are more selective for cyclic GMP as substrate (Shenolikar, et al., 1985; Sharma and Wang, 1986a; Sharma and Wang, 1986b; and see Table 1.2). This raised the possibility that there was more than one activity present in Peak I.

3.4: THERMAL STABILITY OF THE CYCLIC GMP AND CYCLIC AMP PHOSPHODIESTERASE ACTIVITIES OF DE-52 PEAK-I.

The thermal stabilities of the cyclic AMP and cyclic GMP PDE activities of Peak I were assessed at 60°C. Figure 3.5 shows that the cyclic AMP and cyclic GMP PDE activities decayed with kinetics indicative of single sites. However, the half lives for decay of activity were different for the two activities. Cyclic GMP PDE activity decayed with a half life ($t_{0.5}$) of 125 seconds +/- 12 seconds (n=3, +/- SEM) whilst cyclic AMP PDE activity decayed with a $t_{0.5}$ of 260 seconds +/- 15 seconds (n=3, +/- SEM). These results implied that there were indeed two separate activities, which were almost entirely selective for cyclic AMP and cyclic GMP respectively, since the

kinetics of decay suggested the presence of single selective activities for hydrolysis of these two cyclic nucleotides.

3.5: CHROMATOGRAPHY OF PEAK-I ON AFFI-GEL BLUE.

It has been shown that bovine brain isozymes of the Ca^{2+}/calmodulin-stimulated phosphodiesterase can be bound to the dye ligand column Affi-gel Blue (Morril, et al., 1979; Sharma, et al., 1980). This matrix contains the dye-ligand Cibacron Blue 3GA covalently coupled to either agarose or Sepharose. If both the cyclic AMP and cyclic GMP PDE activities of Peak I were associated with the Ca^{2+}/calmodulin-stimulated phosphodiesterase then both of these activities should bind to this column.. It was thus decided to apply DE-52 Peak I to this matrix.

Figure 3.6 shows the results of a typical experiment. Chromatography of DE-52 Peak I on Affi-gel Blue, in the presence of 5 mM $MgCl_2$, resulted in the apparent separation of the cyclic AMP and cyclic GMP PDE activities of Peak I. Whilst the Affi-gel Blue retained 78% +/- 10% (n=5, +/- SEM) of the cyclic GMP PDE activity, only 14% +/- 10% (n=5, +/- SEM) of the cyclic AMP PDE activity was retained. The 'unbound' activity had a cAMP:cGMP activity ratio of 4.29 +/- 0.34 (n=5, +/- SEM), which differed significantly from the cAMP:cGMP ratio for the Peak I activity which was 1.07 +/- 0.09 (n=5, +/- SEM)..

The amount of 'Ca^{2+}/calmodulin-stimulated' activity was calculated by subtracting the PDE activity of Peak I in the absence of Ca^{2+}/calmodulin from the PDE activity obtained in the presence. When this was performed, it was apparent that the majority of the 'Ca^{2+}/calmodulin-stimulated' cyclic AMP and cyclic GMP PDE activities were retained by the matrix (retentions were 97% +/- 1.34% (n=4, +/- SEM) for cyclic GMP and 98% +/- 1.25% (n=3, +/- SEM) for cyclic GMP).

These results indicated that the Peak I activity observed on DE-52 anion-exchange profiles actually consisted of two separate activities, which hydrolysed predominantly cyclic AMP and cyclic GMP respectively and that the Ca^{2+}/calmodulin-stimulated activity was primarily associated with the cyclic GMP PDE activity and not the cyclic AMP PDE activity of Peak I.

An alternative explanation of the results, however, was that Affi-gel Blue chromatography may have removed a factor (e.g an enzyme subunit) whose function was to control the substrate specificity of the activity. In the presence of the putative 'subunit', the enzyme would hydrolyse both cyclic AMP and cyclic GMP and show responsiveness to Ca^{2+}/calmodulin and it's absence the activity would hydrolyse cyclic AMP selectively and show little or no Ca^{2+}/calmodulin-sensitive activity. In order to address this possibility it was necessary to demonstrate that the cyclic GMP activity could be retrieved from the Affi-gel Blue column.

3.6: ELUTION OF THE PHOSPHODIESTERASE ACTIVITY OF PEAK-I FROM AFFI-GEL BLUE.

A number of attempts were made to elute the 'bound' cyclic GMP PDE activity from the Affi-gel Blue matrix. Washing the column extensively in the equilibration buffer eluted only a small amount of the cyclic AMP and cyclic GMP PDE activities (20% +/- 3% (n=3, +/- SEM) and 16.8% +/- 5.9% (n=3, +/- SEM) of the total cyclic AMP and cyclic GMP activities were retrieved respectively (Figure 3.6). Such eluted activity represented only 1.2% +/- 0.6% (n=3, +/- SEM) and 9% +/- 2.9% (n=3 +/- SEM) of the total bound cyclic AMP and cyclic GMP 'Ca^{2+}/calmodulin-stimulated' PDE activities respectively.

Batch elution experiments, from small quantities of Affi-gel Blue, were performed in some instances (see Section 2.13 for general method). In these experiments, only binding and elution of cyclic

GMP PDE activity was assessed. Identical binding of cyclic GMP PDE activity could be achieved under batch binding conditions to that achieved under 'column' conditions (see Figure 3.7 for a typical experiment).

Individual portions of the matrix, containing bound phosphodiesterase activity, were washed in equilibration buffer (which contained approximately the same buffer composition as the pooled fractions containing Peak I phosphodiesterase namely, 50 mM Tris-HCl pH 7.5, 5 mM Benzamidine hydrochloride, 0.2 mM PMSF, 2 μM Leupeptin, 0.1 mM EGTA, 0.1 mM dithiothreitol and 0.15 M NaCl) containing in addition 5 mM $MgCl_2$..

Elution of bound cyclic GMP phosphodiesterase activity was attempted using equilibration buffer, in the presence of EDTA (in place of 5 mM $MgCl_2$), in the presence of high salt and in the presence of high cyclic nucleotide concentrations.

Figure 3.7 summarises the elution conditions attempted to retrieve either bound cyclic GMP PDE activity or Ca^{2+}/calmodulin stimulated cyclic GMP PDE activity from Affi-gel Blue. The activity could not be eluted in 1 mM EDTA indicating that Mg^{2+} ions were not necessary to achieve binding of the cyclic GMP PDE activity to the matrix. Elution in the presence of high sodium chloride concentrations were also unsuccessful as was elution using high concentrations of cyclic GMP or cyclic AMP. In this latter instance the eluted material was dialysed against equilibration buffer to remove the cyclic nucleotides prior to assay of phosphodiesterase activity. In such instances, a 24 hour dialysis of a 2 ml sample against 4 x 500 ml changes of equilibration buffer, caused the loss of approximately 29% +/- 13.3% (n=3, +/- SEM) of the cyclic GMP phosphodiesterase activity of Peak I. This loss of activity was not sufficient to account for the low activities recovered from the Affi-gel Blue indicating that the cyclic GMP phosphodiesterase activity had remained bound to the column,

even in the presence of high concentrations of it's substrates cyclic GMP and cyclic AMP.

3.7: MONO Q ANION-EXCHANGE CHROMATOGRAPHY OF A RAT LIVER SOLUBLE FRACTION.

Since the cyclic GMP phosphodiesterase activity of Peak I could not be retrieved from Affi-gel Blue very readily, it was decided to attempt to separate the cyclic nucleotide phosphodiesterases contained in the rat liver soluble fraction on the high resolution anion-exchange matrix, Mono Q.

The soluble fraction from rat liver, prepared under identical conditions to the DE-52 experiments, was subjected to chromatography on a Mono Q anion-exchange column. Binding of the cyclic AMP and cyclic GMP PDE activities to the column were routinely very high; 98% +/- 1 % (n=10, +/- SEM) and 97% +/- 1.7% (n=10, +/- SEM) for cyclic AMP and cyclic GMP phosphodiesterase activity respectively.

A sodium chloride gradient was used to elute the bound phosphodiesterase activity. A number of preliminary experiments were performed to determine the shape of the gradient that gave optimal separation of the phosphodiesterase activities. The programme used is given in Table 3.2.

Figure 3.8 shows a typical profile obtained using approximately half the supernatant prepared from one rat liver. At 1 μM substrate, five major peaks of activity were eluted between 0 and 0.5 M NaCl, termed PDE MQ-I to PDE MQ-V inclusive. Collectively, these represented 92% +/- 0.05% (n=10, +/- SEM) of the cyclic AMP PDE activity and 94% +/- 0.03% (n=10, +/-SEM) of the cyclic GMP PDE activity applied to the column. The NaCl concentration required to elute the PDE MQ-Peaks were 0.1 M, 0.15 M, 0.25 M, 0.35 M and 0.5 M respectively. A small peak of cyclic AMP and cyclic GMP PDE activity was usually observed eluting on the shoulder of PDE MQ-III but the yield of this activity was very variable so no

studies were performed on it's properties. PDE MQ−V was routinely observed to start eluting at approximately 0.4 M NaCl but could be eluted as a sharper peak of activity by rapidy increasing the gradient to 0.5 M NaCl (see Figure 3.8).

3.8: CHARACTERISATION OF RAT LIVER PDE MQ−PEAKS.

The average total cyclic AMP and cyclic GMP PDE activity associated with each of the individual PDE MQ−Peaks was calculated as was the ratio of cyclic AMP:cyclic GMP hydrolysis at 1 μM. The results are presented in Table 3.3.

PDE MQ−I was shown to be capable of hydrolysing both cyclic AMP and cyclic GMP, although it was much more selective for cyclic AMP since it had a cAMP:cGMP hydrolytic ratio of 5.4. This was very similar to the ratio for the 'unbound' fraction prepared from DE−52 Peak I by Affi−gel Blue chromatography, which was 4.3 (see Section 3.5). Based on the average yield of activity for PDE MQ−I it can be estimated that this Peak contributes approximately 23% of the total cyclic AMP hydrolysis observed in the rat liver supernatant.

PDE MQ−II was highly selective for cyclic GMP as substrate (cAMP:cGMP hydrolysis ratio of 0.13). It was estimated that this Peak contributed 20% of the total soluble cyclic GMP PDE activity but only 2% of the total soluble cyclic AMP PDE activity.

The majority of the rat liver soluble cyclic AMP and cyclic GMP PDE activity was found to be associated with PDE MQ−III. This activity could hydrolyse both cyclic nucleotides but was more selective for cyclic GMP since the substrate ratio was 0.46. It was estimated to contribute 45% of the total cyclic AMP hydrolysis and as much as 74% of the total cyclic GMP hydrolysis in the rat liver soluble fraction.

PDE MQ−IV and PDE MQ−V were both selective for cyclic AMP as a substrate (the substrate ratios were 8.98 and 10.0

respectively). They both contributed a similar amount of the total cyclic AMP hydrolysis (14.3% and 16.2% respectively). Taken together, 30% of the total cyclic AMP hydrolysis of the supernatant was provided by the cyclic AMP-specific class of phosphodiesterase.

The PDE MQ-Peaks were further characterised by analysing the response to the effectors, Ca^{2+}/calmodulin and cyclic GMP. These were investigated in an anlagous manner to the analysis performed for the DE-52 Peaks (see Section 3.3). In all cases, effects were monitored at 1 μM substrate using either saturating levels of Ca^{2+}/calmodulin (50 μM Ca^{2+} and 2 μg/ml calmodulin) and 2 μM cyclic GMP. Table 3.4 shows a summary of the response of the individual Peaks to these effectors.

Ca^{2+}/calmodulin, together, stimulated the cyclic AMP and cyclic GMP PDE activities of PDE MQ-II some 4.8 and 2.2 fold respectively. In contrast, the cyclic AMP PDE activity of PDE MQ-I was not significantly stimulated by these agents, however, the cyclic GMP PDE activity could be stimulated some 0.7 fold, although in some instances this stimulation was either smaller or not observed at all. It seemed likely that this effect was due to a small amount of co-elution of PDE MQ-I and PDE MQ-II. Ca^{2+}/calmodulin did not significantly stimulate the PDE activities of any of the other PDE MQ-Peaks indicating that PDE MQ-II contained all of the soluble Ca^{2+}/calmodulin activity. Additionally, this activity was selective for cyclic GMP under both basal and calmodulin stimulated conditions, at least at 1 μM substrate.

Cyclic GMP stimulated markedly the cyclic AMP hydrolysis of PDE MQ-III (6.3 fold stimulation), confirming the presence of the cyclic GMP-stimulated phosphodiesterase in the soluble fraction of rat liver. Both of the cyclic AMP-specific Peaks were inhibited by 2 μM cyclic GMP although the effects were small (10% and 17% inhibition for PDE MQ-IV and PDE MQ-V respectively).

The observed characteristics of PDE MQ-I and PDE MQ-II were consistent with the notion that there were two activities present in the DE-52 Peak I which selectively hydrolysed cyclic AMP and cyclic GMP respectively. Chromatography on the high resolution anion-exchange column, Mono Q, could apparently resolve these activities from each other giving rise to PDE MQ-I and PDE MQ-II.

In order to confirm that these resolved Peaks did indeed correspond to the activities that would be found in DE-52 Peak I, it was decided to assess their chromatographic properties on Affi-gel Blue. It would be predicted that PDE MQ-I would possess the same properties as the 'cyclic AMP' component of the DE-52 Peak I activity and hence would be unable to bind to Affi-gel Blue. Similarly, PDE MQ-II should possess the same properties as the 'cyclic GMP' component of the DE-52 activity and should thus be capable of binding to Affi-gel Blue very tightly. Figure 3.9 shows the result of a typical experiment in which PDE MQ-I and PDE MQ-II were batch bound to Affi-gel Blue. Whilst 99% +/- 0.47 (n=3, +/- SEM) of the cyclic GMP and 71% +/- 9.8% (n=3, +/- SEM) of the cyclic AMP PDE activity of PDE MQ-II could be bound to Affi-gel Blue only 25% +/- 5.0% (n=3, +/- SEM) of the cyclic AMP and 44% +/- 14% (n=3, +/- SEM) of the cyclic GMP PDE activity of PDE MQ-I could be bound to Affi-gel Blue. In a single experiment, nearly 100% of the 'Ca^{2+}/calmodulin-stimulated' cyclic AMP and cyclic GMP PDE activities of PDE MQ-II were retained on this matrix. These results indicated that PDE MQ-I and PDE MQ-II did indeed correspond to the cyclic AMP and cyclic GMP PDE components of DE-52 Peak I.

3.9: <u>ELUTION OF THE CYCLIC GMP PDE ACTIVITY OF PDE MQ-II FROM AFFI GEL BLUE.</u>

Attempts to elute the bound cyclic GMP PDE activity from Affi-gel Blue were unsuccessful. In a single experiment, 0.75 ml of

PDE MQ–II containing 46 +/– 2.51 pmol/min of cyclic GMP PDE activity was bound to 100 µl of Affi–gel Blue and elution was attempted using a variety of elution procedures (see Section 2.13 for general method). The results are shown in Table 3.5.

The use of high salt (0.5 M or 1.0 M NaCl) either in the presence of 5 mM $MgCl_2$ or 2 mM EDTA eluted less than 10% of the bound activity. Elution in the presence of Ca^{2+}/calmodulin and 2 mM EDTA was also ineffective (8.4% of the activty was eluted). No cyclic GMP PDE activity could be detected when the Affi–gel Blue was assayed for activity. These results implied that binding of PDE MQ–II to the dye matrix column Affi–gel Blue was not dependent on the presence of Mg^{2+} ions. The inability to detect any activity on the column itself could have implied that binding of the enzyme had occurred via the catalytic site (or sterically near to it).

The ability of PDE MQ–I and PDE MQ–II to bind to Affi–gel Blue in the presence of 2 mM EDTA (in place of 5 mM $MgCl_2$) was also investigated. In a single experiment, the properties of these activities were not altered. 24% of the cyclic AMP and 21% of the cyclic GMP PDE activity of PDE MQ–I were retained on Affi–gel Blue under these conditions, whilst 100% of the cyclic AMP and 92% of the cyclic GMP PDE activity of PDE MQ–II were retained. In this experiment, 2 mM EDTA did not have any direct effect on the PDE activity of PDE MQ–I (cyclic AMP and cyclic GMP PDE activities were 92% and 108% of control values). The activity of PDE MQ–II was inhibited slightly by 2 mM EDTA (cyclic AMP and cyclic GMP PDE activities were 84% and 88% of control values). In the presence of 5 mM $MgCl_2$, the cyclic AMP and cyclic GMP PDE activities of PDE MQ–I were 103% and 132% of control values, whilst for PDE MQ–II the cyclic AMP and cyclic GMP PDE activities were 75% and 82% of control values.

Together, these results were taken to indicate that binding of PDE MQ–II to Affi–gel Blue was extremely difficut to reverse,

presumably as a result of the high affinity of the enzyme for the dye ligand Cibacron Blue 3GA.

3.10: EFFECT OF CALCIUM ON MONO Q ANION-EXCHANGE CHROMATOGRAPHY OF RAT LIVER SUPERNATANT.

The presence of PDE MQ-I in rat liver soluble fractions has not previously been observed by other workers (Russel, et al., 1973; Strewler, et al., 1983). This could have been related to the different preparation conditions employed in this study. Strewler et al., (1983) used $CaCl_2$ in the homogenisation buffer. When the rat liver soluble fraction was prepared and chromatographed in Buffers containing calcium they did not observe any activity corresponding to PDE MQ-I. In addition, the chromatographic mobility of the Ca/calmodulin-sensitive form was altered in the presence of calcium. In the presence of calcium the activity eluted at higher salt concentrations than in the presence of the calcium chelator EGTA (Strewler, et al., 1983). This was presumed to be due to the interaction of the phosphodiesterase with calmodulin. Since calmodulin is an acidic protein, such an interaction would be expected to increase the NaCl concentration required to elute the phosphodiesterase activity.

Figure 3.10(a) shows that essentially the same Mono Q profile was obtained when the rat liver supernatant was prepared and chromatographed in the presence of 100 µM $CaCl_{2+}$ as in the presence of 100 µM EGTA. PDE MQ-I was clearly present and in addition there was no evidence for a mobility shift in the Ca^{2+}/calmodulin-stimulated phosphodiesterase (PDE MQ-II). Presumably Mono Q chromatography was sufficient to dissociate the calmodulin:PDE interaction so that these migrate as independent entities.

3.11: MONO Q ANION-EXCHANGE PROFILE OF RAT LIVER SUPERNATANT ASSESSED AT 100μM SUBSTRATE.

The Mono Q profile obtained when the phosphodiesterase assay was performed using 100 μM substrate was clearly different to that obtained when the assay was performed at 1 μM substrate. Peaks corresponding to PDE MQ-I, PDE MQ-II and PDE MQ-III were observed, however the activities corresponding to PDE MQ-IV and PDE MQ-V were not. Importantly, no additional peaks were observed.

3.12: INHIBITOR SENSITIVITY OF RAT LIVER MONO Q PEAKS.

The cyclic AMP hydrolysis of the rat liver-derived phosphodiesterases, PDE MQ-I to PDE MQ-V inclusive, were analaysed in response to the inhibitors IBMX, milrinone and Ro-20-1724. IBMX is considered to be a non-specific inhibitor of phosphodiesterases whilst milrinone and Ro-20-1724 are considered selective for the cyclic AMP-specific class of phosphodiesterase (see Section 1.8). The structures of IBMX, milrinone and Ro-20-1724 are shown in Figures 1.3, 1.6(a) and 1.6(b) respectively.

3.12.1: Method for determination of IC_{50}s.

Dose response curves were constructed at a fixed cyclic AMP concentration of 1 μM and the % inhibition of cyclic AMP hydrolysis determined for each individual drug concentration. Since all the drugs were diluted from 25 mM stocks prepared in dimethylsuphoxide (DMSO) it was necessary to prepare DMSO correction curves. The cyclic AMP hydrolysis of each PDE MQ-Peak was analysed in the presence of DMSO concentrations that corresponded to the concentration that would be present in a given concentration of inhibitor. The average effect on cyclic AMP hydrolysis was determined and the appropriate correction curve constructed. These curves are shown in the Appendix (Figure I-V). On the assumption that the

effects of DMSO and the inhibitor were additive, the inhibition obtained for each drug was corrected for the effect of DMSO.

3.12.2: IC_{50}s for rat liver derived PDE MQ-Peaks.

Representative inhibition curves for each of the individual Peaks are shown in Figures 3.11-3.15 whilst Table 3.6 shows the concentrations of drugs required for half-maximal inhibition of cyclic AMP phosphodiesterase activity (IC_{50}) of each Peak, determined at 1 μM substrate.

PDE MQ-I was found to be very insensitive to each of the three inhibitors tested, including the non-specific phosphodiesterase inhibitor IBMX. On this basis, this activity was termed the IBMX-insensitive form.

PDE MQ-II was found to be most sensitive to inhibition by IBMX (IC_{50} of 5.6 μM) and least sensitive to inhibition by Ro-20-1724 (IC_{50} > 500 μM). Milrinone was found to inhibit, although the IC_{50} for inhibition (145 μM) was approximately 10-fold greater than the IC_{50} for the two cyclic AMP-selective Peaks.

The cyclic AMP PDE activity of PDE MQ-III was found to be insensitive to both milrinone and Ro-20-1724 (in both instances the (IC_{50} values were greater than 500 μM). IBMX was found to be capable of inhibition, although the IC_{50} for inhibition (97 μM) was at least 4 fold greater than for any of the other PDE MQ-Peaks.

The cyclic AMP-selective Peaks, PDE MQ-IV and PDE MQ-V, were found to be inhibited by both IBMX and milrinone with similar sensitivities. The IC_{50}s for IBMX action (14 μM and 11 μM respectively) were comparable to that for it's action against PDE MQ-II (IC_{50} of 5.6 μM). The IC_{50}s for milrinone action against these Peaks (10 μM and 17 μM respectively) were at least 10 fold greater than for any of the other Peaks. PDE MQ-IV and PDE MQ-V were found to differ markedly, however, in their response to Ro-20-1724

with PDE MQ-IV exhibiting poor sensitivity (IC_{50} 190 μM) and PDE MQ-V showing a much greater sensitivity (IC_{50} 1.6 μM).

3.13: SUBCELLULAR FRACTIONATION OF RAT HEPATOCYTE-DERIVED PHOSPHODIESTERASE ACTIVITY.

Mammalian liver is a highly heterogenous organ containing a number of distinct cell types including, in addition to hepatocytes, Kupffer cells (Wardle, 1987). Isolated livers from freshly sacrificed animals will also contain substantial amounts of trapped blood. This is a consequence of the fact that the liver receives a substantial blood flow in accord with it's central role in metabolism. Homogenates of rat liver will presumably contain phosphodiesterases derived from these multiple cell types.

It is possible to isolate hepatocytes by performing collagenase digestion of rat liver (Smith, et al., 1978; Elliot, et al., 1976; Heyworth, et al., 1983d). Such preparations are substantially enriched in hepatocytes and contain very little or no blood-derived cells. Due to the apparently complex number of phosphodiesterase forms observed in the soluble fraction of rat liver and in particular, the observation that a novel IBMX-insensitive phosphodiesterase is observed which has not been previously observed, it is possible that the multiplicity of phosphodiesterase forms observed may be due to the multiplicilty of cell types in rat liver homogenates. With this in mind, it was decided to assess the number and type of soluble phosphodiesterases present in preparations of rat hepatocytes.

3.13.1: Fractionation of rat hepatocyte-derived phosphodiesterase activity.

Fractionation of hepatocytes into a 'washed' P1 fraction (low speed pellet) and P2 and S2 fractions (high speed pellets and supernatants) was performed (see Section 2.8). An analysis of the phosphodiesterase activities contained in these fractions indicated that,

at 1 μM substrate, 50% of the cyclic AMP PDE activity (7,576 pmol/min out of 15,183 pmol/min) and 45% of the cyclic GMP PDE activity (11,749 pmol/min out of 26,619 pmol/min) observed in the homogenate were contained in the soluble fraction. The yield of cyclic AMP PDE activity during the fractionation procedure was 108% and for cyclic GMP PDE activity was 89%.

The Ca^{2+}/calmodulin– and cyclic GMP–sensitivity of the phosphodiesterase activity contained in the homogenate and the various fractions was assessed using saturating levels of Ca^{2+} (100 μM) and calmodulin (2 μg/ml) and 2 μM cyclic GMP respectively. It was observed that only the soluble cyclic GMP PDE activity demonstrated any noticeable sensitivity to Ca^{2+}/calmodulin (1.4 fold stimulation) whilst cyclic GMP–stimulated cyclic AMP PDE activity was observed in both the particulate and soluble fractions. The soluble cyclic AMP PDE activity was stimulated approximately 3 fold. These results are summarised in Figure 3.16(a) and (b).

3.14: MONO Q ANION–EXCHANGE CHROMATOGRAPHY OF RAT HEPATOCYTE–DERIVED SOLUBLE PHOSPHODIESTERASES.

Chromatography of a rat hepatocyte high speed supernatant on a Mono Q column, using identical experimental conditions to those used for rat liver supernatants, resulted in a broadly similar elution profile to that seen with rat liver supernatant (Figure 3.17). Again, five major peaks of activity were observed. An activity corresponding to PDE MQ–I, the IBMX–insensitive phosphodiesterase was present and chromatographed in an identical position to that of the rat liver derived PDE MQ–I. This proved that the presence of this previously unobserved activity was not due to the multiplicity of cell types in rat liver. As with the rat liver PDE MQ–Peaks, an analysis of the ratio of cyclic AMP:cyclic GMP hydrolysis at 1 μM substrate was carried out. The results of this analysis are summarised in Table 3.7. There were several noticeable differences between hepatocyte and liver derived

activities (compare Tables 3.3 and 3.7). Firstly, a lower yield of phosphodiesterase activity was obtained from hepatocytes than from liver. On average the levels of total soluble cyclic AMP phosphodiesterase activity from hepatocytes was approximately one third of the level obtained from whole liver, whilst hepatocyte-derived cyclic GMP phosphodiesterase activity was approximately 40% of the liver activity. Secondly, the proportion of the total activity contributed by each PDE MQ-Peak was different from that observed in rat liver. PDE MQ-I was now observed to contribute approximately 40% of the total cyclic AMP PDE activity (compared with 23% in liver), whilst PDE MQ-III only contributed some 10% of the cyclic AMP PDE activity (compared with 45% in liver). The two cyclic AMP-specific activities were present in approximately equal amounts, as they were in liver, but were now found to form almost 50% of the total cyclic AMP phosphodiesterase activity (compared with 30% in liver). Thirdly, the substrate selectivity of PDE MQ-III was significantly different from that in liver; the substrate selectivity ratio for cyclic AMP hydrolysis:cyclic GMP hydrolysis was 0.15 +/- 0.015 in hepatocytes, compared to 0.46 +/- 0.06 in liver.

3.15: RESPONSE OF RAT HEPATOCYTE-DERIVED PDE MQ-PEAKS TO EFFECTORS.

In an analagous manner to the rat liver-derived PDE MQ-Peaks, an analysis of the responses of the cyclic AMP and cyclic GMP PDE activity to the agents Ca^{2+}/calmodulin and to cyclic GMP was carried out. Once again saturating levels of Ca^{2+}/calmodulin (100 μM and 2 μg/ml respectively) and 2 μM cyclic GMP were used to determine the effects. Table 3.4 summarises the responses and also draws a comparison between hepatocyte and liver derived forms.

3.15.1: Effectors on rat hepatocyte-derived PDE MQ-I.

PDE MQ-I was found to be insensitive to Ca^{2+}/calmodulin when cyclic AMP was the substrate but exhibited a small sensitivity

when cyclic GMP was the substrate (1.28 fold stimulation above control values). This value was smaller than the value observed with the rat liver peak (1.72 fold stimulation of control values). The cyclic GMP PDE activity was not found to be affected by 2 µM cyclic GMP.

3.15.2: Effectors on rat hepatocyte-derived PDE MQ-II.

Ca^{2+}/calmodulin-stimulated PDE activity was found to be associated with PDE MQ-II, as was observed for the rat liver-derived activity. Both the cyclic AMP and cyclic GMP PDE activities were stimulated to very similar extents to the rat liver-derived forms. Once again, the fold stimulation for cyclic AMP as substrate (3.83) was greater than that observed with cyclic GMP as substrate (2.26).

An analysis of both the calmodulin- and Ca^{2+}-dependence for stimulation of cyclic GMP phosphodiesterase activity of PDE MQ-II was carried out, to allow comparisons to be drawn between this activity and previously purified and characterised forms.

Figure 3.18 shows the calmodulin dependency in the presence of saturating Ca^{2+} (100 µM), whilst Figure 3.19 shows the Ca^{2+} dependency in the presence of saturating calmodulin (2 µg/ml). The EC_{50} for calmodulin stimulation of cyclic GMP PDE activity was 28 ng/ml +/- 3 ng/ml (n=4, +/- SEM), this corresponds to an EC_{50} of 1.64nM +/- 0.18nM. In the absence of Ca^{2+}, a small stimulation of cyclic GMP PDE activity could be observed (approximately 1.10-1.15 fold stimulation above controls). This Ca^{2+}-independent action of calmodulin was, however, small compared to the fold stimulation for it's Ca^{2+}-dependent action (3.3 fold). Ca^{2+} was found to be capable of stimulating the cyclic GMP PDE activity, but only in the presence of calmodulin. The EC_{50} for it's action at saturating levels of calmodulin was 18.8 µM +/- 2.1 µM (n=3, +/- SEM). The calmodulin stimulation of both cyclic AMP and cyclic GMP hydrolysis by PDE MQ-II was inhibited by the calmodulin antagonist 5-Iodo-C8 in a dose dependent manner as shown in Figures 3.59(a) and 3.59(b). The IC_{50} for this effect was

13 μM +/- 3.1 μM when cyclic GMP was the substrate and 16.33 μM +/- 1.92 μM when the substrate was cyclic AMP. This antagonist had no significant effect on the basal cyclic AMP and cyclic GMP hydrolysis up to a maximal concentration of 100 μM.

3.15.3: Effectors on rat hepatocyte-derived PDE MQ-III.

The substrate selectivity ratio of hepatocyte derived PDE MQ-III was different to that of the rat liver derived form (0.15 compared with 0.46). Despite this, the cyclic AMP hydrolytic component was stimulated to a very similar extent to that of the rat liver-derived form (5.92 fold stimulation compared to 6.32 fold stimulation for the rat liver-derived form). Further analysis of the effect of cyclic GMP on the cyclic AMP PDE activity of this Peak revealed that cyclic GMP had a biphasic effect on the cyclic AMP PDE activity (Figure 3.20). At 1 μM cyclic AMP, low levels of cyclic GMP could stimulate the cyclic AMP PDE activity, with up to 10 fold stimulations observed at 10 μM cyclic GMP. Higher doses of cyclic GMP were capable of inhibiting cyclic AMP PDE activity. The EC_{50} for stimulation of cyclic AMP hydrolysis was 0.466 μM +/- 0.098 μM (n=4, +/- SEM), whilst the IC_{50} for inhibition of cyclic AMP hydrolysis was found to be 43 μM +/- 9.8 μM (n=4, +/- SEM).

3.15.4: Effectors on rat hepatocyte-derived PDE MQ-IV and V.

The cyclic AMP PDE activity of the cyclic AMP-specific activities PDE MQ-IV and PDE MQ-V, were found to be insensitive to cyclic GMP at a concentration of 2 μM (Table 3.4). Further analysis of the effect of cyclic GMP on these two activities revealed that the IC_{50}s for inhibition of cyclic AMP PDE activity were 214 μM +/- 50 μM and 570 μM +/- 87 μM for PDE MQ-IV and PDE MQ-V respectively (Figure 3.63 and Table 3.8) when assessed at 1 μM substrate. This implied that these two activities belonged to the cyclic GMP-insensitive subclass of cyclic AMP-specific

phosphodiesterases (Yamamoto, et al., 1984; Pyne, et al., 1987b; Weishaar, et al., 1987a; Weishaar, et al., 1987b).

3.16: KINETIC ANALYSIS OF RAT HEPATOCYTE-DERIVED PDE MQ PEAKS.

An analysis of the substrate utilisation of PDE MQ-I to PDE MQ-V was carried out in order to obtain K_m values for cyclic AMP and cyclic GMP. V_{max} values were also determined and in instances where the kinetics of hydrolysis of both substrates determined, a ratio of the respective V_{max} values calculated.

3.16.1: Methods for determination of kinetic parameters of PDE MQ-Peaks.

This was performed by conservatively pooling activities from Peaks in order to minimise any possible contamination with neighbouring activities. In the case of PDE MQ-I, fractions were first pooled and then passed over a column of Affi-gel Blue in the presence of 5 mM $MgCl_2$. This was performed to remove any residual PDE activity contributed by PDE MQ-II which binds tightly to this matrix (see Section 3.8 and Figure 3.9). Kinetic data were initially analysed by Lineweaver-Burk analysis and K_m and V_{max} values estimated from the plots where applicable. The estimates of the V_{max} value were further validated by constructing both Hanes plots and Eadie-Hofstee plots of the data and an average V_{max} value determined. Hill plots were constructed using the averaged V_{max} value and the degree of co-operativity determined as well as an estimate of the K_m. Absolute V_{max} values could not be determined since the preparations were not pure. In instances where the V_{max} values for both cyclic AMP and cyclic GMP were determined, a ratio of the respective V_{max} values was calculated (PDE MQ-I and PDE MQ-III). The results of a number of kinetic analyses are summarised in Table 3.8 and representative plots shown in Figures 3.21-3.38.

3.16.2: Kinetics of rat hepatocyte-derived PDE MQ-I.

PDE MQ-I was found to hydrolyse both cyclic AMP and cyclic GMP with linear kinetics, as assessed by Lineweaver-Burk analysis (Figures 3.21 and 3.23 and Table 3.8). Hill plots of the kinetic data were also linear with slopes of 0.97 and 1.04 for cyclic AMP and cyclic GMP respectively, indicating that hydrolysis of these two cyclic nucleotides obeyed hyperbolic kinetics (Figures 3.22 and 3.24). Estimates of the K_m values for both substrates determined from both Lineweaver-Burk plots and Hill plots were similar (Table 3.8). Cyclic AMP was hydrolysed with a K_m of 25 μM to 31 μM and cyclic GMP with a K_m of 237 μM to 246 μM. The ratio of V_{max} for cyclic AMP:Vmax for cyclic GMP was 1.07 +/- 0.14 (n=3, +/- SEM).

3.16.3: Kinetics of rat hepatocyte-derived PDE MQ-II.

PDE MQ-II hydrolysed cyclic GMP with apparently non-linear kinetics as determined by Lineweaver-Burk analysis, with evidence of both high and low affinity components, (Figure 3.25). In addition, the kinetic data was plotted using both Hanes and Eadie-Hofstee plots (Figure 3.26(a) and (b)). These further confirmed the non-linear kinetics for cyclic GMP hydrolysis since they more readily showed evidence of apparent negative co-operativity. The limiting K_m values, deduced from Lineweaver-Burk plots, were 1.9 μM (K_{m1}) and 11 μM (K_{m2}) respectively (Figure 3.25 and Table 3.8). The Hill plots were linear with slopes that were less than 1 (0.72) and gave a K_m value of 5.1 μM for cyclic GMP hydrolysis (Figure 3.27 and Table 3.8). The kinetics of cyclic AMP hydrolysis of PDE MQ-II were not determined due to the low activity observed with this cyclic nucleotide as substrate (see Table 3.7 and Figure 3.17)

3.16.4: Kinetics of rat hepatocyte-derived PDE MQ-III.

This activity hydrolysed both cyclic AMP and cyclic GMP with positively cooperative kinetics. Lineweaver-Burk plots of cyclic AMP hydrolysis were non-linear (Figure 3.28). The fitted line did not intercept on the 1/[cAMP] axis so a 'K_m' value could not be

determined from this plot. The fitted line did, however, indicate that a 'limiting' V_{max} value could be estimated, since the fitted line 'levelled' off (see inset of Figure 3.28). Hanes and Eadie–Hofstee plots of cyclic AMP hydrolysis were also distinctly non–linear (Figure 3.29), producing bi–phasic curves typical of enzymes obeying positively co–operative kinetics (see Whalin, et al., 1989). Hill plots for cyclic AMP hydrolysis were linear over the range between 10 and 90% of the V_{max} value (+1 to −1 on the y–axis), giving an estimated K_m of 37.7 μM for cyclic AMP hydrolysis with a Hill Coefficient of 1.62 (Table 3.8 and Figure 3.30).

Cyclic GMP was also hydrolysed with positively cooperative kinetics. Lineweaver–Burk analysis of this data once again showed plots that were typical of enzymes obeying positively cooperative kinetics. There was no intercept on the 1/[cGMP] axis and the fitted line also 'levelled off', allowing an estimate of the V_{max} value to be calculated (Figure 3.31 and inset of Figure 3.31). Hanes and Eadie–Hofstee plots were markedly non–linear and were of a very similar shape to those obtained when cyclic AMP was the substrate (Figure 3.32). Hill plots of the data were constructed using an estimate of the V_{max} from Lineweaver–Burk plots and also a value from V (Velocity) versus log [cGMP] plots. These plots were linear and gave an estimate of the K_m for cyclic GMP of 35.5 μM with a Hill coefficient of 1.22 (Figure 3.33 and Table 3.8). The ratio of the V_{max} for cyclic AMP hydrolysis to the V_{max} for cyclic GMP hydrolysis was found to be 0.31 +/− 0.07 (n=4, +/− SEM).

3.16.5 Kinetics of rat hepatocyte–derived PDE MQ–IV.

This activity has been shown to exhibit a greater selectivity for cyclic AMP as substrate than for cyclic GMP (Table 3.7) so kinetic parameters were only determined with cyclic AMP as substrate. Lineweaver–Burk plots of the kinetic data were non–linear with evidence of both high and low affinity components (Figure 3.34). Estimates of the K_{m1} (high–affinity) and K_{m2} (low–affinity)

parameters gave values of 0.8 μM and 8.0 μM respectively. When the data was plotted as both Eadie–Hofstee and Hanes plots the non-linear nature of the kinetics was more apparent, particularly when Eadie–Hofstee plots were used. Estimates of the limiting K_m and V_{max} parameters gave very similar values to those obtained from Lineweaver–Burk plots (Figures 3.34, 3.35(a) and 3.35(b)). Hill plots of the data using an averaged V_{max} value determined from the separate plots gave a straight line between −1 and +1 on the y-axis (i.e. at velocities between 10% and 90% of the V_{max}). The estimated Hill coefficient was 0.69 and the estimated K_m value was 6.33 μM (Figure 3.36 and Table 3.8).

3.16.6: Kinetics of rat hepatocyte-derived PDE MQ-V.

As for PDE MQ-IV, this activity has been shown to exhibit a greater selectivity for cyclic AMP as substrate than for cyclic GMP (Table 3.7) so that only the kinetics of cyclic AMP hydrolysis were determined. Lineweaver–Burk plots of the kinetic data were linear giving an estimated K_m value of 1.02 μM for cyclic AMP as substrate. Hill plots of the data were also linear and gave a value for the Hill coefficient of 1.01 with an estimated K_m value of 0.62 μM. There was, however, some variability in the slope of the Hill plots for this activity, so that in some instances, despite the apparent linearity of Lineweaver–Burk plots, Hill coefficients with values below 1 were obtained (Figure 3.38). When this was the case, value of the Hill coefficient was only ever estimated to be as low as 0.8. This may have reflected a partial coelution of PDE MQ-IV with PDE-MQ-V, although this possibility could not be directly tested.

3.17: MAGNESIUM DEPENDENCY OF PDE MQ-PEAKS.

The metal ion Mg^{2+} is commonly employed in assays for phosphodiesterase activity and is considered to be essential to support the cyclic nucleotide hydrolytic capacity of phosphodiesterases. It was decided to assess the dependency of the individual PDE-MQ-Peaks on

Mg^{2+} in order to confirm this commonly held assumption. Phosphodiesterase assays were performed under basal conditions (i.e. in the absence of any other effectors) in the presence of increasing concentrations of magnesium and the degree of hydrolysis of cyclic AMP or cyclic GMP determined.

PDE MQ–II to PDE MQ–V inclusive were found to be almost entirely dependent on the addition of Mg^{2+} to support cyclic nucleotide hydrolysis. The EC_{50}s for Mg^{2+} stimulation of phosphodiesterase activity by PDE MQ–II to PDE MQ–V were estimated from the dose response curves shown in Figures 3.39–3.41 and summarised in Table 3.9. In the absence of Mg^{2+}, PDE MQ–II exhibited a cyclic GMP phosphodiesterase activity that was 14% +/− 1% (n=3, +/− SEM) of the fully simulated activity, indicating that some Mg^{2+}–independent activity could be exhibited by this form. PDE MQ–III and PDE MQ–V were similar in this respect, exhibiting 19% +/− 2% and 5% +/− 1.4% (n=3, +/− SEM) of fully stimulated levels respectively. The phosphodiesterase activity of PDE MQ–IV was found to be absolutely dependent on the presence of Mg^{2+}, with virtually no activity observed in it's absence.

On the other hand, PDE MQ–I was found to be nearly fully active in the absence of Mg^{2+}, although further activity could be stimulated in the presence of increasing concentrations of Mg^{2+}. In the absence of added Mg^{2+}, PDE MQ–I was found to exhibit an activity that was 80% +/− 3% (n=3, +/− SEM) of the maximal activity observed. Further to this, it was shown that the cyclic AMP PDE activity of PDE MQ–I was still fully active in the presence of 10 mM EDTA (Figure 3.39). The EC_{50}s for Mg^{2+} stimulation of activity for PDE MQ–II, PDE MQ–IV and PDE MQ–V were similar with values of 20 μM +/− 2 μM, 50 μM +/− 7 μM and 40 μM +/− 6 μM respectively (n=3, +/− SEM). PDE MQ–III exhibited a significantly larger EC_{50} value of 193 μM +/− 18 μM. These results are summarised in Table 3.9.

3.18: EFFECT OF INHIBITORS ON RAT HEPATOCYTE-DERIVED PDE MQ-PEAKS.

Given that some differences existed between the PDE MQ-Peaks derived from rat hepatocytes and those derived from rat liver, particularly with respect to PDE MQ-III, it was decided to assess the pharmacological characteristics of these resolved activities using an extended range of well characterised phosphodiesterase inhibitors. In addition to the inhibitors used for characterisation of the rat liver derived activities (IBMX, milrinone and Ro-20-1724), the phosphodiesterase inhibitors, ICI 63197, ICI 118233, zaprinast and rolipram were used. ICI 63197 has been shown to exert a more selective inhibitory effect on a purified preparation the rat liver 'peripheral' plasma membrane phosphodiesterase as compared to the rat liver 'dense-vesicle' phosphodiesterase (Pyne, et al., 1987a). ICI 118233 is a cardiotonic agent that specifically interacts with the cyclic GMP-inhibited subclass of cyclic AMP-specific phosphodiesterase, whilst rolipram has been shown to interact with the cyclic GMP-insensitive subclass of cyclic AMP-specific phosphodiesterase (Schwabe, et al., 1976; Bergstrand, et al., 1977; Henkel-Tigges and Davis, et al., 1990). Zaprinast is a derivative of IBMX that exerts some selectivity for cyclic GMP-specific phosphodiesterases including at least some of the Ca^{2+}/calmodulin-stimulated isozymes (Bergstrand, et al., 1978; Quade, et al., 1984). The characteristics of these inhibitors are described in Section 1.8. The structures of ICI 118233, ICI 63197, rolipram and zaprinast are shown in Figures 1.6(e), 1.6(d) and 1.4(a).

The cyclic AMP phosphodiesterase activity of the individual PDE MQ-peaks was assessed in the presence of increasing concentrations of these inhibitors and the IC_{50} determined in exactly the same manner as for the rat liver activities. Whilst cyclic AMP was normally used to assess the activity of the individual peaks, cyclic GMP was used instead in some instances. This was the case when the

phosphodiesterase activity of PDE MQ-II was assessed in the presence of ICI 118233, ICI 63197, zaprinast, and rolipram.

The IC_{50} values are summarised and compared to the comparable values for rat liver-derived PDE MQ-Peaks in Table 3.6. The data for ICI 63197 inhibition of phosphodiesterase activity are given in the text. Representative inhibition curves are shown in Figures 3.42-3.53.

As for rat liver, PDE MQ-I was found to be insensitive to any of the inhibitors tested (Figures 3.42, 3.43 and 3.52). Whilst in all instances the IC_{50} values were greater than 500 μM, this maximal concentration of some inhibitors did produce some inhibition of phosphodiesterase activity. The extent of this inhibition were in the range 10% to 20% maximal effect when the inhibitors were IBMX, milrinone, and Ro-20-1724. Larger effects were observed with zaprinast and rolipram where the maximal effects were in the range 40% to 50%.

PDE MQ-II was found to be similar to the rat liver-derived form, although it was found to be marginally more sensitive to both milrinone and Ro-20-1724. The cyclic GMP-selective inhibitor zaprinast was found to inhibit the cyclic GMP PDE activity with an IC_{50} of 4.5 μM. PDE MQ-IV showed some sensitivity to this inhibitor although the IC_{50} for inhibition in this instance was approximately 70 fold higher than for PDE MQ-II (IC_{50} value of 300 μM) (Figures 3.44 and 3.45). The IC_{50} for ICI 63197 action against this activity was 317 μM +/- 49 μM (n=3, +/- SEM) (Figure 3.52).

PDE MQ-III was found to be very insensitive to any of the inhibitors tested with only IBMX showing a very weak inhibitory response. The IC_{50} in this case was 323 μM which was some 3 fold lower than the value for the rat liver-derived activity (Figures 3.46 and 3.47). This difference may be related to the different substrate selectivity of this Peak in hepatocytes compared to liver. The IC_{50} for ICI 63197 action was greater than 500 μM (Figure 3.52). The maximal

inhibition in this case was approximately 20%. It was noted that small activatory effects could be observed in the presence of milrinone and IBMX, rolipram and zaprinast. The magnitude of these effects were only in the range of 30% to 50% which is much smaller than the stimulation obtained in the presence of a maximal concentration of cyclic GMP which was about 10-fold (see Figure 3.20).

The cyclic AMP-selective activities, PDE MQ-IV and PDE MQ-V, were found to be sensitive to inhibition by IBMX and milrinone with similar IC_{50} values to the rat liver-derived forms. Whilst in rat liver Ro-20-1724 displayed a clear selectivity for PDE MQ-V over PDE MQ-IV (100 fold more potent) this difference was not as clear for the hepatocyte forms although there was still a 10 fold difference in the IC_{50} values (13 μM compared with 1.6 μM). ICI 118233 was not found to be inhibitory for either of these two forms or any of the other forms. The cyclic GMP PDE selective inhibitor zaprinast was found to exert only a small inhibitory effect on PDE MQ-IV and none on PDE MQ-V. Both PDE MQ-IV and PDE MQ-V were found to be very sensitive to rolipram with PDE MQ-V showing a greater sensitivity than PDE MQ-IV (IC_{50} values of 0.095 μM and 0.5 μM respectively) (Figures 3.48, 3.49, 3.50 and 3.51). Similarly, ICI 63197 was found to be capable of inhibiting both PDE MQ-IV and PDE MQ-V. The IC_{50} values in this instance were 2.1 μM +/- 0.6 (n=3, +/- SEM) and 6.6 μM +/- 0.3 (n=3 +/- SEM) for PDE MQ-IV and PDE MQ-V respectively (Figure 3.53).

3.19: FURTHER CHARACTERISATION OF PDE MQ-PEAKS.

3.19.1: Further characterisation of PDE MQ-I and PDE MQ-II.

The phosphodiesterase activities of rat hepatocyte-derived PDE MQ-I and PDE MQ-II were further characterised by determining the molecular weights of these activities by gel filtration on calibrated Sephacryl S-200 and S-300 gel filtration columns respectively (see

Section 2.17). The calibration curves shown in Figures 3.54 and 3.57 show that the S-200 column is suitable to separate proteins over a molecular weight range of 12,500 to 66,000, whilst the S-300 column is suitable to separate proteins over a range of 12,500 to 410,000. Chromatography of PDE MQ-I on the Sephacryl S-200 column yielded a single peak of cyclic AMP phosphodiesterase activity that corresponded to a molecular weight of 33-kDa +/- 3-kDa (n=3, +/- SEM) (Figure 3.55). In contrast, chromatography of PDE MQ-II on the Sephacryl S-300 column yielded a peak of activity that had evidence of two closely eluting cyclic GMP phosphodiesterase activities (Figure 3.58). These activities were estimated to have molecular weights of 237-kDa +/- 13-kDa (n=3, +/- SEM) and 190-kDa +/- 5-kDa (n=3, +/- SEM) in three separate determinations. The yields of phosphodiesterase activity were very low in both procedures. It was not possible to estimate a precise value however, since the column profiles from both determinations were assayed for two hours instead of the usual ten minutes. This was performed to ensure that activity could be detected.

The ability of the cyclic nucleotides cyclic GMP and cyclic CMP to inhibit the cyclic AMP phosphodiesterase activity of PDE MQ-I was determined. Figure 3.56 shows that both of these cyclic nucleotides were capable of inhibiting this activity although the IC_{50}s determined for this process were high. The IC_{50} for cyclic GMP was found to be 850 μM +/- 57 μM (n=3, +/- SEM), whilst the IC_{50} for cyclic CMP was found to be 413 μM +/- 27 μM (n=3, +/- SEM). The IC_{50} for this action of cyclic GMP is also shown in Table 3.9 where it is compared with cyclic GMP-dependent-inhibition of the other phosphodiesterases.

The calmodulin antagonist, 5-Iodo-C8, has been shown to be a relatively selective inhibitor of calmodulin stimulated bovine heart cyclic nucleotide phosphodiesterase (MacNeil, et al., 1988). The data shown in Figure 3.59 show that this calmodulin antagonist could inhibit the Ca^{2+}/calmodulin-stimulated portion of the cyclic GMP and cyclic

AMP phosphodiesterase activity of PDE MQ-II. The IC_{50}s for this action of 5-Iodo-C8 were found to be 13 μM +/- 3.1 μM (n=3, +/- SEM) when cyclic GMP was the substrate and 16.33 μM +/- 1.92 μM when cyclic AMP was the substrate. This antagonist had no significant effect on the basal cyclic AMP and cyclic GMP hydrolysis up to a maximal concentration of 100 μM.

3.19.2: Further characterisation of PDE MQ-IV and PDE MQ-V.

From the data presented in Table 3.4 the cyclic AMP phosphodiesterase activities of PDE MQ-IV and PDE MQ-V were shown to be insensitive to inhibition by 2 μM cyclic GMP. At this level of cyclic GMP and a substrate concentration of 1 μM the maximal inhibitions observed were 19% and 23% respectively. The data presented in Figure 3.63 show that cyclic GMP could inhibit the cyclic AMP phosphodiesterase activity of PDE MQ-IV and PDE MQ-V with IC_{50} values of 214 μM +/- 50 μM and 570 μM +/- 87 μM respectively. These values are quoted in Table 3.9 which also draws a comparison with the effect of cyclic GMP on the other activities. This analysis further confirmed that these two activities were distinct from the cyclic GMP-inhibited subclass of cyclic AMP-specific phosphodiesterase.

3.20: THE SOLUBLE PHOSPHODIESTERASES IN OTHER RAT TISSUES.

Given the large number of soluble activities that were observed in the soluble fraction derived from both rat liver and hepatocytes, it was decided to see if this multiplicity of phospodiesterase activities was also observed in the soluble fractions of other tissues. Soluble fractions were prepared from brain, kidney and adipose tissue under identical conditions to those used for the preparation of hepatocyte and liver soluble extracts. For the case of adipocytes, the initial homogenisation was carried out at room temperature to avoid coaggulation of fats occuring. Thereafter, all procedures were carried

out at 4°C. Soluble fractions were separated using an identical gradient to that employed for the separation of rat liver and hepatocyte soluble extracts.

3.20.1: Adipose tissue soluble phosphodiesterases.

Figure 3.60 shows the Mono Q profile obtained from the rat adipose tissue soluble fraction. Cyclic AMP and cyclic GMP phosphodiesterase activities were determined at 0.1 μM substrate. Three peaks of phosphodiesterase activity were obtained. The first of these eluted at 0.25 M NaCl and formed the vast majority of the cyclic GMP phosphodiesterase activity. The position of this activity was noted to correspond with the position of the cyclic GMP-stimulated phosphodiesterase (PDE MQ-III) in rat liver and hepatocytes (see Figures 3.8 and 3.17) and similarly could hydrolyse both cyclic AMP and cyclic GMP. This activity was also more selective for cyclic GMP as substrate than cyclic AMP as PDE MQ-III was found to be. Despite the similarity of the elution position to PDE MQ-III, this activity did not possess cyclic GMP-stimulated phosphodiesterase activity. 2 μM cyclic GMP stimulated this activity only 1.17 +/- 0.1 fold (n=2, +/- SD). In contrast, saturating levels of Ca^{2+}/calmodulin were noted to stimulate the cyclic GMP phosphodiesterase activity of this peak 3.2 +/- 0.3 fold (n=2, +/- SD). This was taken to imply that the cyclic GMP-stimulated phosphodiesterase was not soluble in rat adipose tissue and that the Ca^{2+}/calmodulin-stimulated phosphodiesterase was the major cyclic GMP phosphodiesterase present. Furthermore, this activity appeared to differ from the rat liver and hepatocyte-derived PDE MQ-II both in it's elution position and in it's substrate selectivity; the liver and hepatocyte activity eluted at a lower ionic strength from this column and was more highly selective for cyclic GMP as substrate. This activity was not characterised in more detail however.

Two peaks of cyclic AMP phosphodiesterase activity were also observed to be present. The first of these (Peak II) eluted at

approximately 0.3 M NaCl, with some evidence of coelution with the cyclic GMP phosphodiesterase (Peak I). The second activity (Peak III) was less abundant than Peak II and was observed to elute at approximately 0.4 M NaCl. Whilst, Peak III appeared to correspond in position to PDE MQ-IV, Peak II did not seem to correspond to any of the PDE MQ-Peaks in hepatocytes although it's position was similar to that of the small peak of activity that was occasionally observed to elute on the shoulder of PDE MQ-III in rat liver soluble profiles. Whilst these activities were not characterised further, there was no evidence for an activity corresponding to the IBMX-insensitive PDE MQ-I.

3.20.2: Brain soluble phosphodiesterases.

Mono Q chromatography of the rat brain soluble fraction separated a large number of phosphodiesterases. Figure 3.61 shows the presence of at least four such activities. Peak I eluted at approximately 0.18 M NaCl and could hydrolyse both cyclic AMP and cyclic GMP with some selectivity for cyclic AMP as substrate. Peak II eluted over a very broad range of NaCl concentrations between approximately 0.2 M and 0.35 M NaCl. This peak hydrolysed both cyclic AMP and cyclic GMP and appeared to be slightly more selective for cyclic GMP. It was noted that the cyclic AMP and cyclic GMP phosphodiesterase phosphodiesterase activities, contained within this peak, did not coelute, with cyclic AMP elute eluting at a slightly higher NaCl concentration. This might imply that there was more than one phosphodiesterase activity present in this peak.

There were two peaks of cyclic AMP phosphodiesterase activity, which appeared to correspond to the positions of PDE MQ-IV and PDE MQ-V respectively. None of these activities were characterised in any detail

3.20.3: Kidney soluble phosphodiesterases.

Analysis of the soluble phosphodiesterases in kidney revealed the presence of four peaks of phosphodiesterase activity. Two of these

were selective for cyclic GMP as substrate and two were selective for cyclic AMP as substrate. The profile obtained was clearly different to that of the rat liver and hepatocyte profiles, since there was no activity that corresponded to PDE MQ-I. These four activities eluted at 0.18 M, 0.24 M, 0.31 M and 0.36 M NaCl respectively. It should be noted that a slightly different gradient was used to elute these activities so that optimal separation of activities could be achieved. Both the cyclic AMP and cyclic GMP phosphodiesterase activities of peak I were stimulated by Ca^{2+}/calmodulin. Cyclic AMP phosphodiesterase activity was increased about 2.5 fold and cyclic GMP phosphodiesterase about 3.5 fold. The cyclic AMP phosphodiesterase activity of peak II was increased about 5 fold using 2 μM cyclic GMP as the effector. The properties of the two cyclic AMP-specific phosphodiesterases were not examined in detail.

FIGURE 3.1:

Ca^{2+} STIMULATION OF RAT LIVER SOLUBLE PHOSPHODIESTERASE ACTIVITY.

A soluble fraction was prepared from rat liver as indicated in Section 2.4. Phosphodiesterase activity was assessed in the presence of increasing concentrations of added calcium. The basal level of EGTA was always 30μM. Cyclic AMP phosphodiesterase activity is indicated by (□) and cyclic GMP phosphodiesterase activity by (■) .The data is expressed in pmol/min/ml as the mean of triplicates ± SD.

FIGURE 3.1:

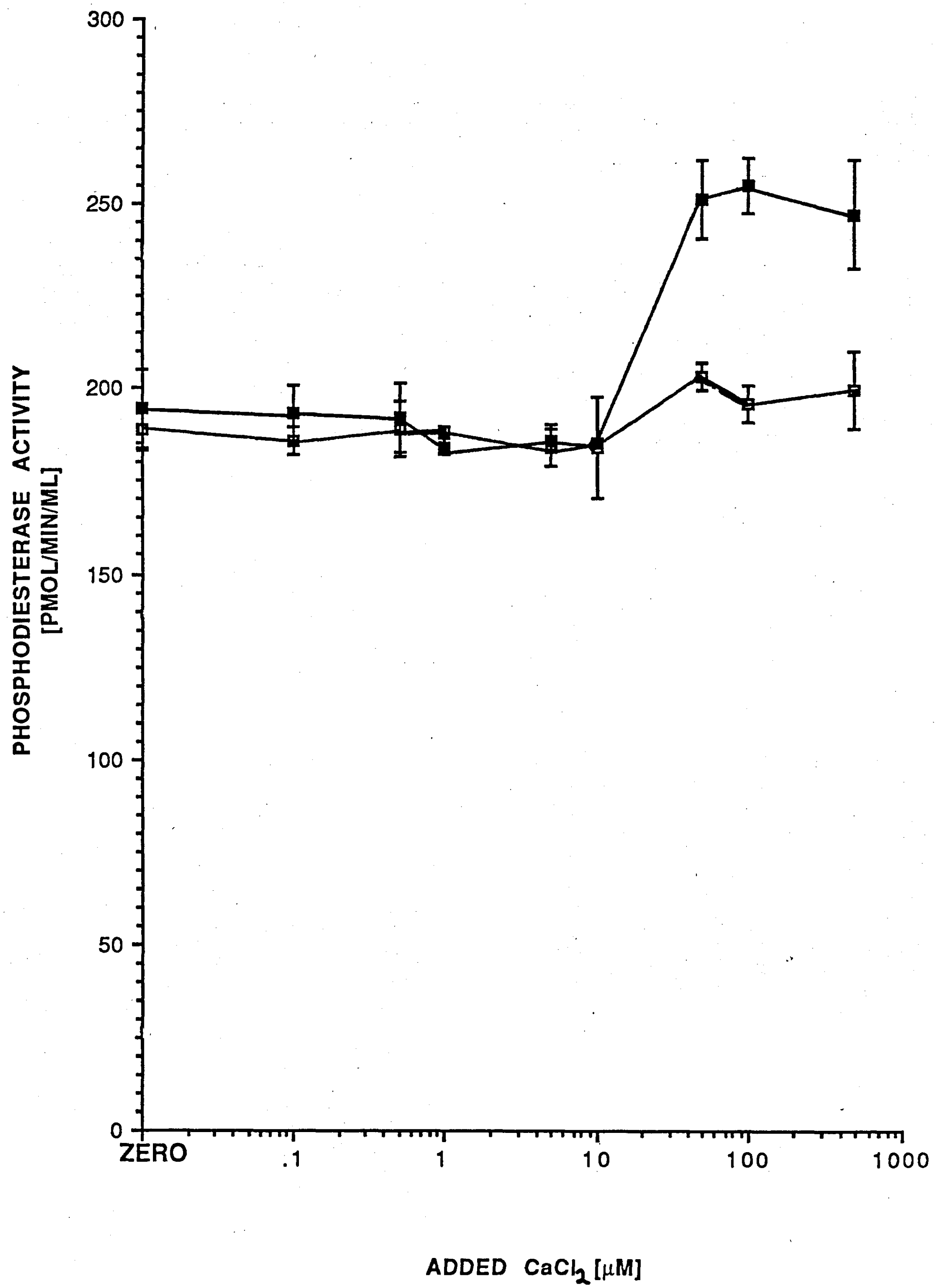


FIGURE 3.2:

EFFECT OF INCREASING EGTA ON RAT LIVER SOLUBLE PHOSPHODIESTERASE ACTIVITY.

A soluble fraction was prepared from rat liver as indicated in Section 2.4. Phosphodiesterase activity was assessed in the presence of increasing concentrations of added EGTA. The basal level of EGTA was always 30μM. Cyclic AMP phosphodiesterase activity is indicated by (□) and cyclic GMP phosphodiesterase activity by (■). The data is expressed in pmol/min/ml as the mean of triplicates ± SD.

FIGURE 3.2:

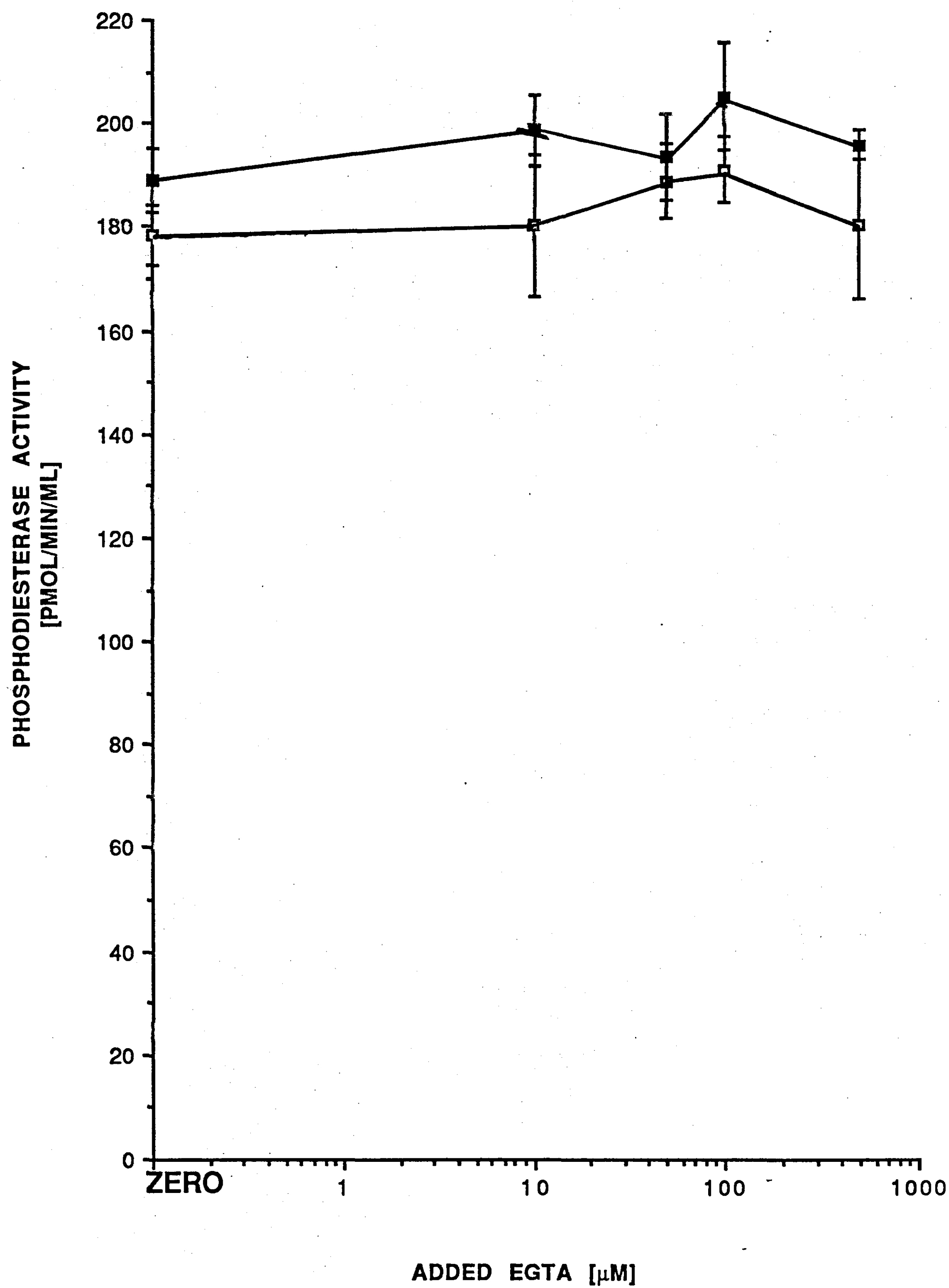


FIGURE 3.3:

REVERSIBILITY OF CALCIUM EFFECT ON RAT LIVER SOLUBLE PHOSPHODIESTERASE ACTIVITY.

A rat liver soluble fraction was prepared as in Section 2.4. Samples were incubated in the absence (▩) or presence (■) (▧) of 2mM $CaCl_2$ at 30°C for the times indicated. Samples were removed, diluted 1/5 in Buffer 'A' (50mM Tris-HCl pH 7.4, 0.1mM EGTA, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2µM Leupeptin, 0.1mM Dithiothreitol) and 25µl assayed for cGMP phosphodiesterase activity either in the presence (▩) (▧) or absence (■) of 2mM EGTA. Results are expressed in pmol/min/ml as mean of triplicate determinations ± SD.

FIGURE 3.3:

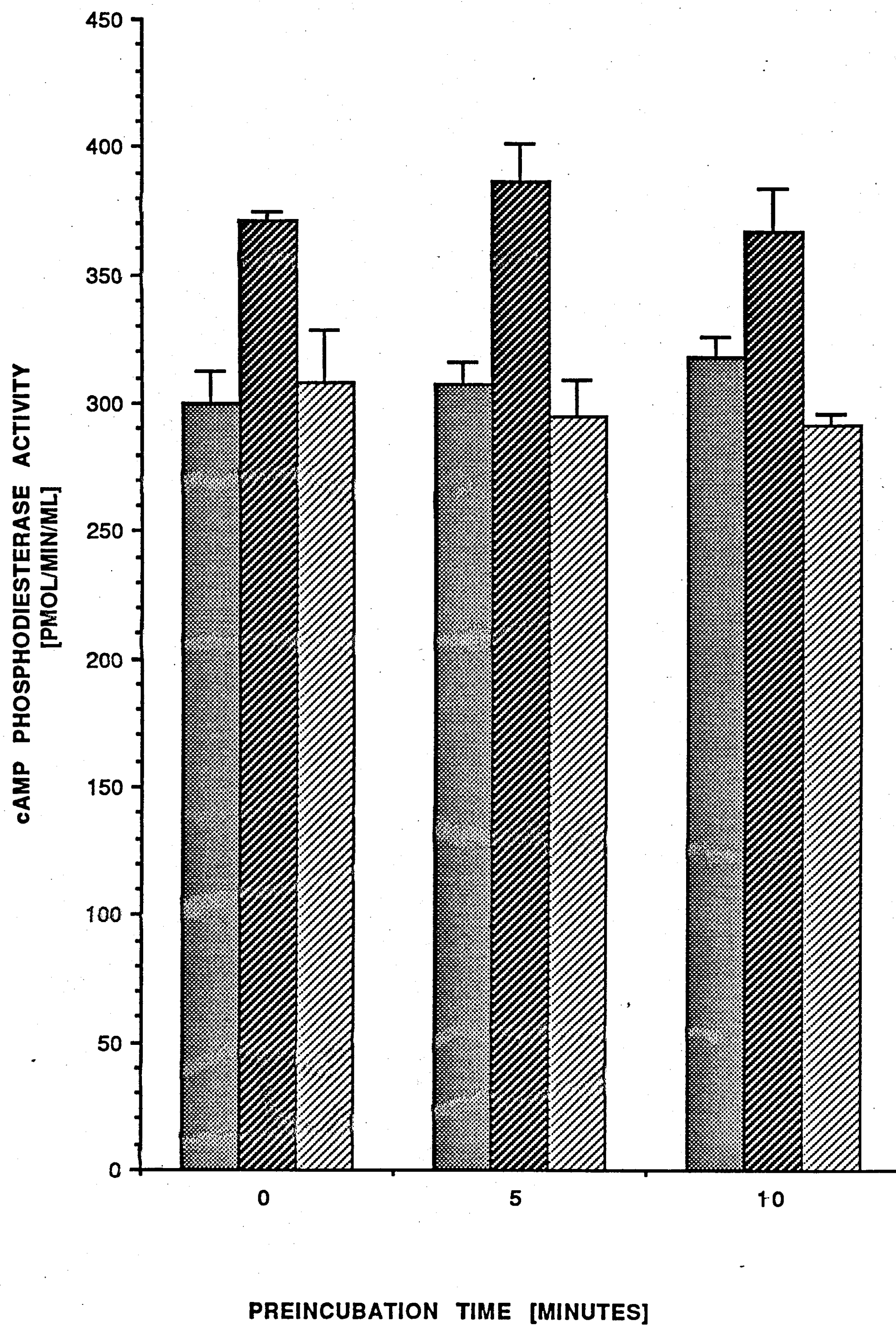


FIGURE 3.4:

SEPARATION OF RAT LIVER SOLUBLE PHOSPHODIESTERASE ACTIVITY BY DE-52 CHROMATOGRAPHY

A soluble rat liver extract (45mls, 263mg of protein) prepared from one rat liver was applied to a column of DE-52 (3.1cm x 1.2cm) (prepared and equilibrated as in Section 2.8.1). The column was loaded and washed as in Section 2.8.2. Phosphodiesterase activity was eluted using a 400ml linear sodium chloride gradient between 0 and 0.5M NaCl contained in Buffer 'A' (50mM Tris-HCl pH 7.4, 0.1mM EGTA, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2μM Leupeptin, 0.1mM Dithiothreitol) and 4ml fractions were collected. 25μl aliquots were removed and assayed for cyclic AMP (□) and cyclic GMP (■) phosphodiesterase activity at 1μM substrate. The sodium chloride gradient is shown as (—). Calmodulin activity was assayed by the protocol described in Section 2.9 and the peak of activity is indicated as the hatched box (▭).

The relative yields of protein were:

Peak I-29mg

Peak II-37mg

Peak III-11mg

FIGURE 3.4:

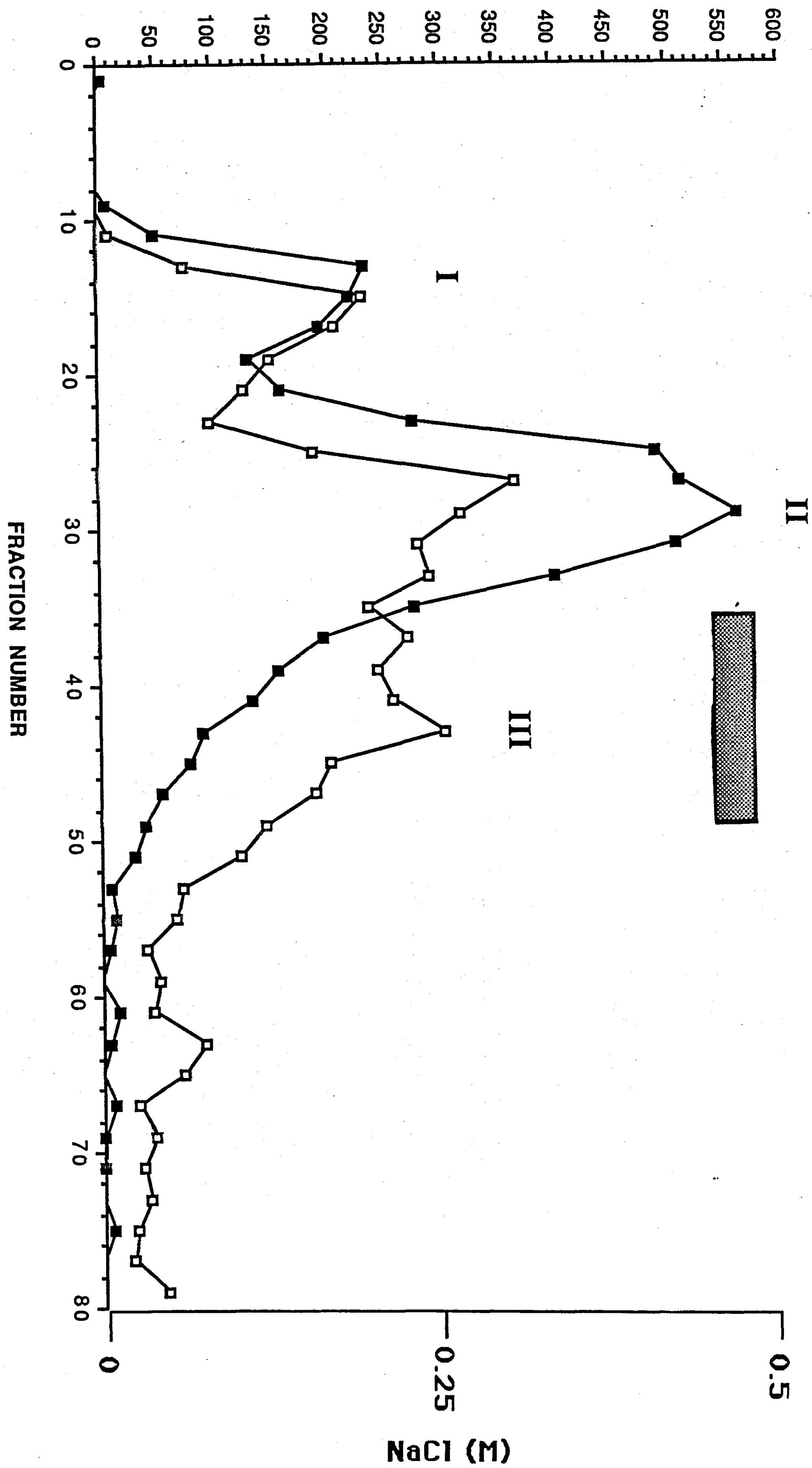


FIGURE 3.5:

THERMAL INACTIVATION OF cAMP AND cGMP PHOSPHODIESTERASE ACTIVITY OF DE-52 PEAK I AT 60°C.

Samples (25μl) of Peak I prepared by DE-52 chromatography were incubated at 60°C for the indicated time before their removal onto ice. Phosphodiesterase activity was then determined at 1μM cGMP (■) and 1μM cAMP (□). The zero time cAMP and cGMP PDE activities were 27 pmol/min/ml and 20 pmol/min/ml respectively. Results are expressed as the log of the % of original activity remaining for each of the individual time points.

FIGURE 3.5:

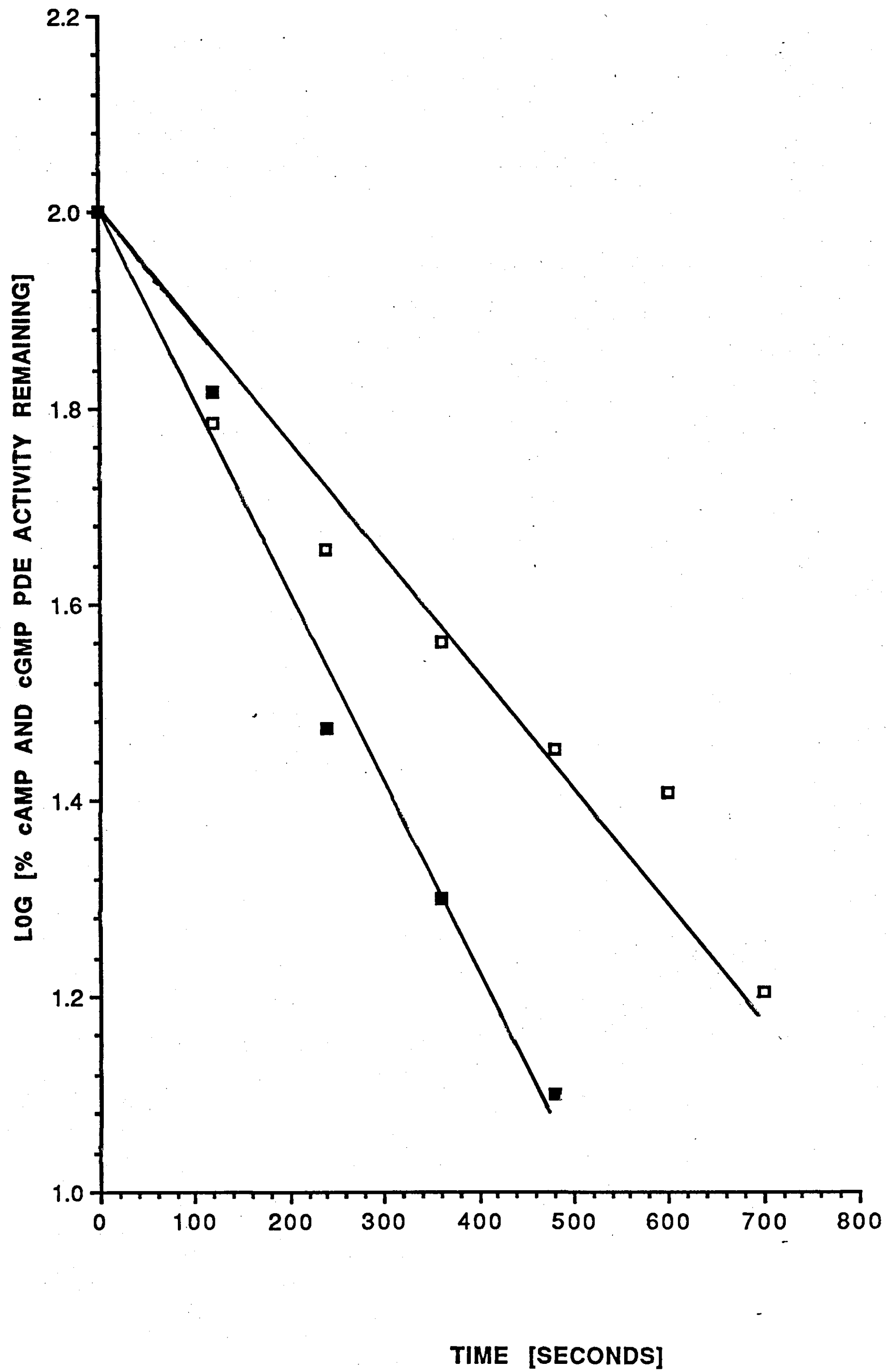


FIGURE 3.6:

CHROMATOGRAPHY OF DE-52 PEAK I ON AFFI-GEL BLUE

Pooled fractions (24mls) from the DE-52 step containing 12mg of protein were made up to 5mM $MgCl_2$ and applied at 0.5ml/min to a column of Affi-gel Blue (1.8cm x 1.1cm) previously equilibrated in Buffer 'A' (50mM Tris-HCl pH 7.4, 0.1mM EGTA, 5mM Benzamidine hydrochloride, 0.2mM PMSF, 2μM leupeptin, 0.1mM Dithiothreitol) containing in addition 0.15M NaCl and 5mM $MgCl_2$. The unbound fraction was collected as a pool and the column was then washed in 8 Bed volumes of the equilibration buffer and this was collected as a pool.

(a) Phosphodiesterase activity was assessed at 1μM cAMP for basal activity (■) and in the presence of 2μg/ml of Calmodulin (□) and 2μg/ml Calmodulin plus 50μM $CaCl_2$ (▨).

(b) Phosphodiesterase activity was assessed at 1μM cGMP for basal activity (■) and in the presence of 2μg/ml of Calmodulin (□) and 2μg/ml Calmodulin plus 50μM $CaCl_2$ (▨)

Values were determined in triplicate and are expressed in pmol/min as the mean ± SD.

FIGURE 3.6(a)

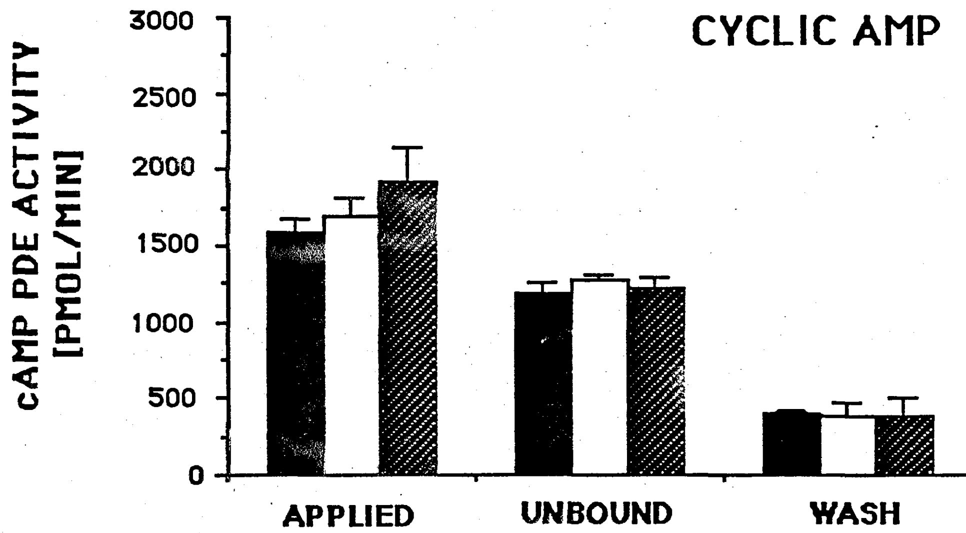


FIGURE 3.6(b)

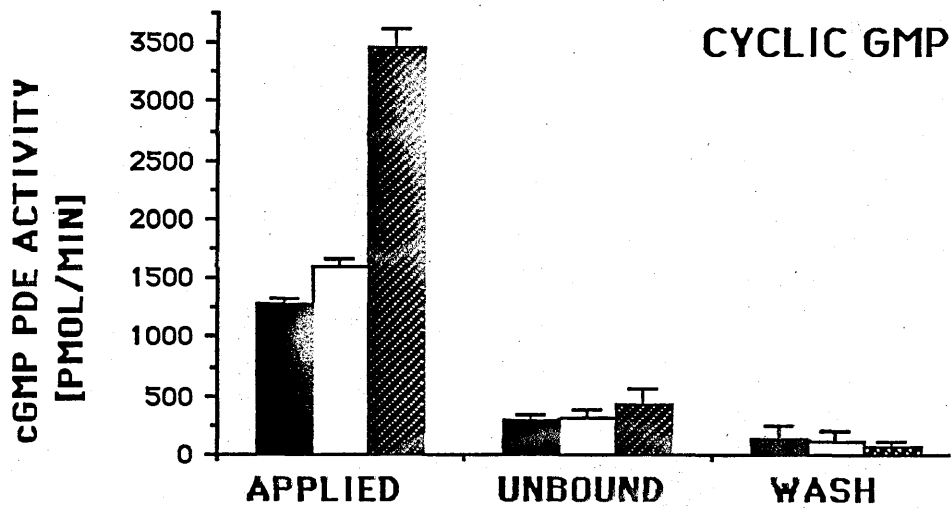


FIGURE 3.7:

ELUTION OF CYCLIC GMP PDE ACTIVITY OF DE-52 PEAK I FROM AFFI-GEL BLUE.

1ml aliquots of DE-52 Peak I (containing 56 pmol/min of cGMP PDE activity each) isolated from a rat liver soluble fraction were made up to 5mM $MgCl_2$ and incubated with 100µl aliquots of Affi-gel Blue prepared and equilibrated as in Section 2.12. Incubation was for 30 minutes at 4^oc on a rotary mixer. Gels were washed in 2 x 1ml aliquots of equilibration buffer (see Section 2.12). Elution of cGMP PDE activity was attempted using 2 x 1ml aliquots of Buffer A (50mM Tris-HCl pH 7.4, 0.1mM EGTA, 5mM Benzamidine, 0.2mM PMSF, 0.1mM dithiothreitol, 2µM Leupeptin) containing the indicated additions.

1: Control cGMP PDE activity.
2: cGMP PDE activity eluted during wash.
3: 0.15M NaCl/1mM EDTA.
4: 0.15M NaCl/5mM $MgCl_2$/10mM cGMP.
5: 0.15M NaCl/5mM $MgCl_2$/10mM cAMP.
6: 0.5M NaCl/5mM $MgCl_2$.
7: 1.0M NaCl/5mM $MgCl_2$.

1ml of DE-52 Peak I contained 56 ± 3.91 pmol/min of cGMP PDE activity and in the presence of saturating Ca^{2+}/Calmodulin exhibited an activity of 156.25 pmol/min.

Cyclic GMP phosphodiesterase activity was assessed either in the absence (▩) or presence of saturating levels of Ca^{2+}/Calmodulin (▧).

FIGURE 3.7:

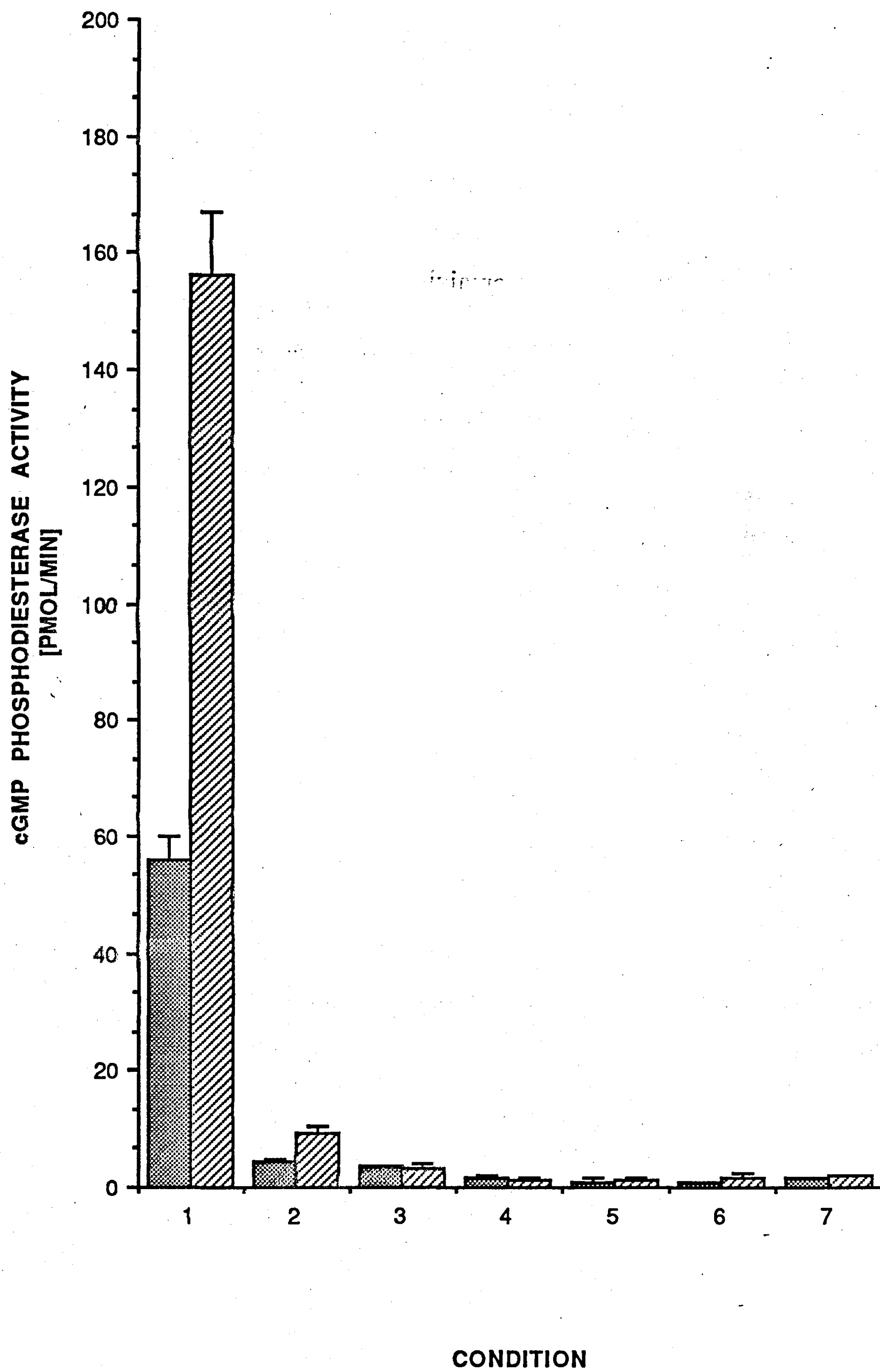


FIGURE 3.8:

MONO Q ANION-EXCHANGE PROFILE OF RAT LIVER SOLUBLE PHOSPHODIESTERASE ACTIVITY.

Approximately half the high speed supernatant prepared from rat liver (20mls containing 120mg of protein) as in Section 2.4 was applied to a Mono Q column at 1ml/min (see Section 2.11). Phosphodiesterase activity was eluted using a sodium chloride gradient at 1ml/min and 1ml fractions collected. Cyclic AMP (□) and cyclic GMP (■) phosphodiesterase activity was determined at 1µM substrate by assaying a 25µl aliquot of every alternate fraction. The phosphodiesterase activity is expressed in pmol/min. The sodium chloride gradient is shown as a solid line (–).

The relative yields of protein were:

PDE-MQ I 1.62mg

PDE-MQ II 1.14mg

PDE-MQ III 0.525mg

PDE-MQ IV 0.098mg

PDE-MQ V 0.110mg

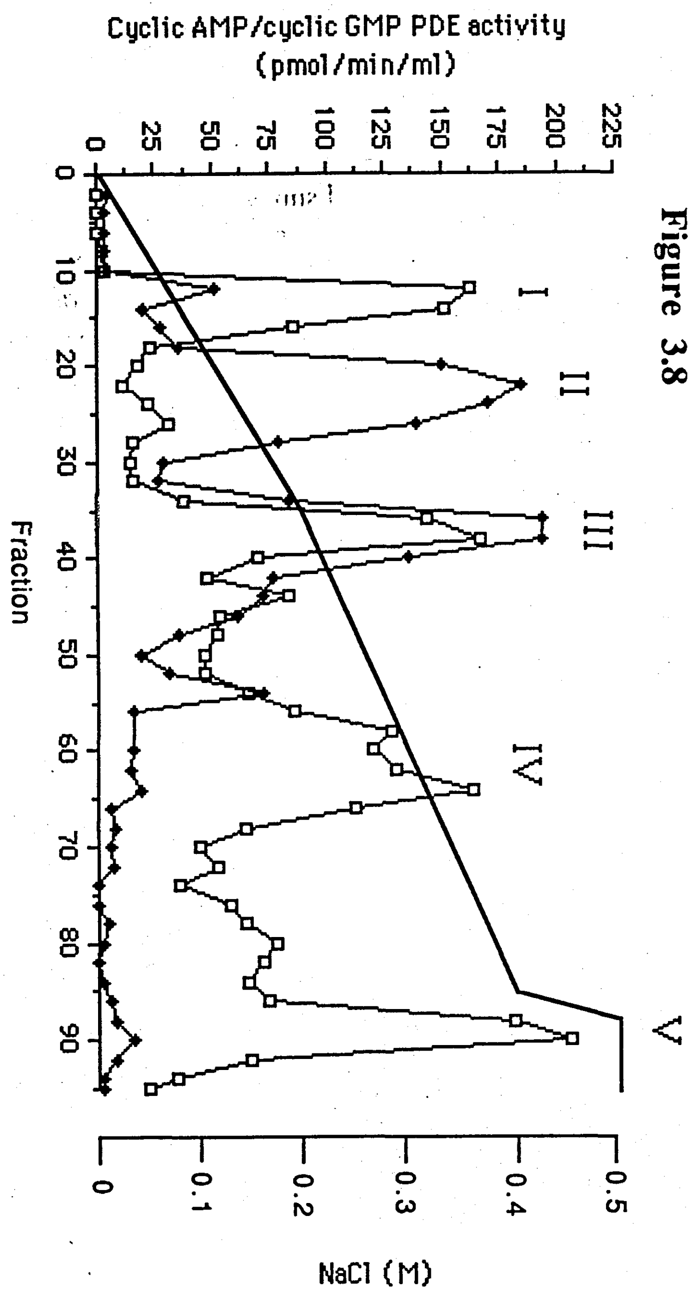


Figure 3.8

FIGURE 3.9:

CHROMATOGRAPHY OF PDE MQ-I AND PDE MQ-II ON AFFI-GEL BLUE.

PDE MQ-I and PDE MQ-II were isolated by Mono Q anion-exchange chromatography of a rat liver supernatant (see Section 2.11). 0.75ml aliquots were made up to 5mM $MgCl_2$ and batch bound to 110µl aliquots of Affi-gel Blue using the method described in Section 2.12. Gels were washed in 2 x 1ml aliquots of the equilibration buffer. Washes and the unbound fraction were pooled to give a total unbound fraction. Phosphodiesterase activity was assessed at 1µM cAMP (▩) and 1µM cGMP (▧). Results are expressed as the mean of triplicates ± SD.

FIGURE 3.9:

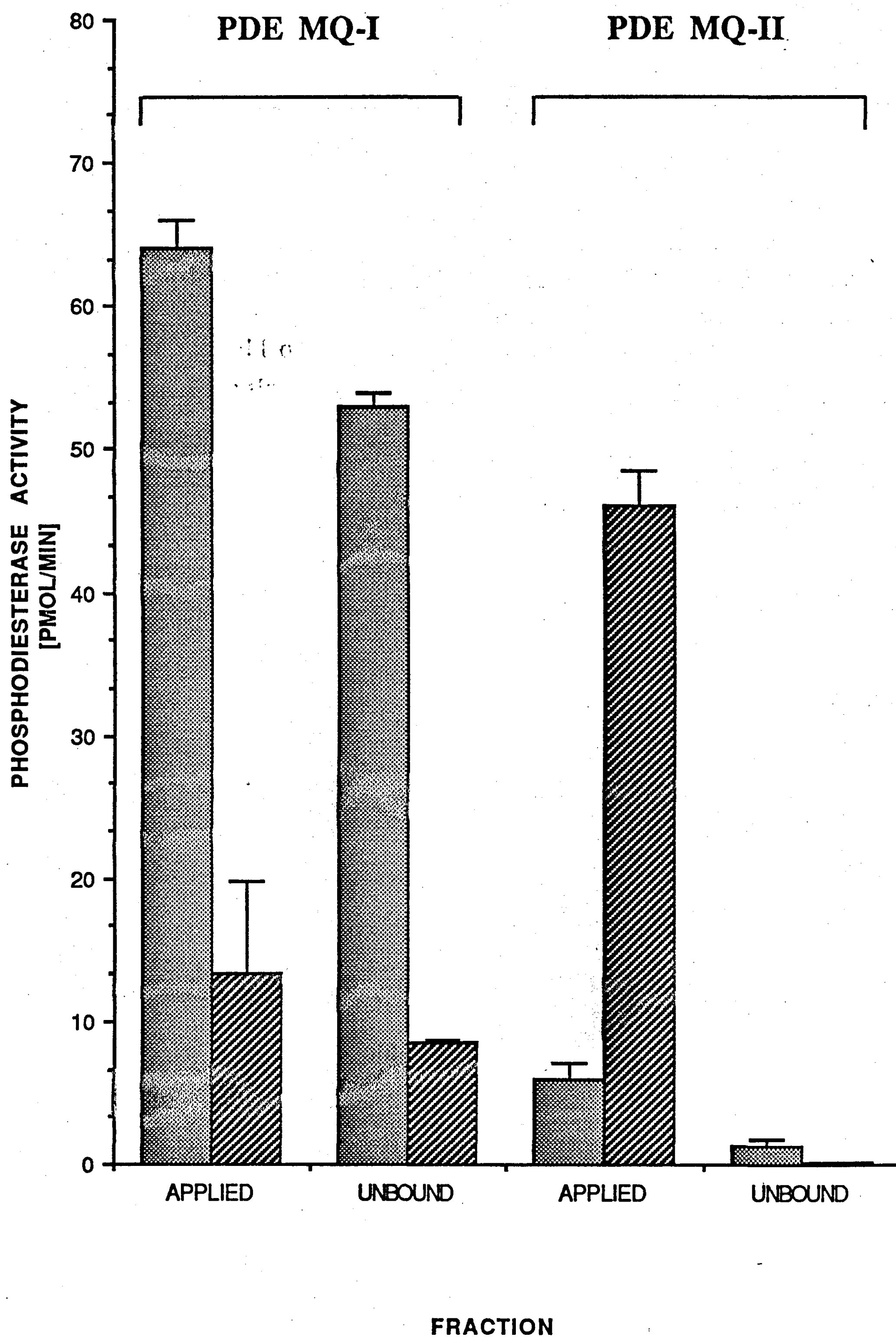


FIGURE 3.10 (a):

MONO Q ANION-EXCHANGE SEPARATION OF RAT LIVER SOLUBLE PDE MQ-PEAKS ISOLATED IN THE PRESENCE OF CALCIUM.

A rat liver supernatant was prepared as in Section 2.4 except that 0.1mM $CaCl_2$ was used in place of 0.1mM EGTA in the homogenisation buffer. 20mls of the supernatant (147mg of protein) were applied to a Mono Q column and activity eluted using an identical protocol to that detailed in Section 2.11 except that 0.1mM EGTA was replaced with 0.1mM $CaCl_2$ in all the column elution buffers. Cyclic AMP (□) and cyclic GMP (■) phosphodiesterase activity was determined at 1µM substrate by assaying a 25µl aliquot of every alternate fraction. Phosphodiesterase activity is expressed in pmol/min/ml.

FIGURE 3.10 (b):

MONO Q ANION-EXCHANGE SEPARATION OF RAT LIVER SOLUBLE PDE MQ-PEAKS ASSESSED AT 100µM SUBSTRATE

A soluble rat liver fraction was prepared as in Section 2.4. 20mls was applied to a Mono Q column and activity eluted as described in Section 2.11. 1ml fractions were collected and cyclic AMP (□) and cyclic GMP (■) phosphodiesterase activity determined at 100µM substrate. Phosphodiesterase activity is expressed in pmol/min/ml.

FIGURE 3.10(a):

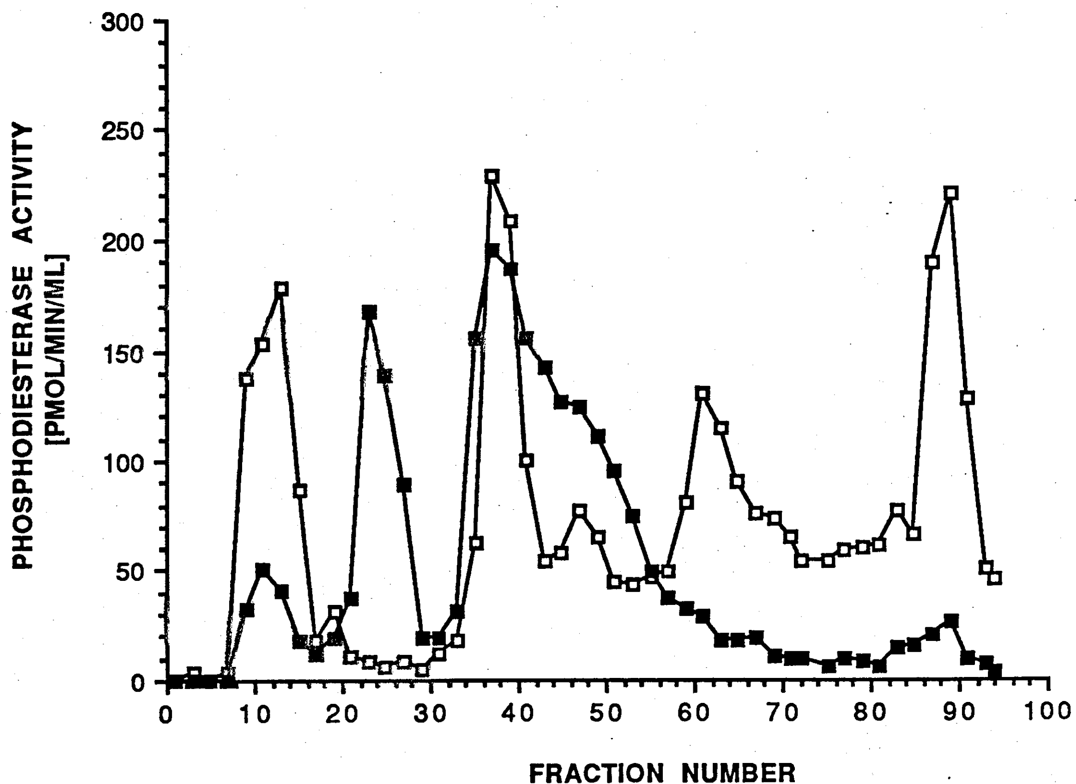


FIGURE 3.10(b):

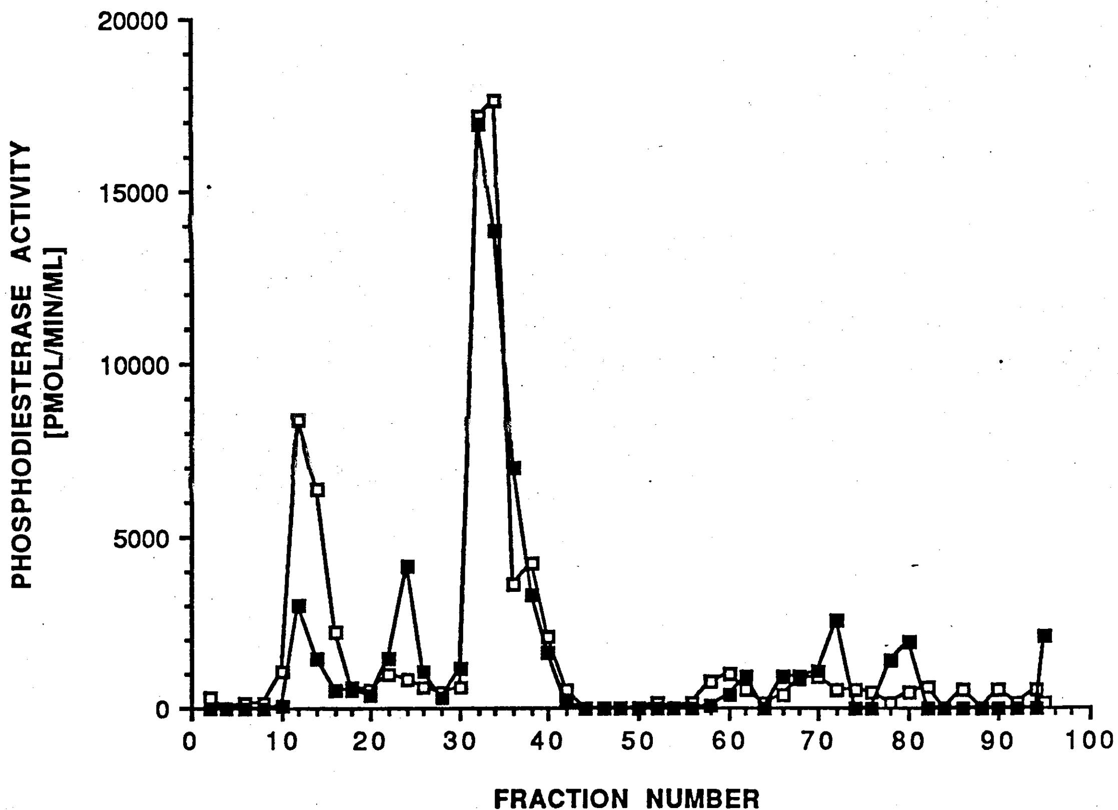


FIGURE 3.11:

INHIBITION OF RAT LIVER PDE MQ-I BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of liver-derived PDE MQ-I was assessed at 1μM substrate in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (1-V). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.11:

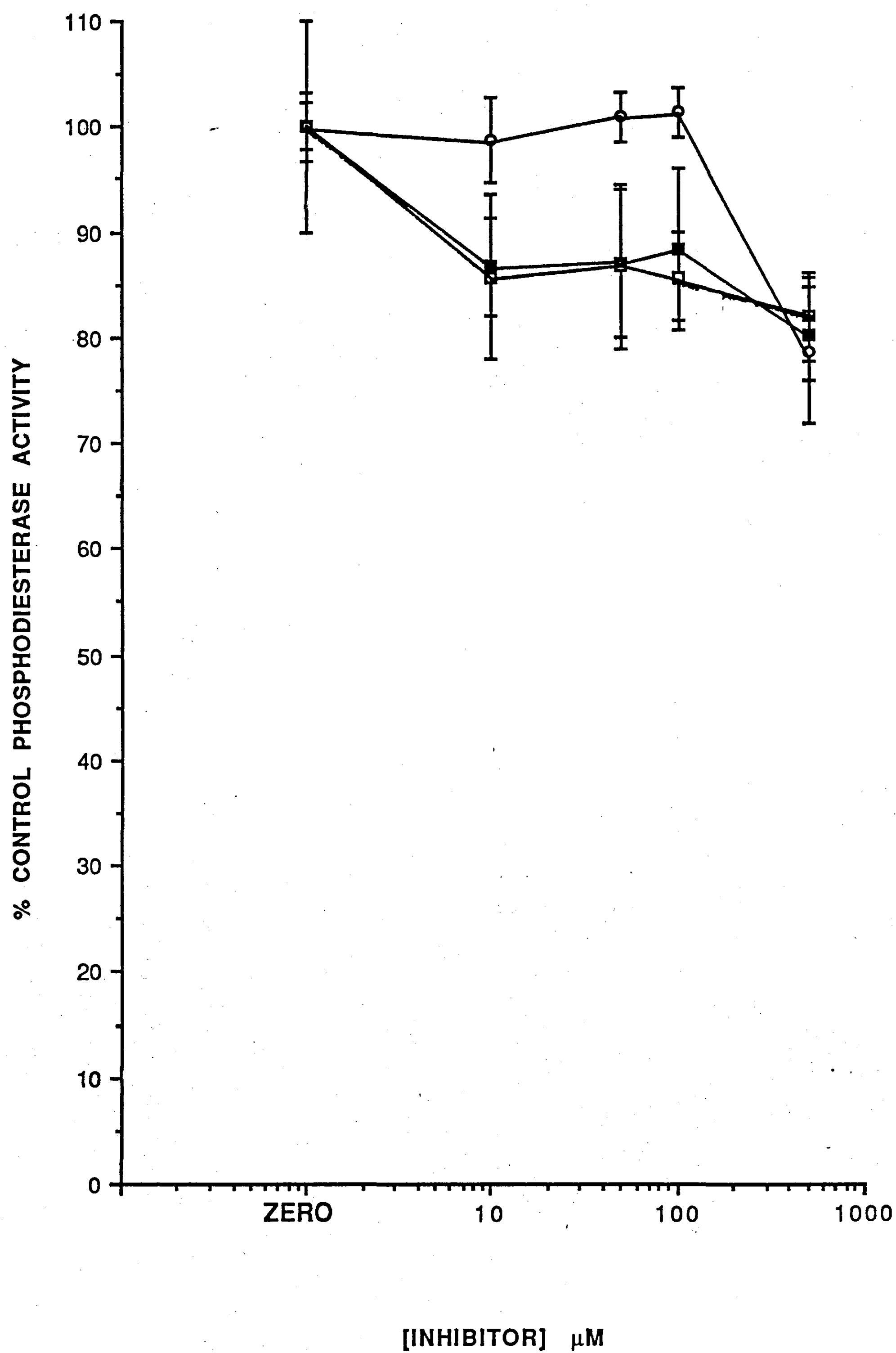


FIGURE 3.12:

INHIBITION OF RAT LIVER PDE MQ-II BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of liver-derived PDE MQ-II was assessed at 1μM substrate in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (1-V). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.12:

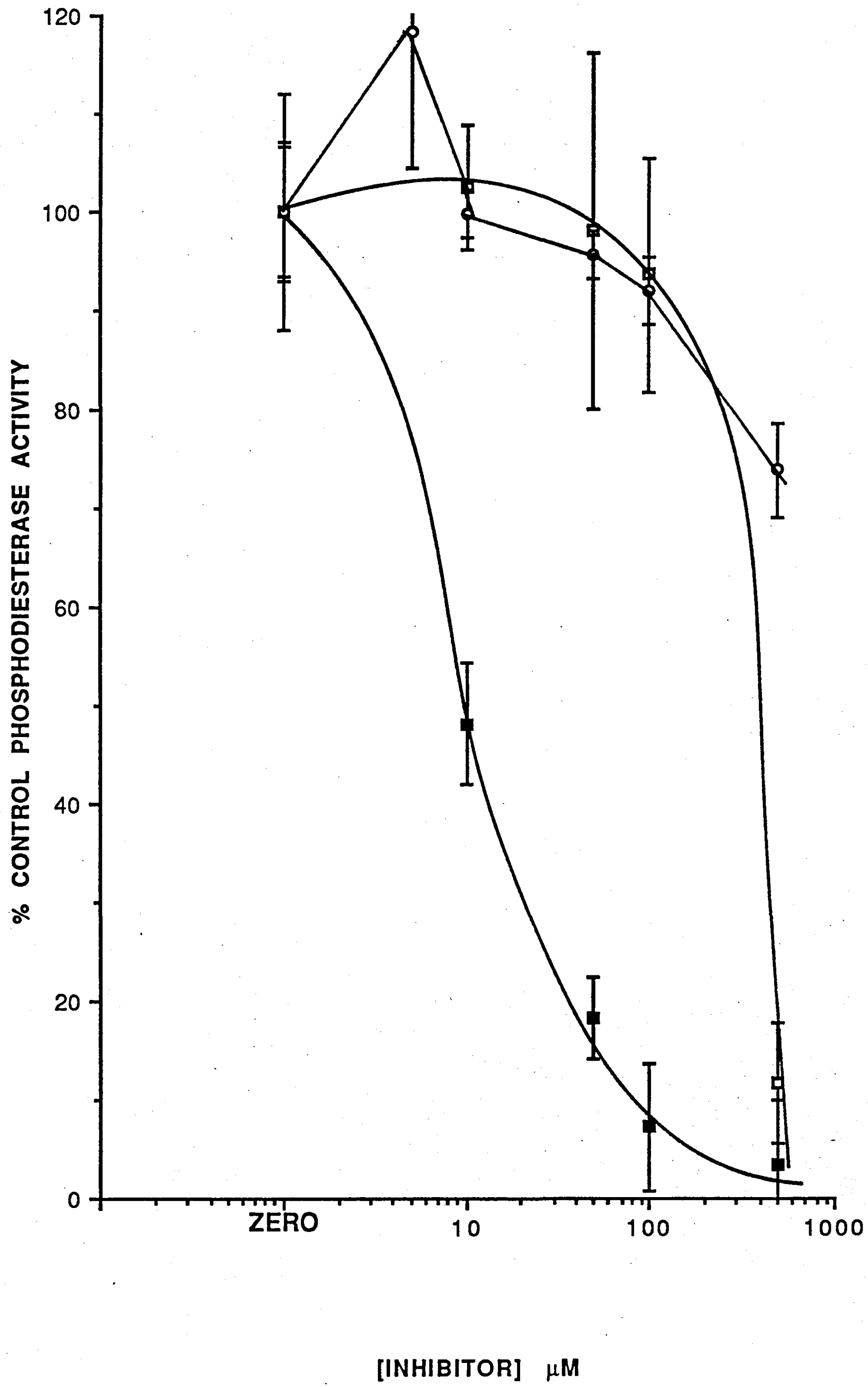


FIGURE 3.13:

INHIBITION OF RAT LIVER PDE MQ-III BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of liver-derived PDE MQ-III was assessed at 1μM substrate in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (1-V). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.13:

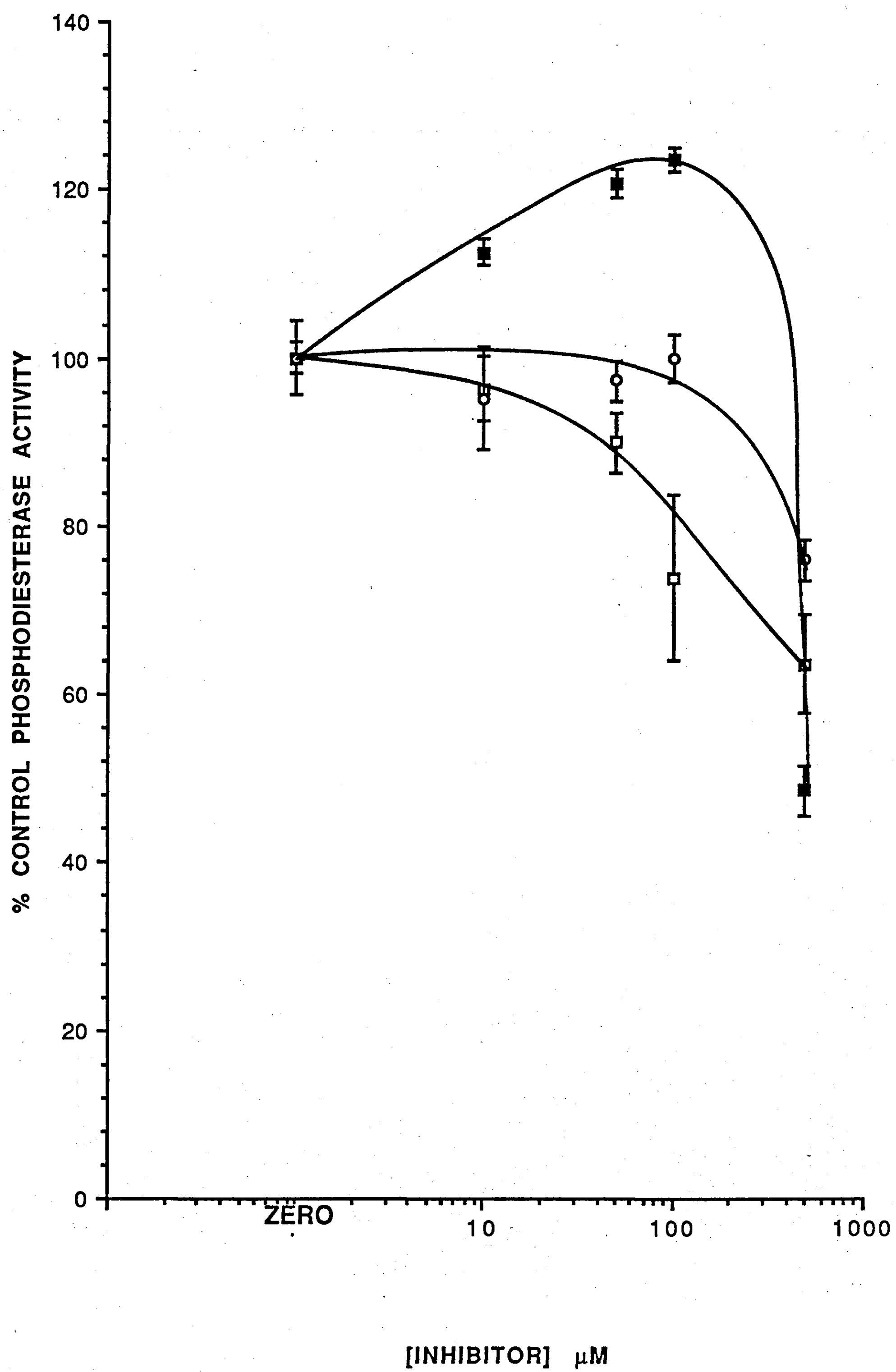


FIGURE 3.14:

INHIBITION OF RAT LIVER PDE MQ-IV BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of liver-derived PDE MQ-IV was assessed at 1µM substrate in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (1-V). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.14:

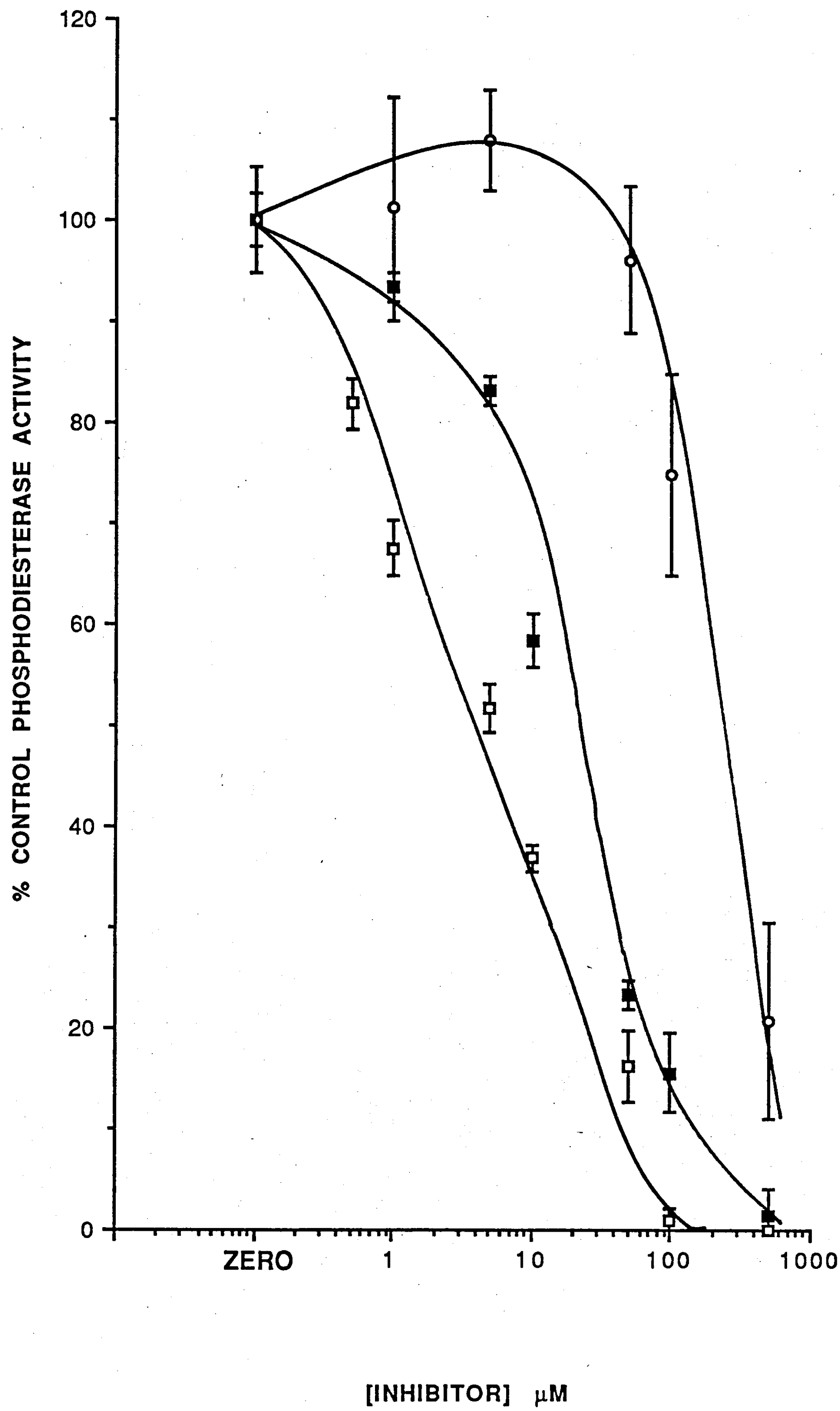


FIGURE 3.15:

INHIBITION OF RAT LIVER PDE MQ-V BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of liver-derived PDE MQ-V was assessed at 1µM substrate in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (1-V). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.15:

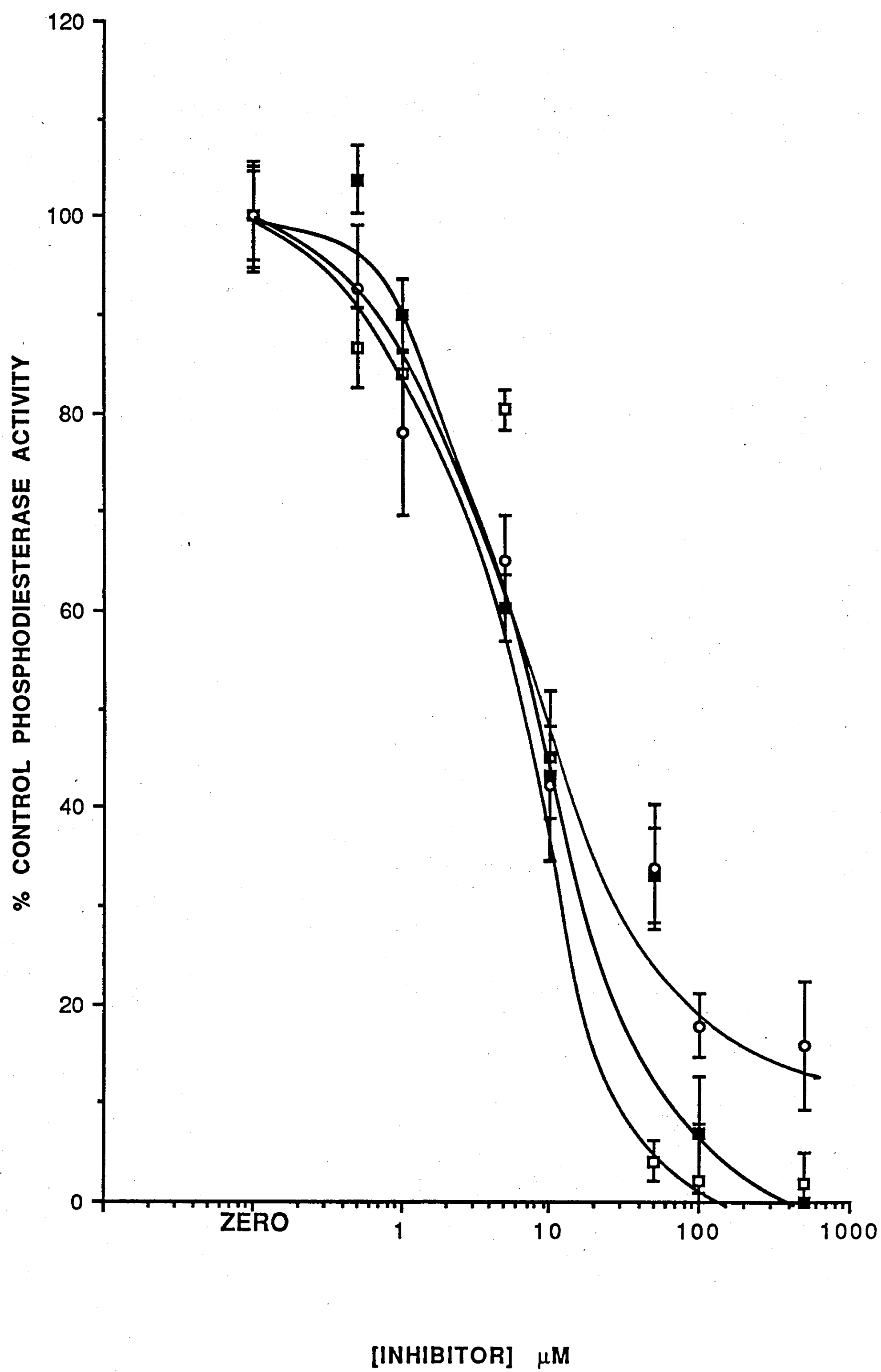


FIGURE 3.16:

DISTRIBUTION OF RAT HEPATOCTYE PHOSPHO-DIESTERASE ACTIVITY.

Hepatocytes were prepared by collagenase digestion from one rat (250-275g) as described in Section 2.5 and homogenised in approximately 50 mls of Buffer 'A' (50mM Tris-HCl pH 7.5, 0.25M Sucrose, 5mM Benzamidine Hydrochloride, 0.2mM PMSF, 20µM Leupeptin, 0.1mM EGTA and 0.1mM dithiothreitol) as detailed in Sections 2.4 and 2.5. The homogenate was spun at 706*gav* for 10 minutes to produce a low speed 'pellet' (P_1) and 'supernatant' (S_1).The supernatant was retained. The pellet was 'washed' by resuspending in a further 40 mls of Buffer 'A' and recentrifuged to produce a further low speed pellet and supernatant. The supernatant was once again retained and the pellet was 'washed' in a further 40 mls of Buffer 'A' and centrifuged to produce a final 'washed' P_1 fraction and a futher low speed supernatant. The three supernatants were centrifuged at 163,750*gav* to produce three high speed supernatants (S_2) and three high speed pellets (P_2).

3.16 (a): DISTRIBUTON OF RAT HEPATOCYTE cAMP PHOSPHODIESTERASE ACTIVITY.

The cyclic AMP phosphodiesterase activity of the individual fractions was assessed at 1µM substrate in the absence (▩) or presence of 2 µg/ml Calmodulin plus 50µM $CaCl_2$ (▧) or 2µM cyclic GMP (▧).

3.16 (b): DISTRIBUTON OF RAT HEPATOCYTE cGMP PHOSPHODIESTERASE ACTIVITY.

The cyclic GMP phosphodiesterase activity of the individual fractions was assessed at 1µM substrate in the absence (▩) or presence of 2 µg/ml Calmodulin plus 50µM $CaCl_2$ (▧).

Fraction 1: Homogenate.
Fraction 2: 'Washed P_1' pellet.
Fraction 3: High speed supernatant (S_2).
Fraction 4: High speed pellet (P_2).
Fraction 5: High speed supernatant from first wash of P_1.
Fraction 6: High speed pellet from first wash of P_1.
Fraction 7: High speed supernatant from second wash of P_1.
Fraction 8: High speed pellet from second wash of P_1

FIGURE 3.16(a):

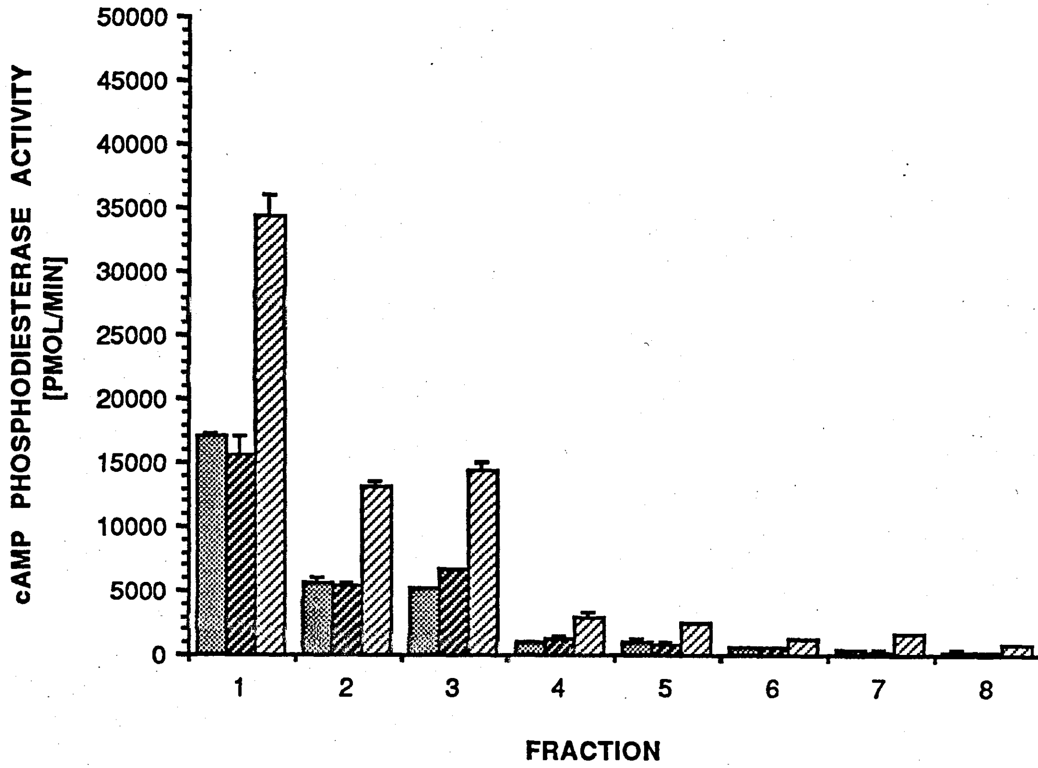


FIGURE 3.16(b):

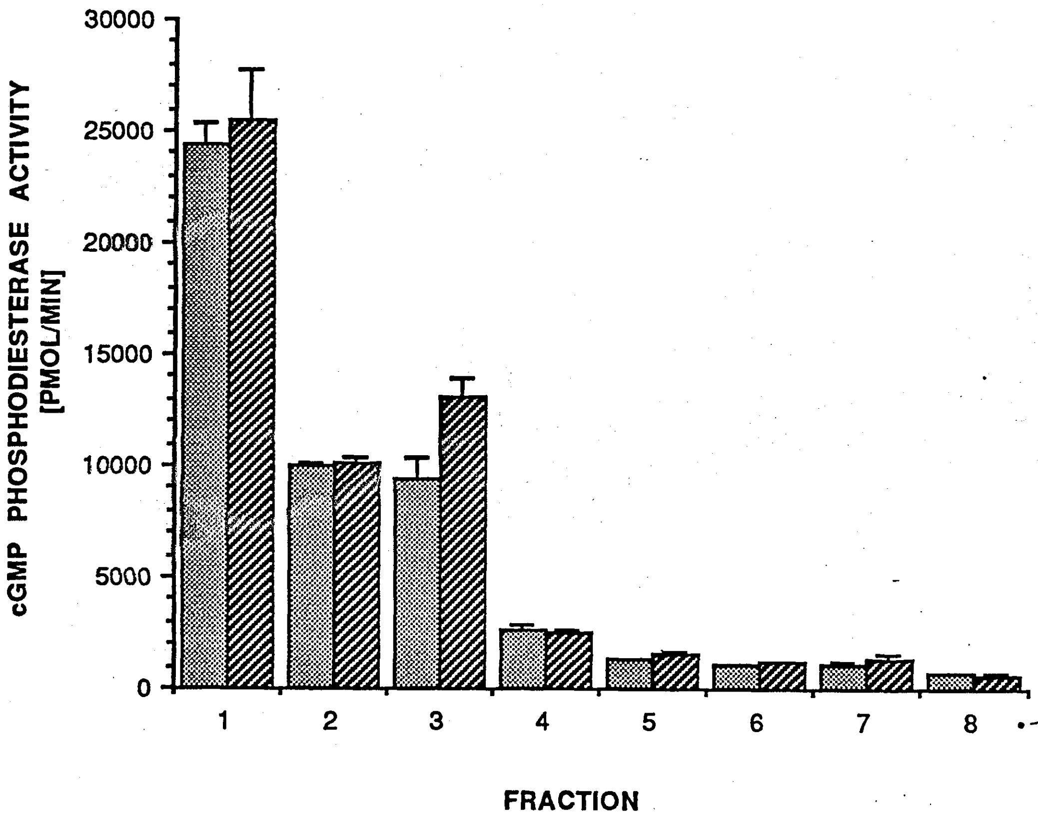


FIGURE 3.17:

MONO Q ANION-EXCHANGE PROFILE OF RAT HEPATOCYTE SOLUBLE PHOSPHODIESTERASE ACTIVITY.

Hepatocytes, prepared by collagenase digestion of one rat liver, were homogenised under isotonic conditions in the presence of protease inhibitors (see Section 2.5). A high speed supernatant was prepared as described in Section 2.4. All of the supernatant prepared from one liver (containing approximately 80mg of protein) was applied to a Mono Q anion-exchange column at 1ml/min (see Section 2.11). Using an identical protocol to that adopted for elution of rat liver-derived soluble phosphodiesterase (see Section 2.11), the phosphodiesterase activity was eluted using a sodium chloride gradient at 1ml/min and 1ml fractions were collected. Cyclic AMP (□) and cyclic GMP (■) PDE activity were determined at 1μM substrate by assaying a 25μl aliquot of every alternate fraction. The sodium chlorlide gradient is shown as (– – –).

The relative yields of protein were as follows;

PDE MQ-I = 0.744mg.

PDE MQ-II = 1.944mg.

PDE MQ-III = 0.9mg.

PDE MQ-IV = 0.923mg.

PDE MQ-V = 0.12mg.

FIGURE 3.17:

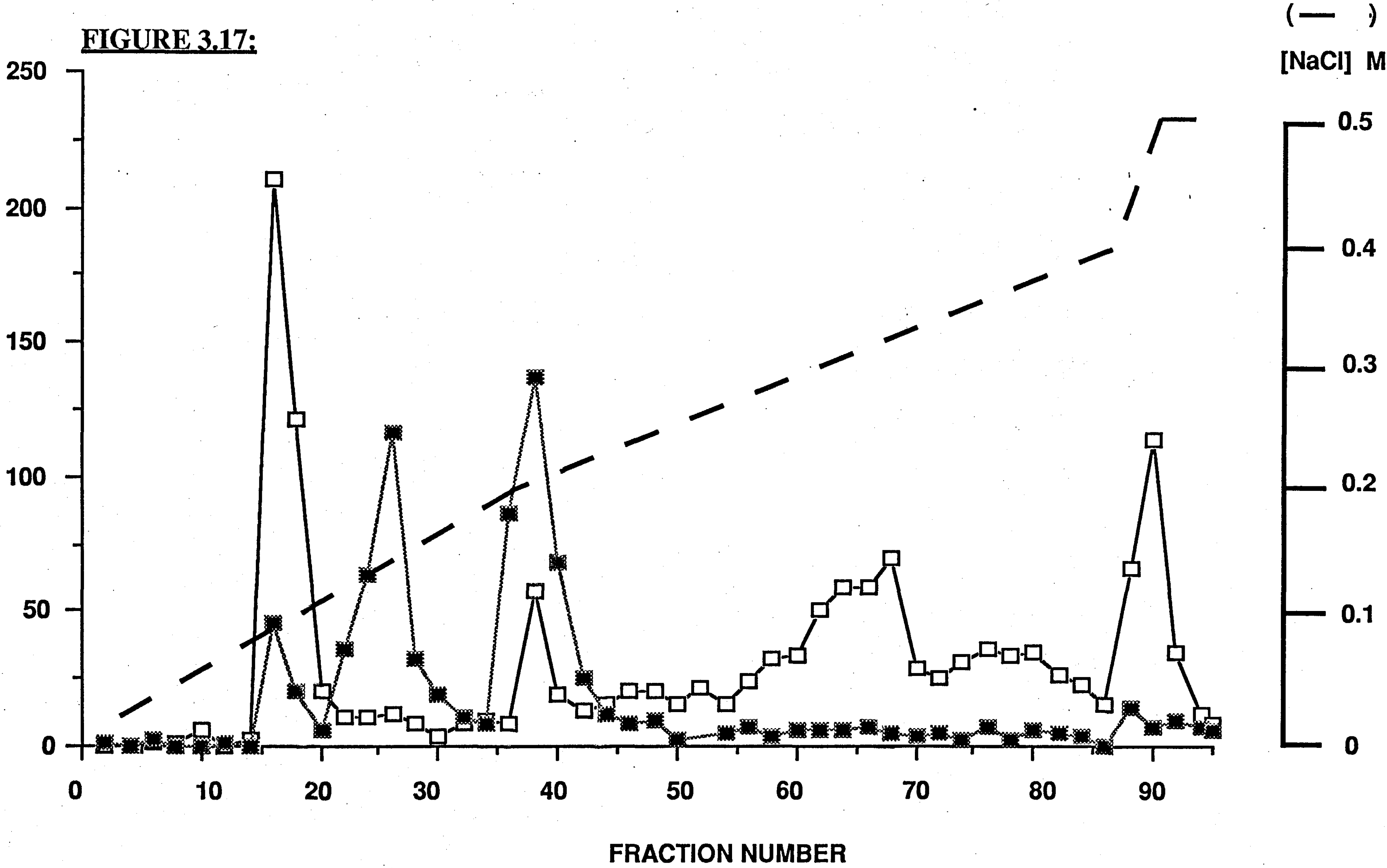


FIGURE 3.18:

DOSE RESPONSE FOR CALMODULIN STIMULATION OF cGMP PHOSPHODIESTERASE ACTIVITY OF PDE MQ-II.

PDE MQ-II, prepared from a rat hepatocyte soluble fraction by Mono Q anion-exchange chromatography, was assayed for cGMP phosphodiesterase activity in the presence (■) or absence (□) of 100μM $CaCl_2$. The Calmodulin concentration was varied between 0 and 10μg/ml. The data is expressed in pmol/min/ml as the mean of triplicates ± SD.

FIGURE 3.18:

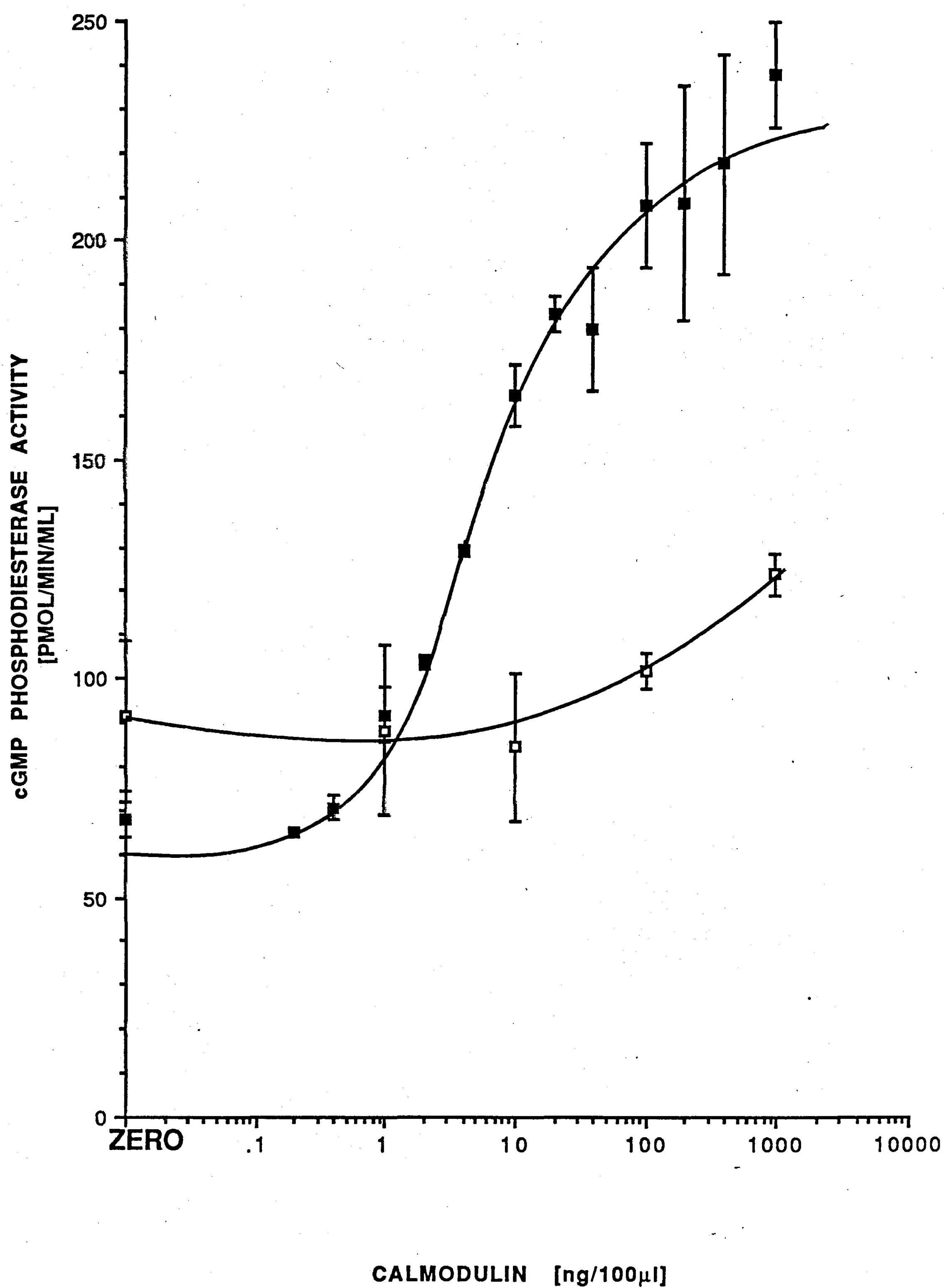


FIGURE 3.19:

DOSE RESPONSE FOR Ca^{2+} STIMULATION OF THE cGMP PHOSPHODIESTERASE ACTIVITY OF PDE MQ-II.

PDE MQ-II was prepared from the soluble fraction of rat hepatocytes by Mono Q anion-exchange chromatography. cGMP phosphodiesterase activity was assessed in the absence (□) and presence (■) of a saturating concentration of Calmodulin (2μg/ml). The added calcium concentration was varied between zero and 500μM. Results are expressed in pmol/min/ml of cGMP phosphodiesterase activity as the mean of triplicates ± SD.

FIGURE 3.19:

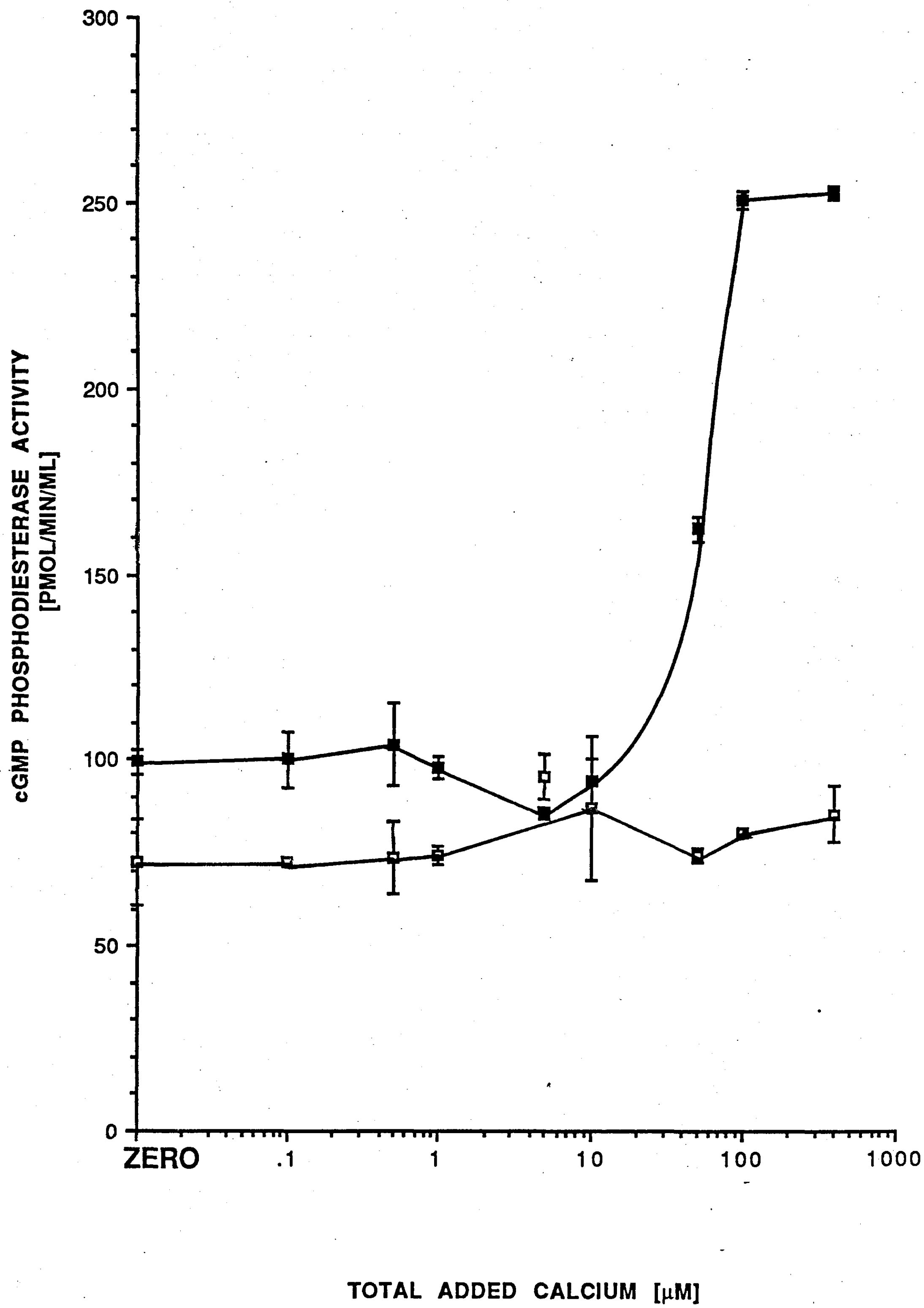


FIGURE 3.20:

DOSE RESPONSE FOR cGMP EFFECTS ON THE cAMP PDE ACTIVITY OF PDE MQ-III.

PDE MQ-III was prepared from the soluble fraction of rat hepatocytes by Mono Q anion-exchange chromatography. The cyclic AMP phosphodiesterase activity was assessed in the presence of increasing concentrations of cyclic GMP. The results are expressed in pmol/min/ml of cAMP phosphodiesterase activity as the mean of triplicate determinations ± SD.

FIGURE 3.20:

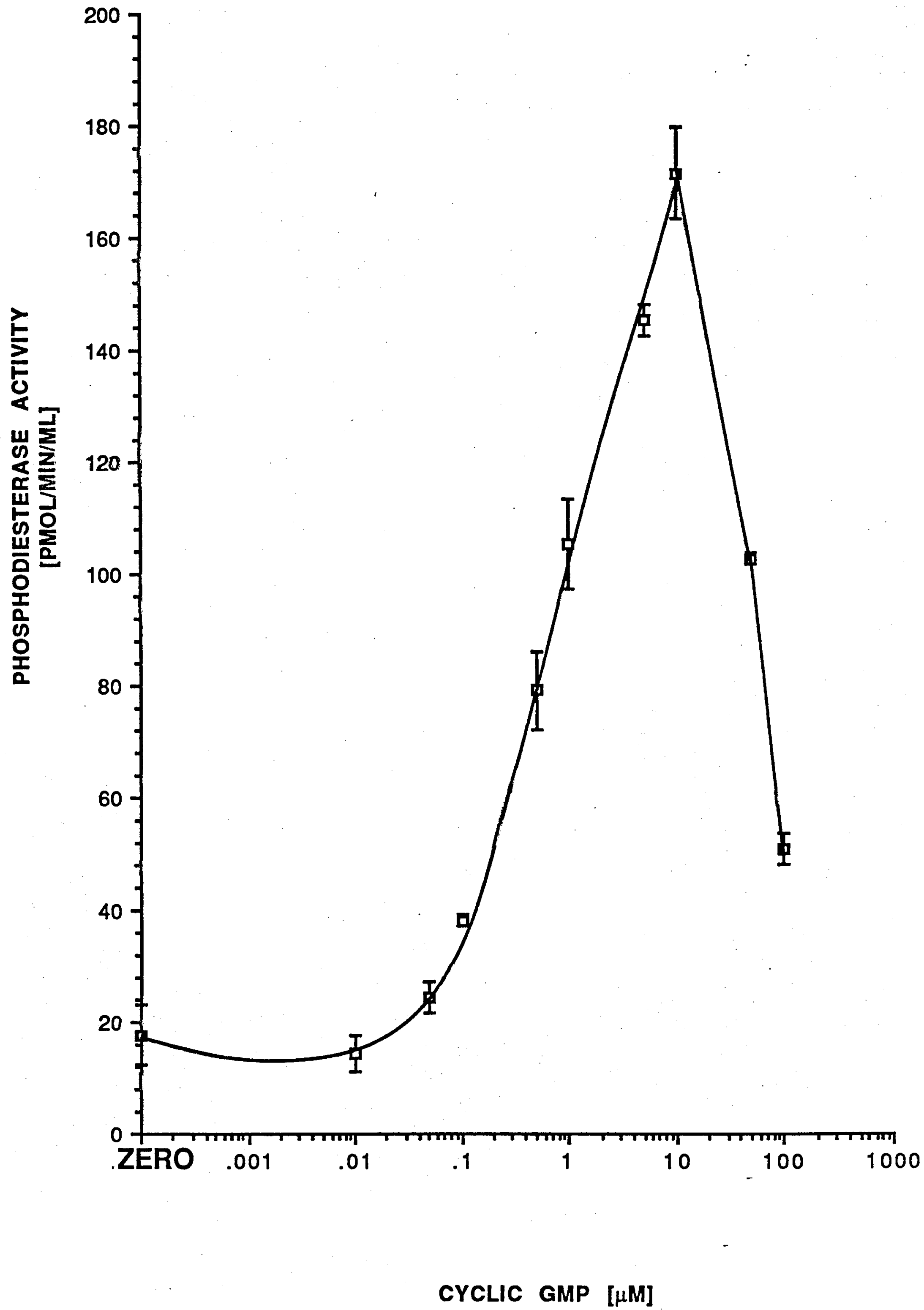


FIGURE 3.21:

LINEWEAVER-BURK PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-I

The cAMP phosphodiesterase activity of PDE MQ-I was assessed over a substrate range of 0.5μM-100μM. The velocity of the reaction was calculated as pmol of cyclic AMP hydrolysed/min/ml of enzyme preparation. Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. The line was fitted by least squares analysis. The estimated V_{max} and K_m values determined from such a plot were 1,052 pmol/min/ml and 24μM respectively.

FIGURE 3.21:

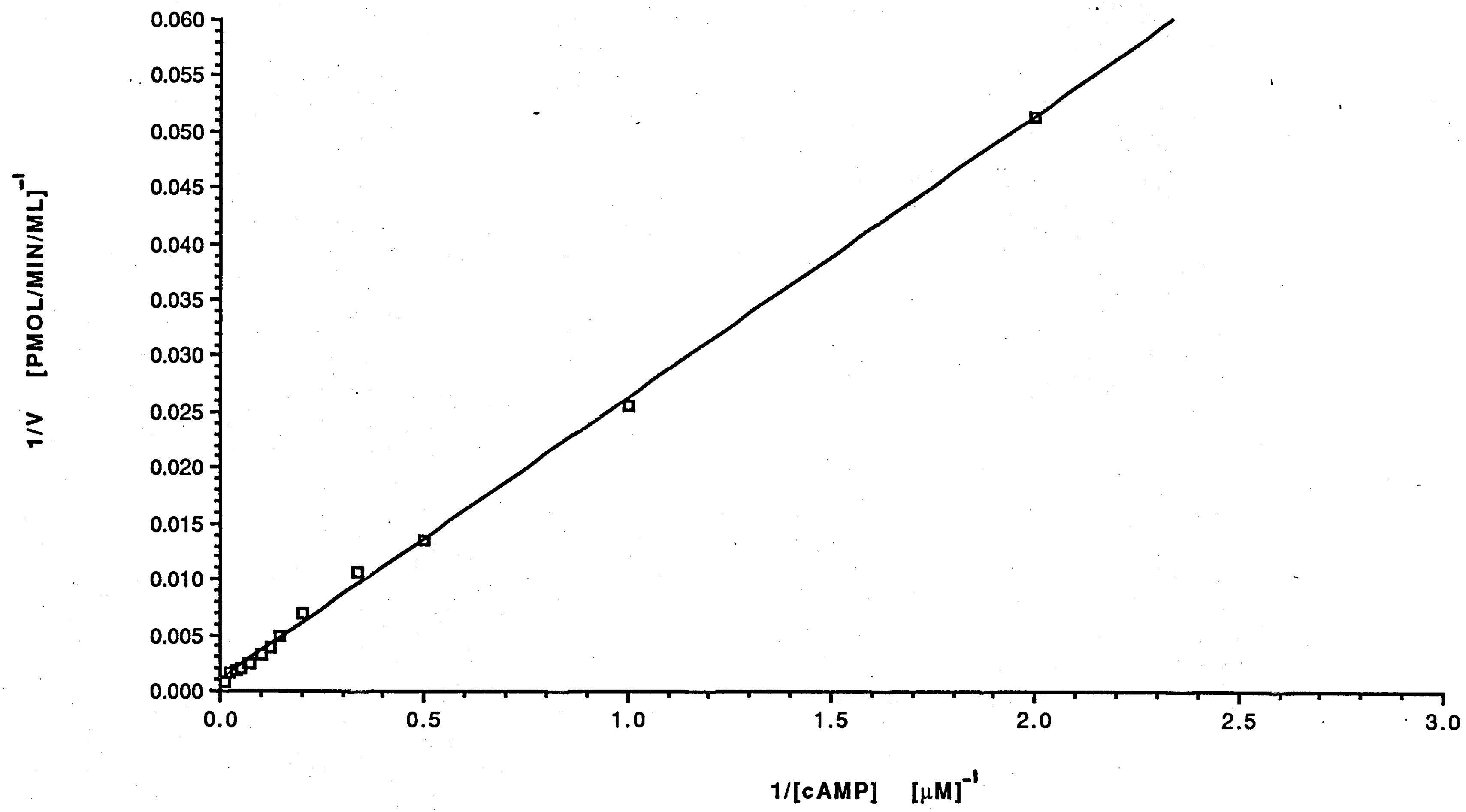


FIGURE 3.22:

HILL PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-I.

Kinetic data were replotted as Hill plots using a value of the V_{max} estimated from Lineweaver-Burk plots, Hanes plots and Eadie-Hofstee plots. The average V_{max} value was calculated to be 1390 pmol/min/ml. This value was used to determine the Hill plot. The slope of the Hill plot gives a value of 1.07 for the Hill Coefficient (h). The estimated K_m value for cAMP from this plot was 32μM.

FIGURE 3.22:

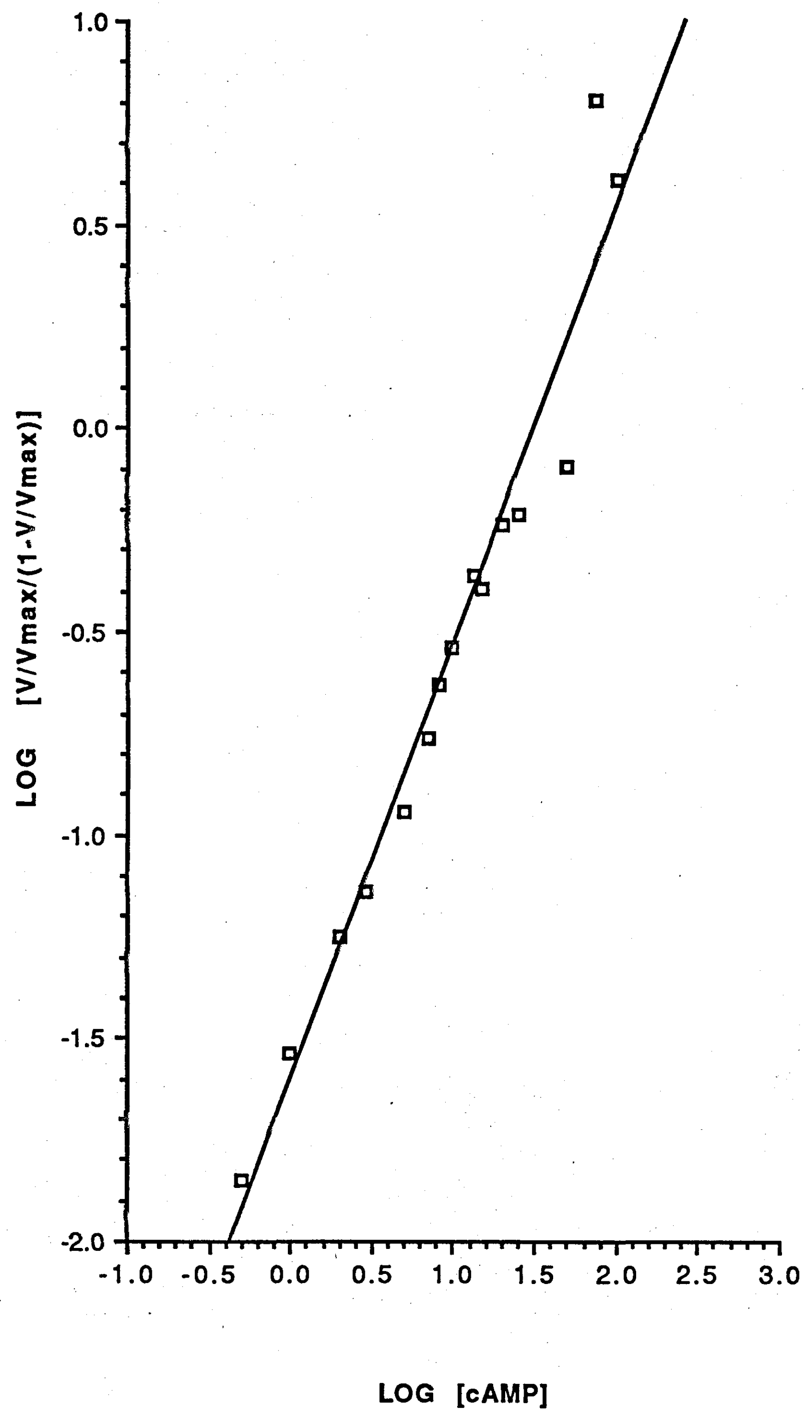


FIGURE 3.23:

LINEWEAVER-BURK PLOT FOR CYCLIC GMP HYDROLYSIS BY PDE MQ-I

The cGMP phosphodiesterase activity of PDE MQ-I was assessed over a substrate range of 1.0μM-500μM. The velocity of the reaction was calculated as pmol of cyclic GMP hydrolysed/min/ml of enzyme preparation. Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. The line was fitted by least squares analysis. The estimated V_{max} and K_m values determined from such a plot were 2,000 pmol/min/ml and 250μM respectively.

FIGURE 3.23:

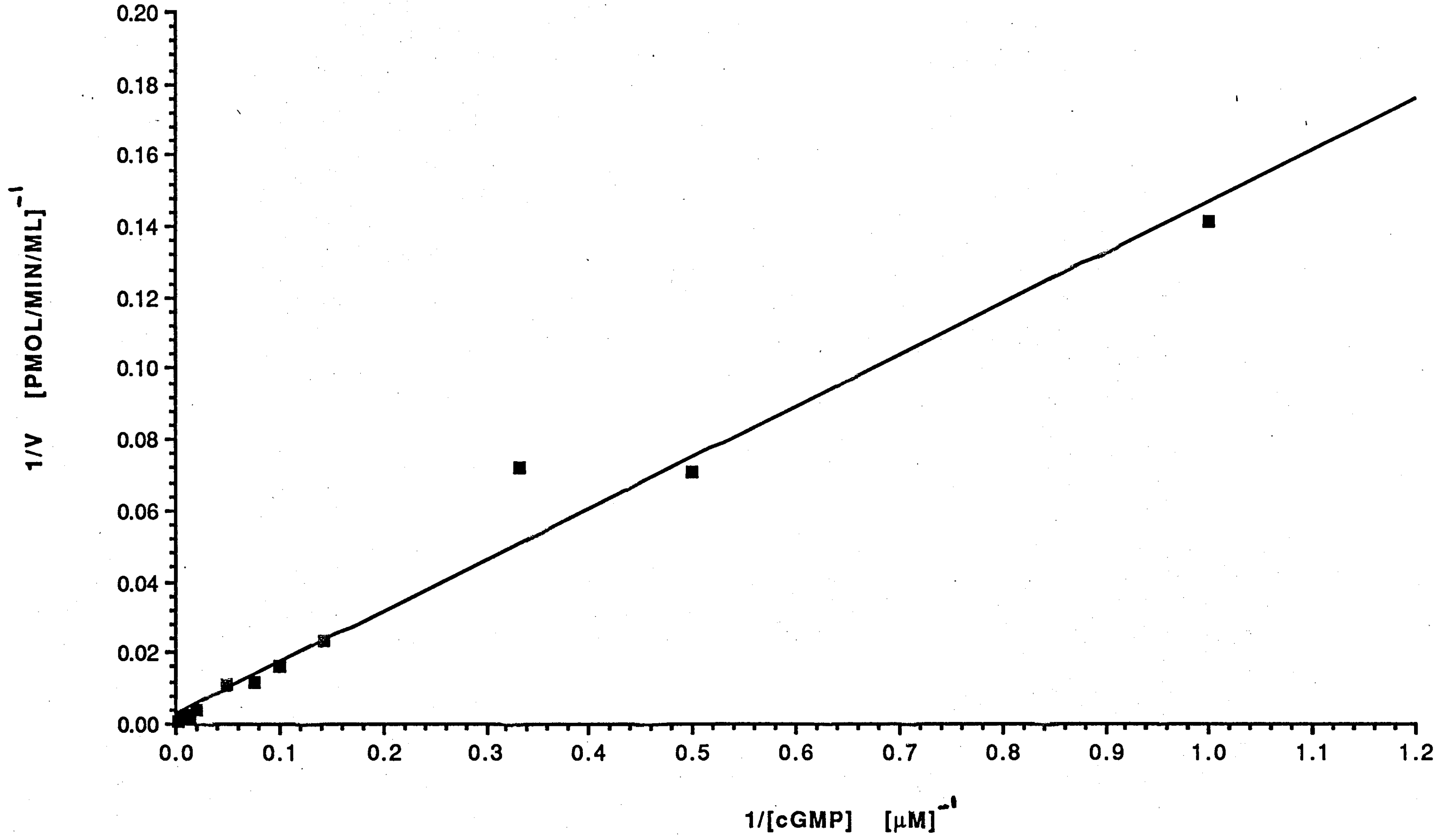


FIGURE 3.24:

HILL PLOT FOR CYCLIC GMP HYDROLYSIS BY PDE MQ-I.

Kinetic data were replotted as Hill plots using a value of the V_{max} estimated from Lineweaver-Burk plots, Hanes plots and Eadie-Hofstee plots. The average V_{max} value was calculated to be 1,775 pmol/min/ml +/- 159. This value was used to determine the Hill plot. The slope of the Hill plot gives a value of 1.01 for the Hill Coefficient (h). The estimated K_m value from this plot was 282μM.

FIGURE 3.24:

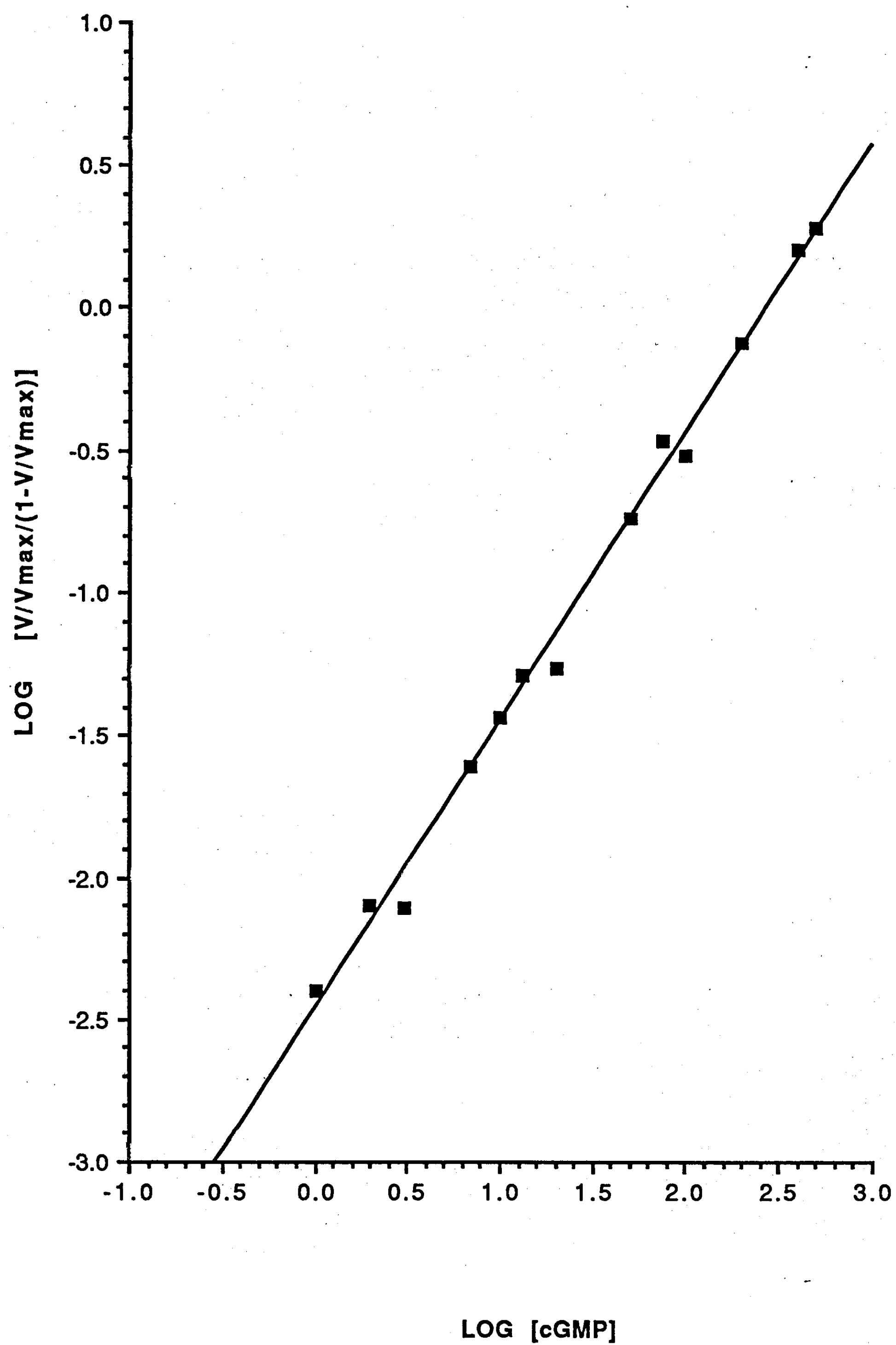


FIGURE 3.25:

LINEWEAVER-BURK PLOT FOR CYCLIC GMP HYDROLYSIS BY PDE MQ-II.

The cGMP phosphodiesterase activity of PDE MQ-II was assessed over a substrate range of 0.1μM-100μM. The velocity of the reaction was calculated as pmol of cyclic GMP hydrolysed/min/ml of enzyme preparation. Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. The limiting K_m and V_{max} values estimated from this plot were, K_{m1} = 2.9μM, K_{m2} = 14μM, V_{max1} = 264 pmol/min/ml and V_{max2} = 563 pmol/min/ml.

FIGURE 3.25:

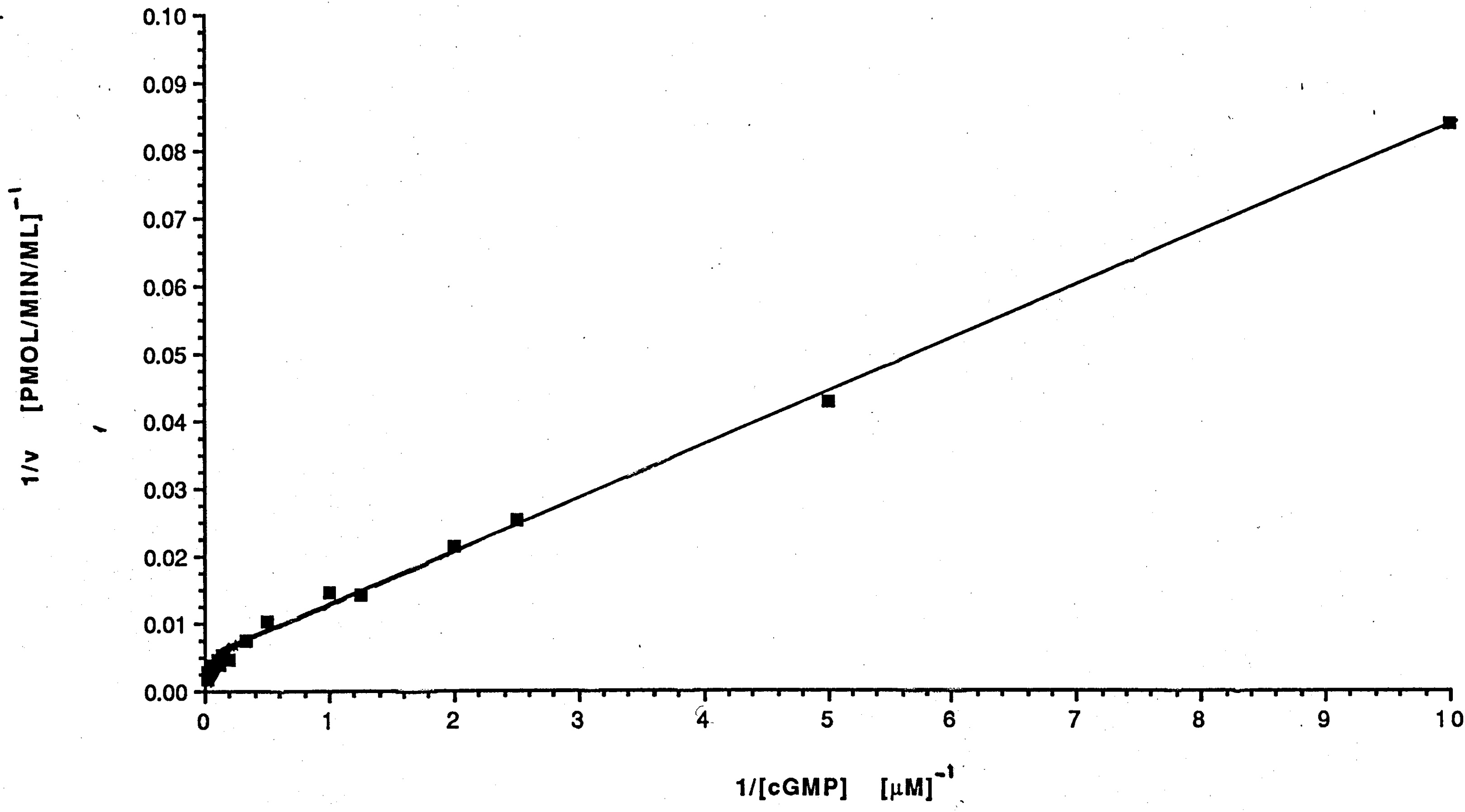


FIGURE 3.26(a):

EADIE-HOFSTEE PLOT FOR cGMP HYDROLYSIS BY PDE MQ-II.

Kinetic data were plotted as velocity (v) against v/[cGMP]. Estimates of the intercepts gave the following limiting values for K_m and V_{max}.

$K_{m1} = 1.6\mu M$, $K_{m2} = 11\mu M$

$V_{max1} = 200$ pmol/min/ml, $V_{max2} = 550$ pmol/min/ml

FIGURE 3.26(b):

HANES PLOT FOR cGMP HYDROLYSIS BY PDE MQ-II.

Kinetic data were plotted as [cGMP]/velocity(v) against [cGMP]. Estimates of the intercepts gave the following limiting values for K_m and V_{max}.

$K_{m1} = 1.2\mu M$, $K_{m2} = 8\mu M$

$V_{max1} = 171$ pmol/min/ml, $V_{max2} = 510$ pmol/min/ml.

FIGURE 3.26(a):

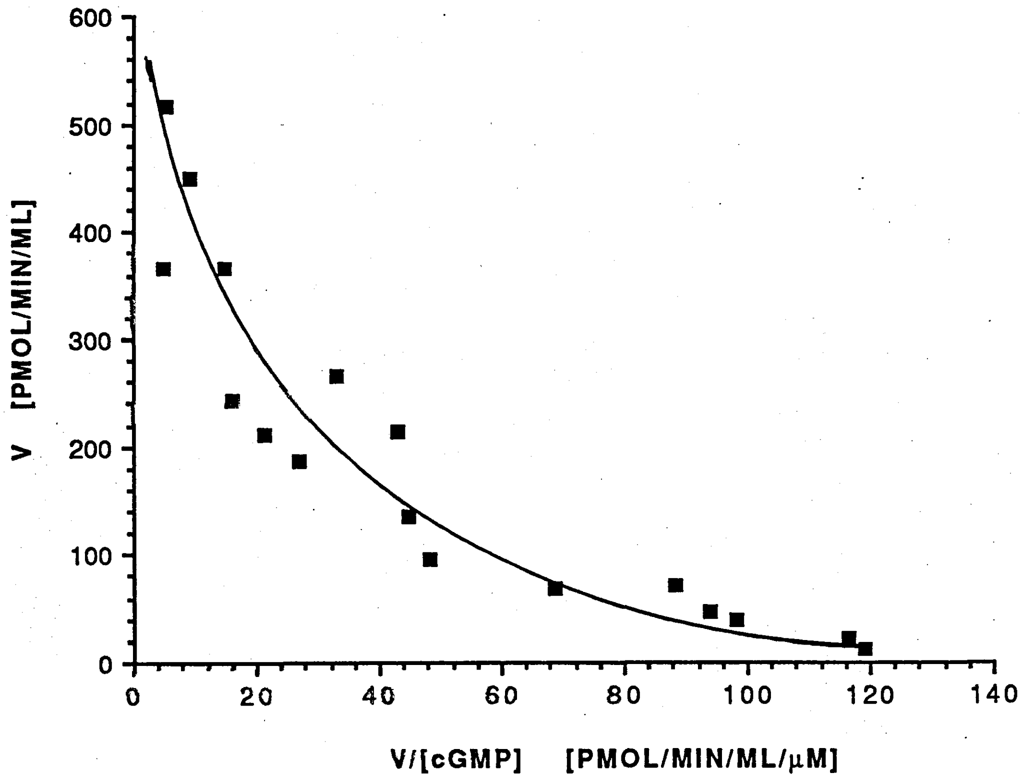


FIGURE 3.26(b):

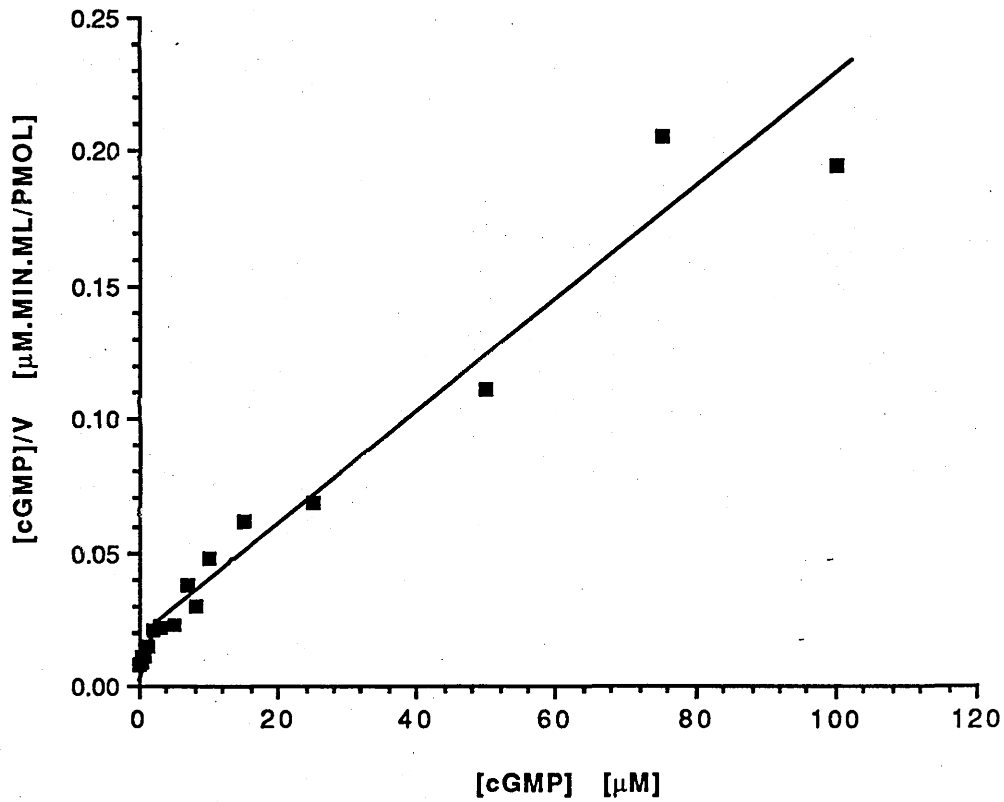
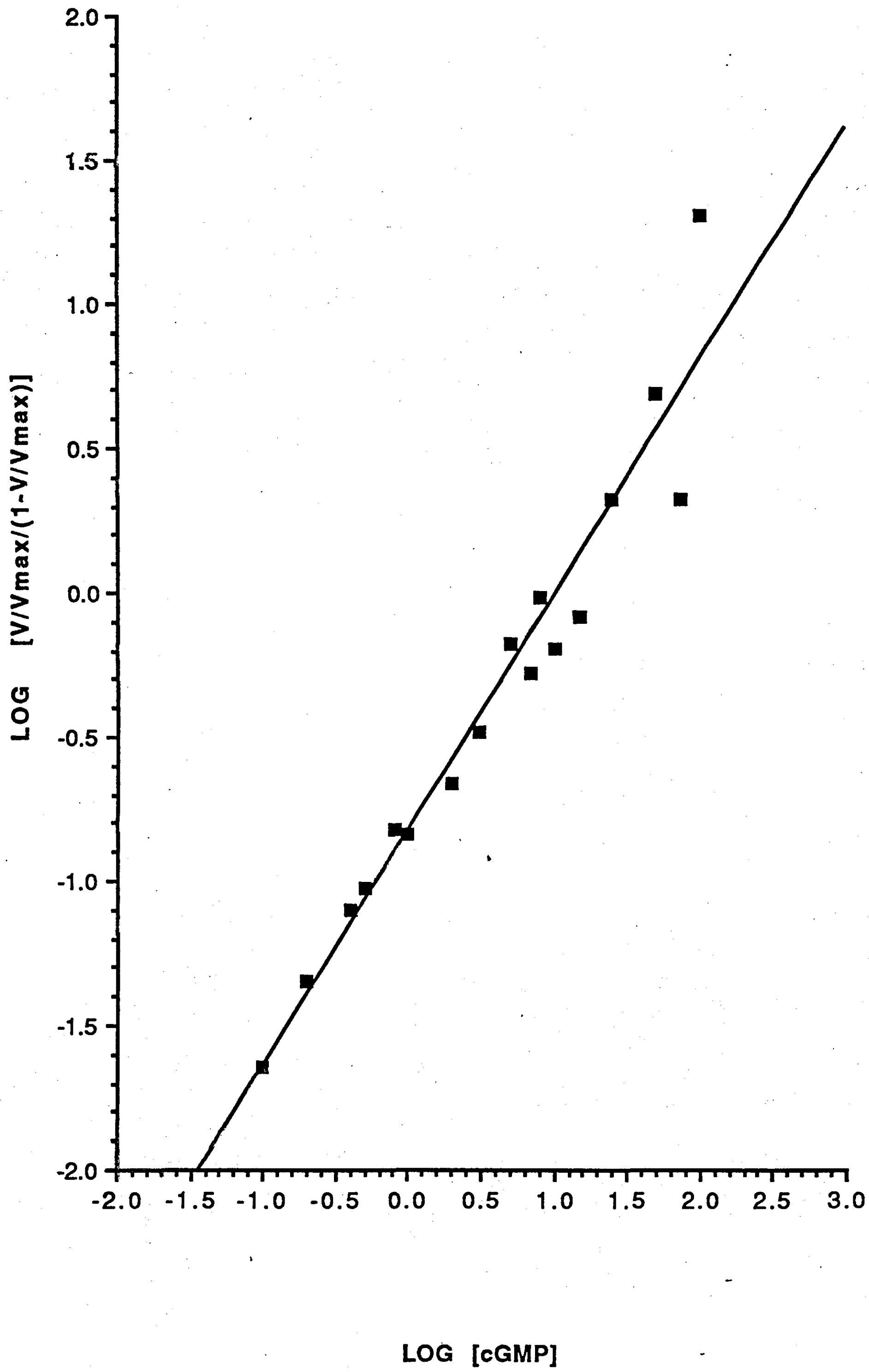


FIGURE 3.27:

HILL PLOT FOR CYCLIC GMP HYDROLYSIS BY PDE MQ-II.

Kinetic data were replotted as Hill plots using the V_{max2} values estimated from Lineweaver-Burk plots, Hanes plots and Eadie-Hofstee plots. The average V_{max2} value was calculated to be 541 pmol/min/ml ± 23 (SD). This value was used to determine the Hill plot. The slope of the Hill plot gives a value of 0.81 for the Hill Coefficient (h). The estimated K_m value from this plot was 7.94μM.

FIGURE 3.27:



LOG [cGMP]

FIGURE 3.28:

LINEWEAVER-BURK PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-III.

The cAMP phosphodiesterase activity of PDE MQ-III was assessed over a substrate range of 0.3μM-700μM. The velocity of the reaction was calculated as pmol of cyclic AMP hydrolysed/min/ml of enzyme preparation. Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. The inset shows the Lineweaver-Burk plot at the maximal levels of cAMP. From this it was estimated that the V_{max} value was 4,000 pmol/min/ml.

FIGURE 3.28:

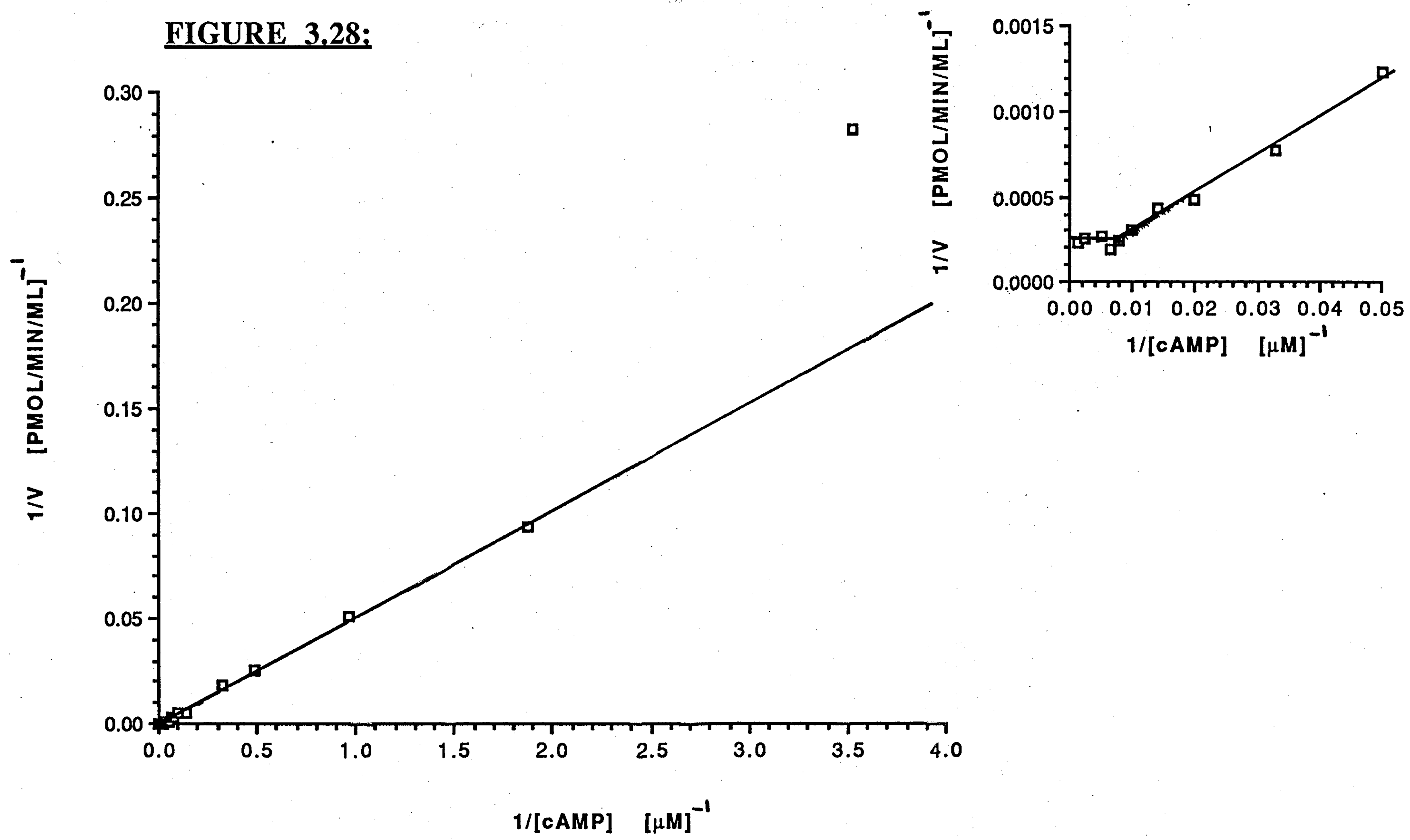


FIGURE 3.29(a) and (b):

HANES AND EADIE-HOFSTEE PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-III.

Kinetic data were plotted both as (a) [cAMP]/velocity (v) against [cAMP] (Hanes plot) and as (b) velocity (v) against v/[cAMP] (Eadie-Hofstee plot).

FIGURE 3.29(a):

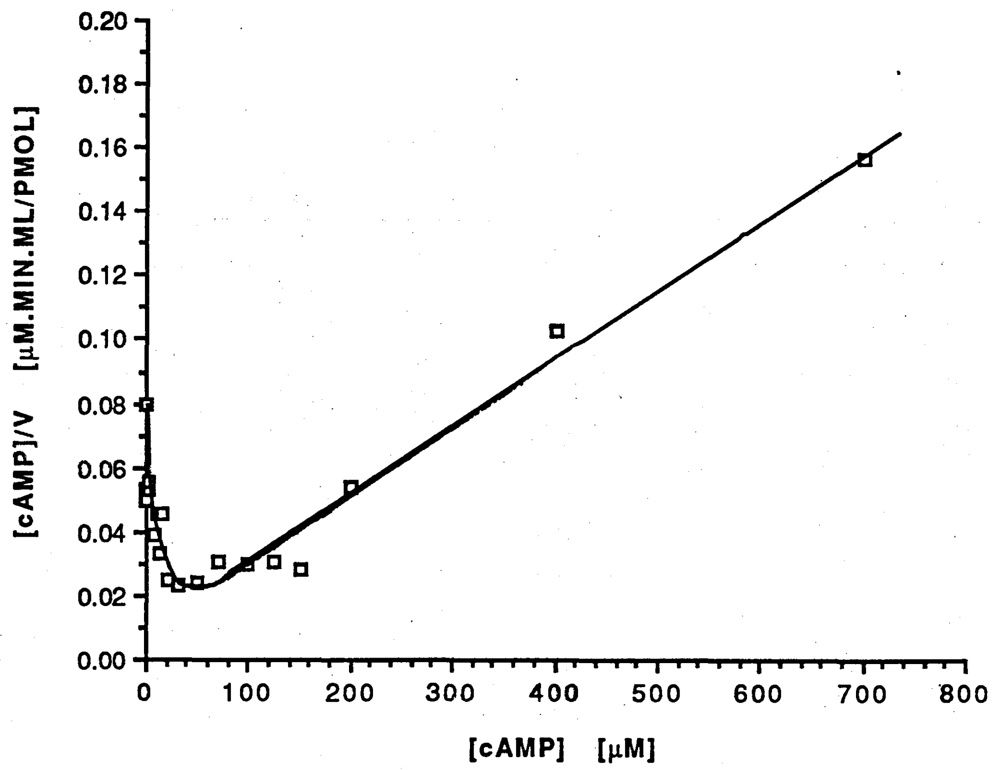


FIGURE 3.29(b):

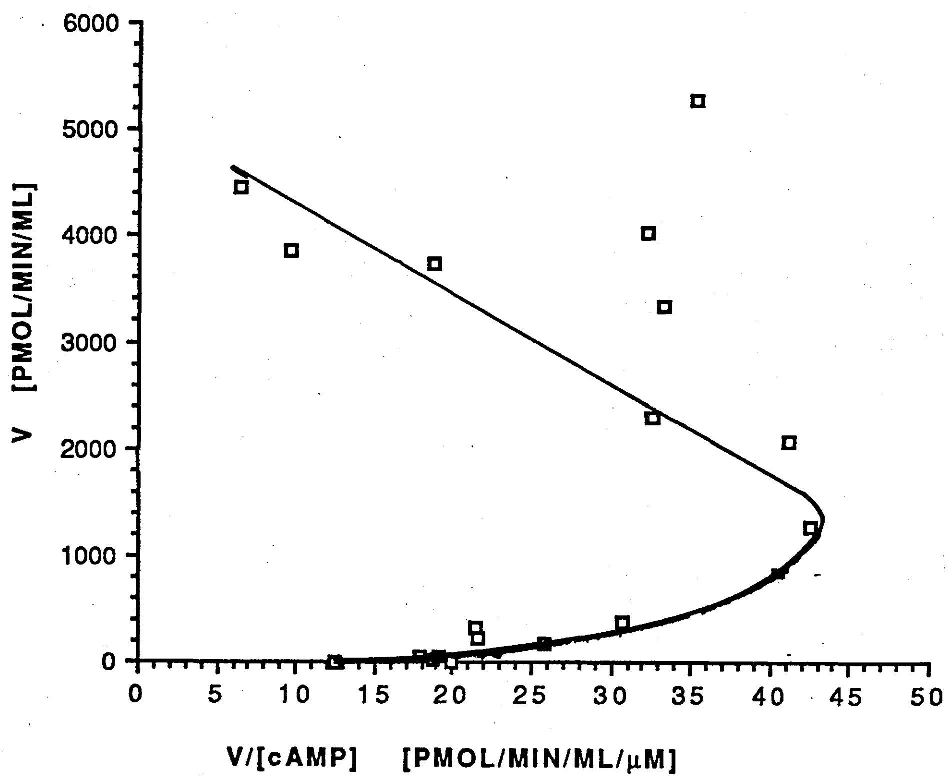


FIGURE 3.30:

HILL PLOT ANALYSIS FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-III.

Kinetic data were replotted as Hill plots using a V_{max} value of 4000 pmol/min/ml estimated both from Lineweaver-Burk plots, and a Velocity versus Log [cAMP] plot. The slope of the Hill plot gives a value of 1.44 for the Hill Coefficient (h). The estimated K_m value from this plot was 50μM.

FIGURE 3.30:

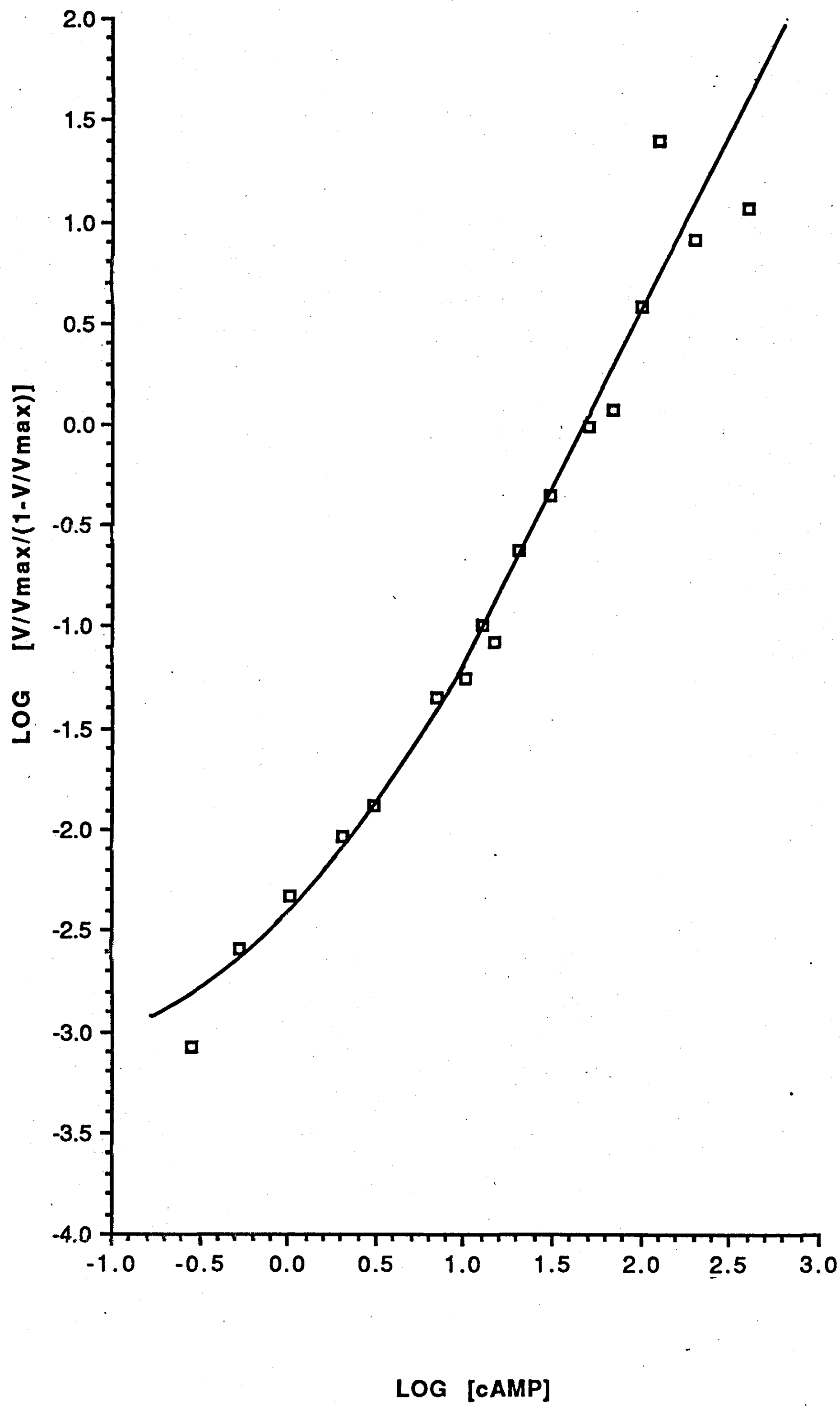


FIGURE 3.31:

LINEWEAVER-BURK PLOT FOR CYCLIC GMP HYDROLYSIS BY PDE MQ-III.

The cGMP phosphodiesterase activity of PDE MQ-III was assessed over a substrate range of 0.13μM-200μM. The velocity of the reaction was calculated as pmol of cyclic GMP hydrolysed/min/ml of enzyme preparation. Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. The inset shows the Lineweaver Burk plot at the maximal levels of cGMP. From this it was estimated that the V_{max} value was 8,500 pmol/min/ml.

FIGURE 3.31:

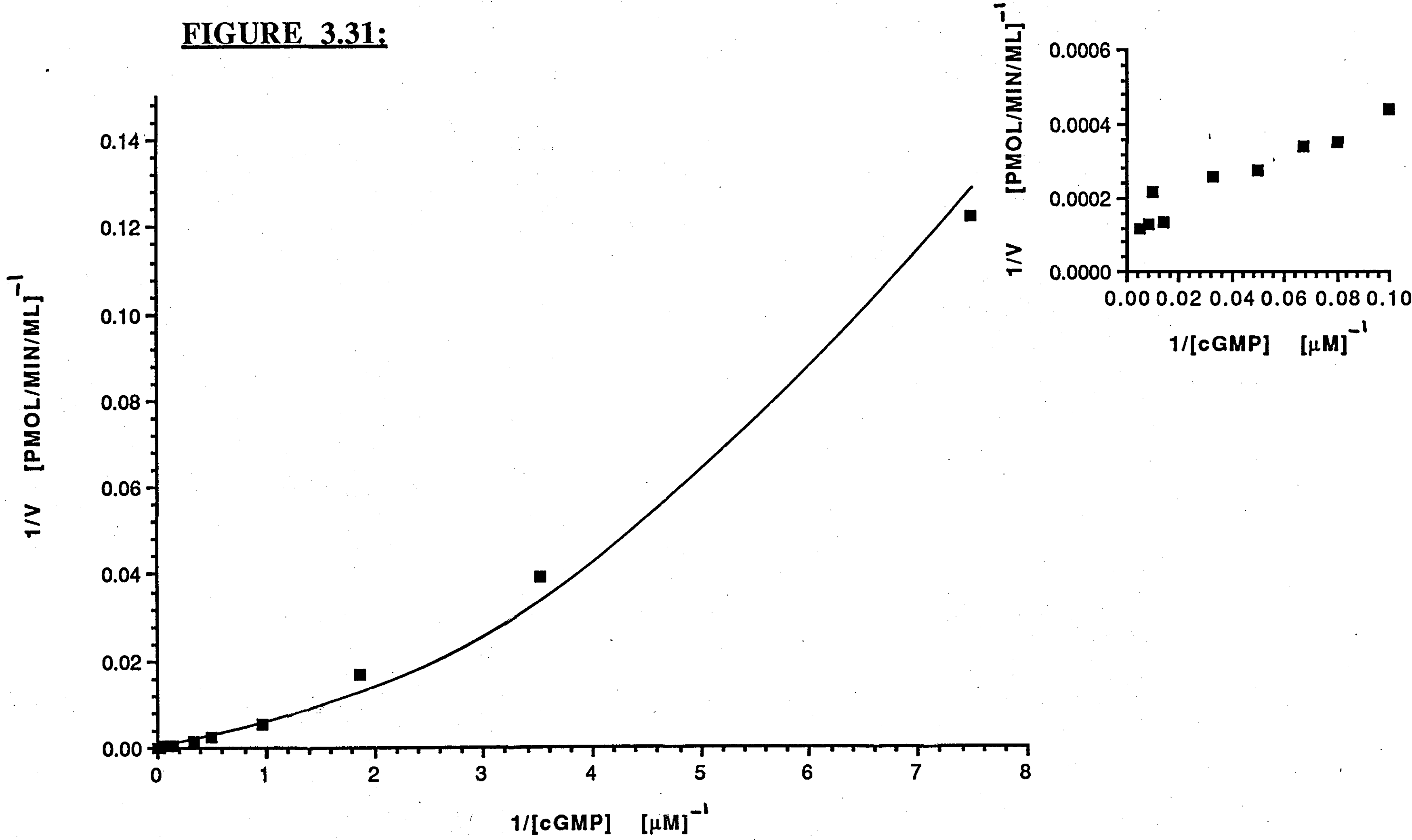


FIGURE 3.32(a) and (b):

HANES AND EADIE-HOFSTEE PLOT FOR CYCLIC GMP HYDROLYSIS BY PDE MQ-III.

Kinetic data were plotted both as (a) [cGMP]/velocity (v) against [cGMP] (Hanes plot) and as (b) velocity (v) against v/[cGMP] (Eadie-Hofstee plot).

FIGURE 3.32(a):

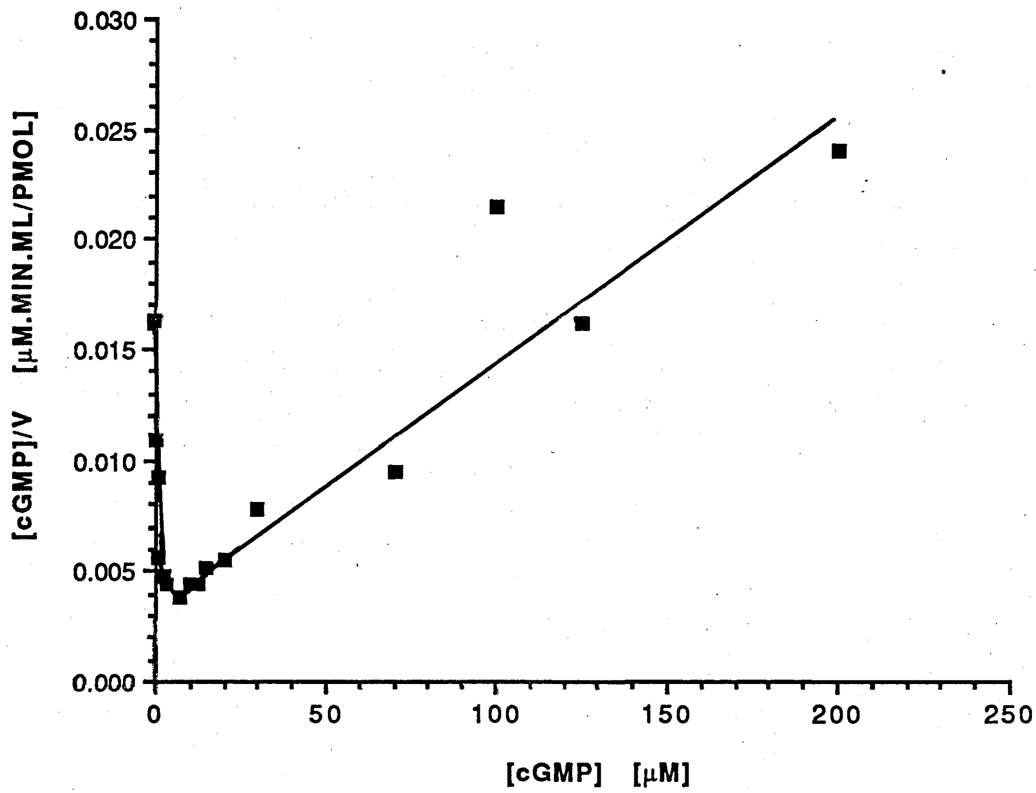


FIGURE 3.32(b):

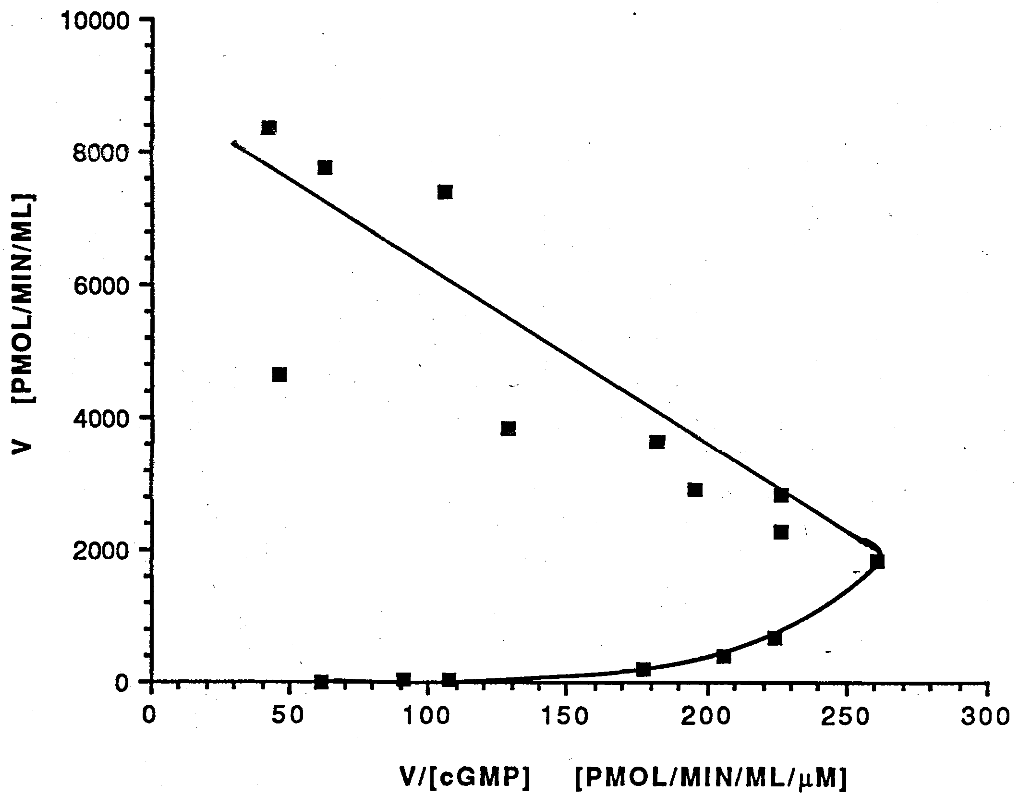


FIGURE 3.33:

HILL PLOT FOR CYCLIC GMP HYDROLYSIS BY PDE MQ-III.

Kinetic data were replotted as Hill plots using a V_{max} value of 4,000 pmol/min/ml estimated both from Lineweaver-Burk plots, and a Velocity versus Log [cGMP] plot. The slope of the Hill plot gives a value of 1.22 for the Hill Coefficient (h). The estimated K_m value from this plot was 32μM.

FIGURE 3.33:

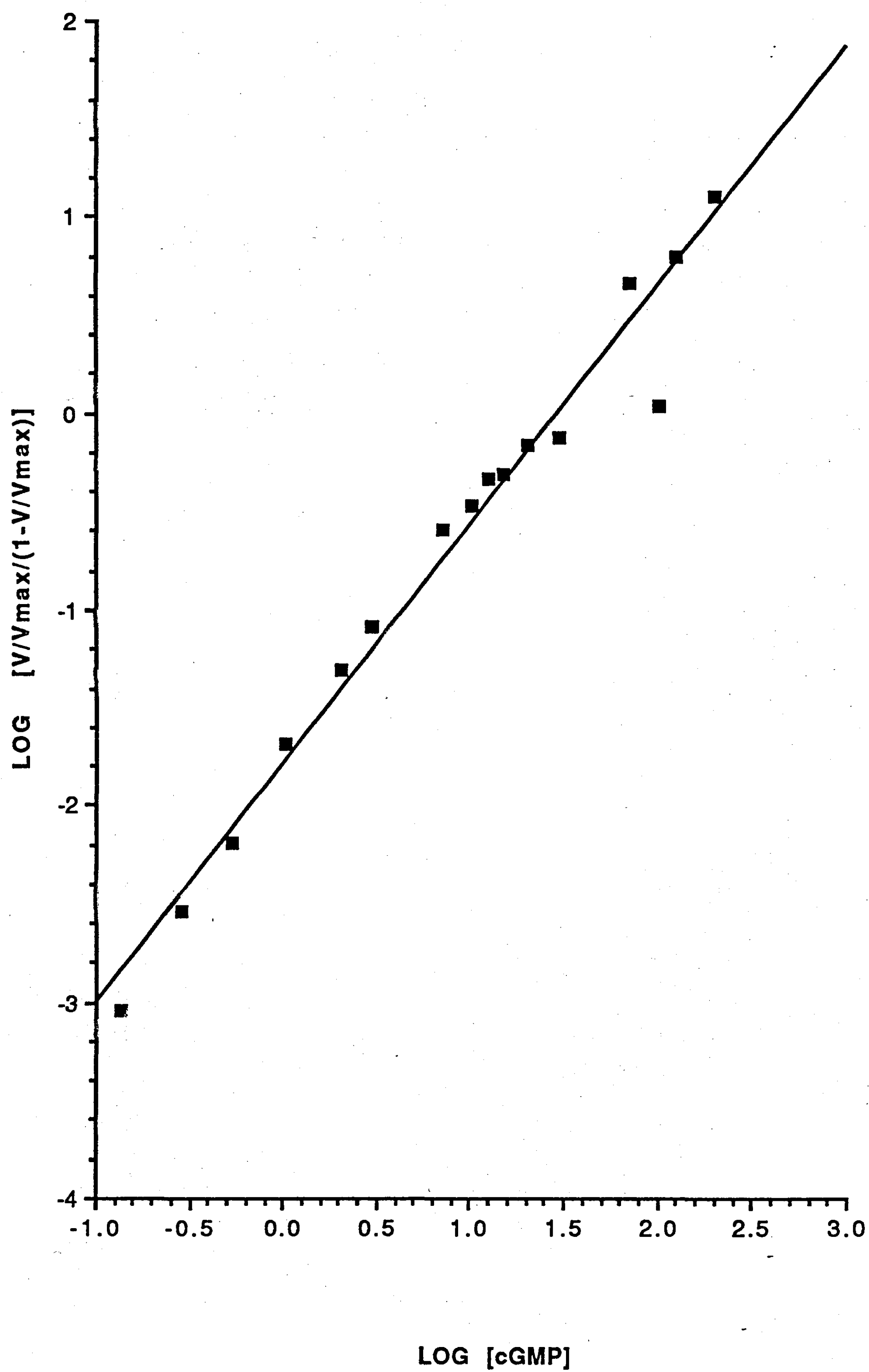


FIGURE 3.34:

LINEWEAVER-BURK PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-IV.

The cyclic AMP phosphodiesterase activity of PDE MQ-IV was assessed over a substrate range of 0.15μM-100μM. The velocity of the reaction was calculated as pmol of cyclic GMP hydrolysed/min/ml of enzyme preparation. Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. The limiting K_m and V_{max} values estimated from this plot were, K_{m1} = 0.95μM, K_{m2} = 6.67μM, V_{max1} = 155 pmol/min/ml and V_{max2} = 261 pmol/min/ml.

FIGURE 3.34:

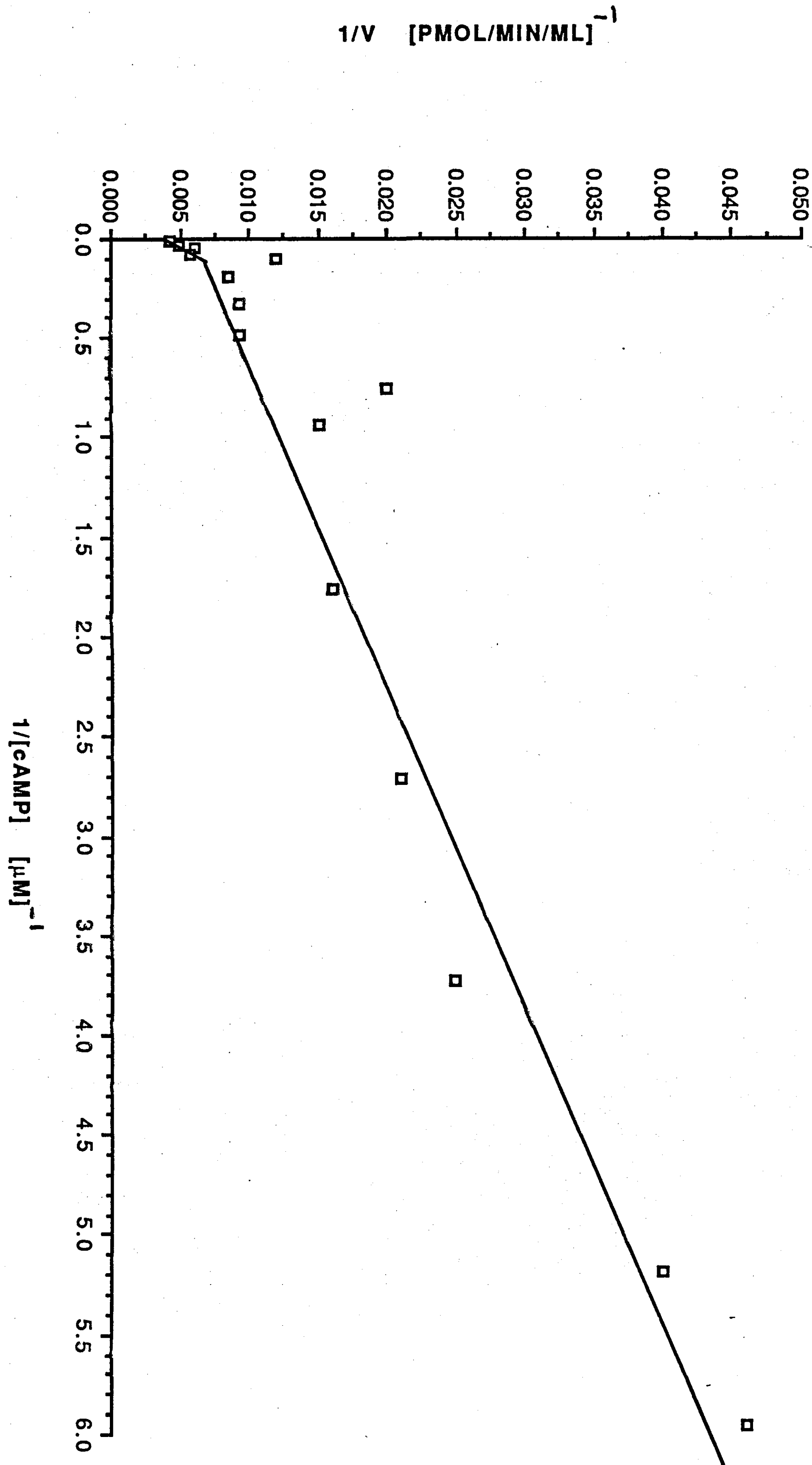


FIGURE 3.35(a):

EADIE-HOFSTEE PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-IV.

Kinetic data were plotted as velocity (v) against v/[cAMP]. Estimates of the intercepts gave the following limiting values for K_m and V_{max};

$K_{m1} = 0.7\mu M$, $K_{m2} = 7.2\mu M$

$V_{max1} = 129$ pmol/min/ml, $V_{max2} = 240$ pmol/min/ml

FIGURE 3.35(b):

HANES PLOT FOR cAMP HYDROLYSIS BY PDE MQ-IV.

Kinetic data were plotted as [cAMP]/velocity(v) against [cAMP]. Estimates of the intercepts gave the following limiting values for K_m and V_{max};
$K_{m1} = 0.8\mu M$, $K_{m2} = 8\mu M$

$V_{max1} = 145$ pmol/min/ml, $V_{max2} = 266$ pmol/min/ml.

FIGURE 3.35(a):

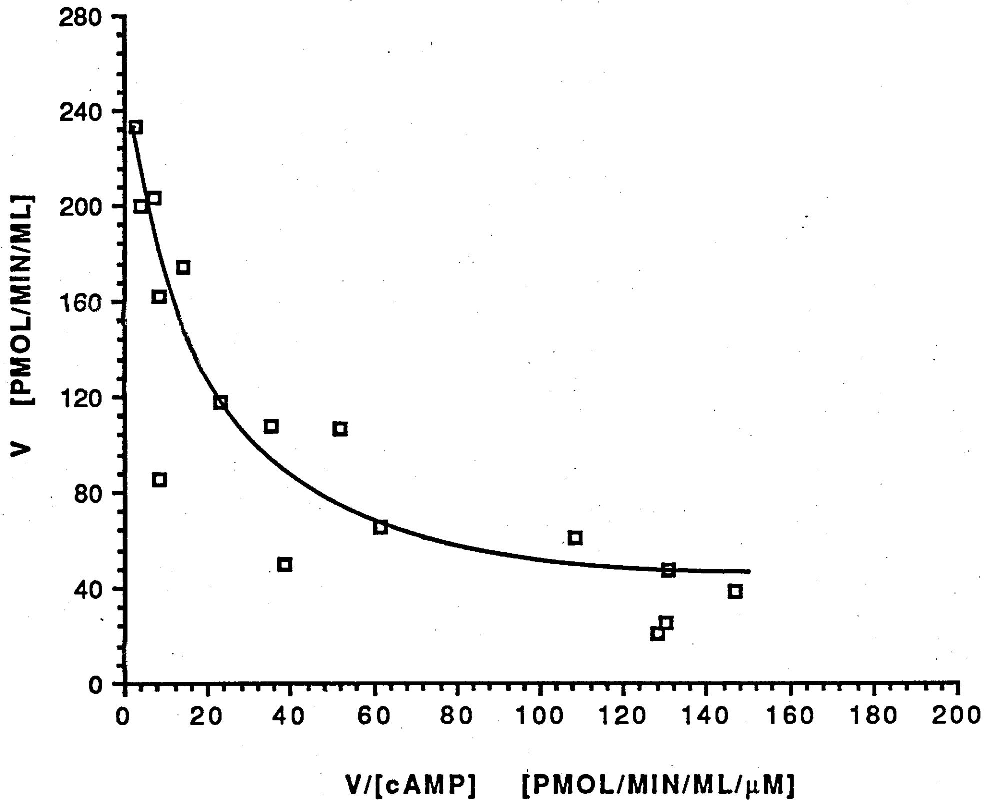


FIGURE 3.35(b):

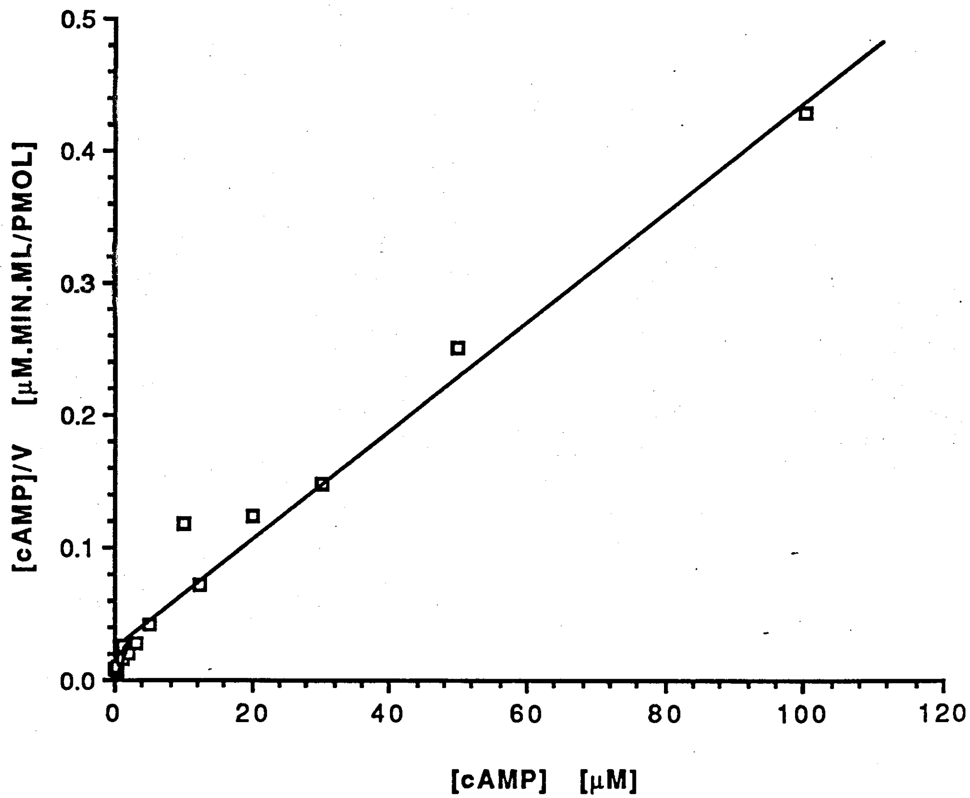


FIGURE 3.36:

HILL PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-IV.

Kinetic data were replotted as Hill plots using a value of the V_{max2} estimated from Lineweaver-Burk plots, Hanes plots and Eadie-Hofstee plots. The average V_{max2} value was calculated to be 270 pmol/min/ml. This value was used to determine the Hill plot. The slope of the Hill plot gives a value of 0.59 for the Hill Coefficient (h). The estimated K_m value from this plot was 7.1μM.

FIGURE 3.36:

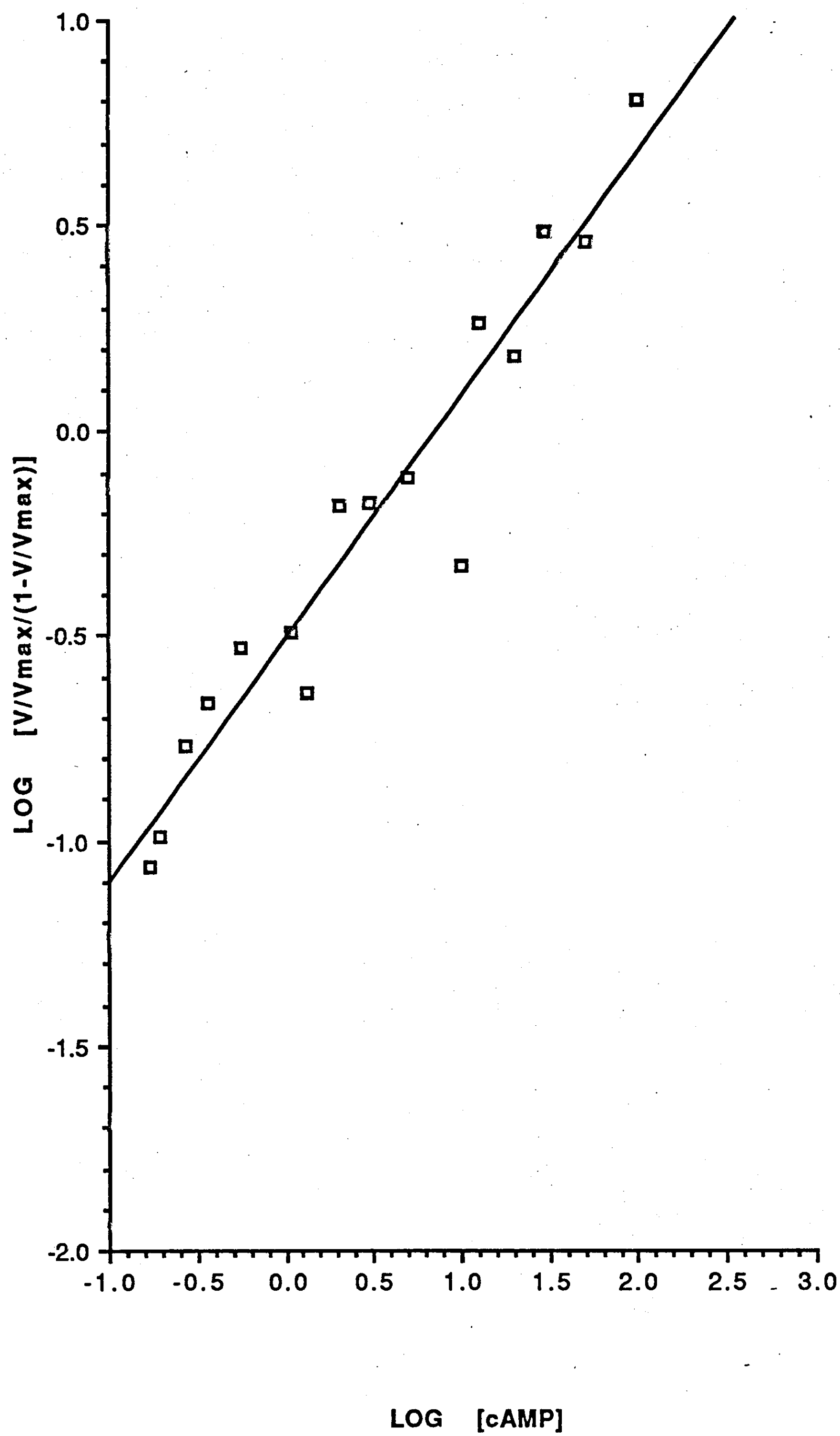


FIGURE 3.37:

LINEWEAVER-BURK PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-V.

The cyclic AMP phosphodiesterase activity was assessed over a substrate range of 0.064µM-20µM. The velocity of the reaction was calculated as pmol of cyclic AMP hydrolysed/min/ml of enzyme preparation. Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration. The line was fitted by least squares analysis. The estimated V_{max} and K_m values determined from such a plot were 152 pmol/min/ml and 0.63µM respectively.

FIGURE 3.37:

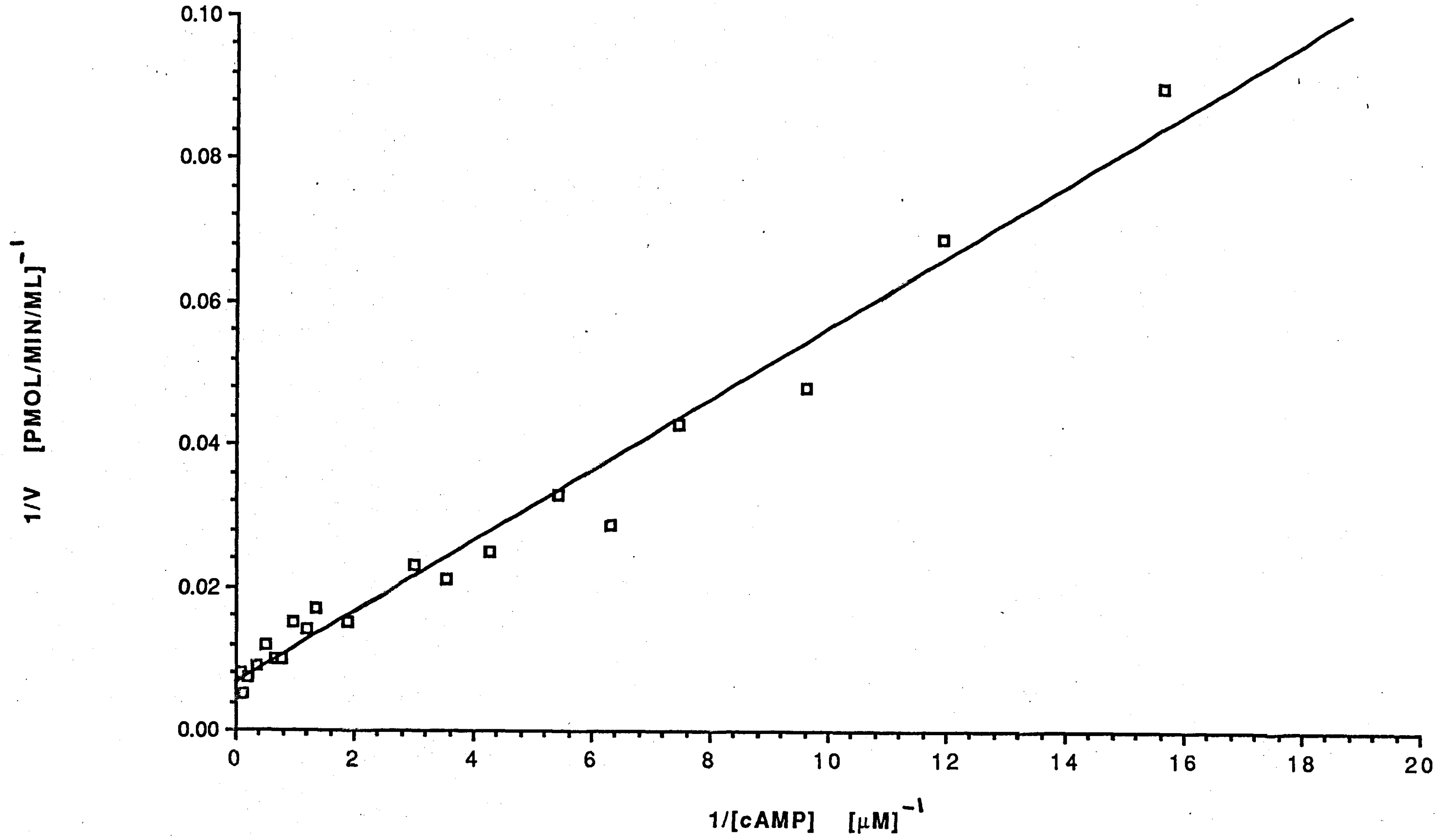


FIGURE 3.38:

HILL PLOT FOR CYCLIC AMP HYDROLYSIS BY PDE MQ-V.

Kinetic data were replotted as Hill plots using a value of the V_{max} estimated from Lineweaver-Burk plots, Hanes plots and Eadie-Hofstee plots. The average V_{max} value was calculated to be 156 pmol/min/ml. This value was used to determine the Hill plot. The slope of the Hill plot gives a value of 0.80 for the Hill Coefficient (h). The estimated K_m value from this plot was 1μM.

FIGURE 3.38:

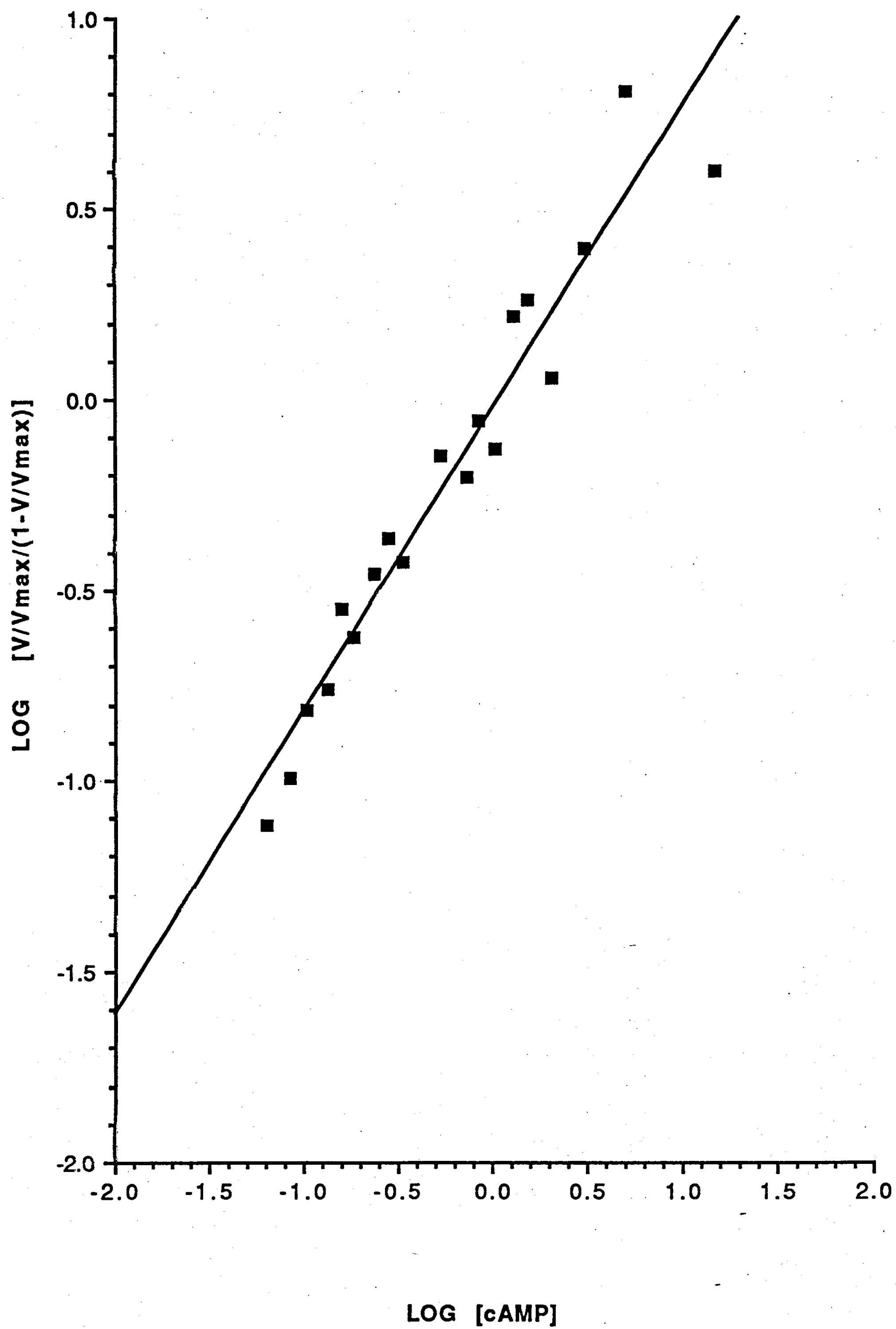


FIGURE 3.39

EFFECT OF MAGNESIUM ON PDE MQ-I AND PDE MQ-II CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY.

The cAMP phosphodiesterase activity of PDE MQ-I (□) and cGMP phosphodiesterase activity of PDE MQ-II (●) were assayed at 1μM substrate in the absence and presence of increasing concentrations of magnesium. Results are expressed as the % of maximal phosphodiesterase activity observed ± SD for triplicates. The basal activities were:

PDE MQ-I 59 pmol/min/ml

PDE MQ-II 18.55 pmol/min/ml.

EFFECT OF EDTA ON THE cAMP PHOSPHODIESTERASE ACTIVITY OF PDE MQ-I.

The cyclic AMP phosphodiesterase activity of PDE MQ-I was assayed at 1μM substrate in the absence and presence of increasing concentrations of EDTA (■). Results are expressed as % of control activity (100%) ± SD for triplicates. The control activity was 54.3 pmol/min/ml.

FIGURE 3.39

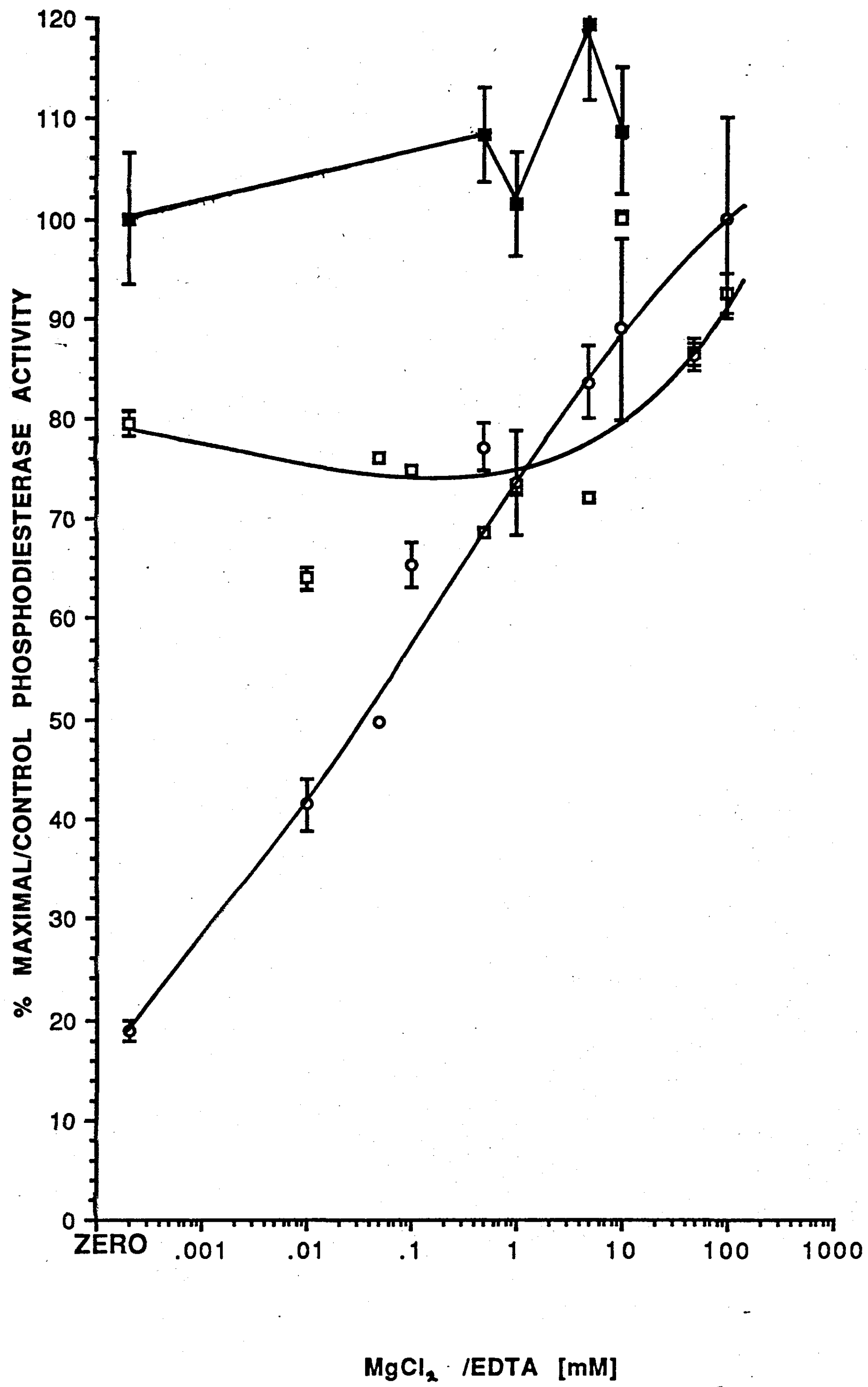


FIGURE 3.40

EFFECT OF MAGNESIUM ON PDE MQ-III AND PDE MQ-IV CYCLIC AMP PHOSPHODIESTERASE ACTIVITY.

The cAMP phosphodiesterase activity of PDE MQ-IV (□) and cGMP phosphodiesterase activity of PDE MQ-III (■) were assayed at 1μM substrate in the absence and presence of increasing concentrations of magnesium. Results are expressed as the % of maximal phosphodiesterase activity observed ± SD for triplicates. The basal activities were:

PDE MQ-III 31 pmol/min/ml.

PDE MQ-IV 0 pmol/min/ml.

FIGURE 3.40

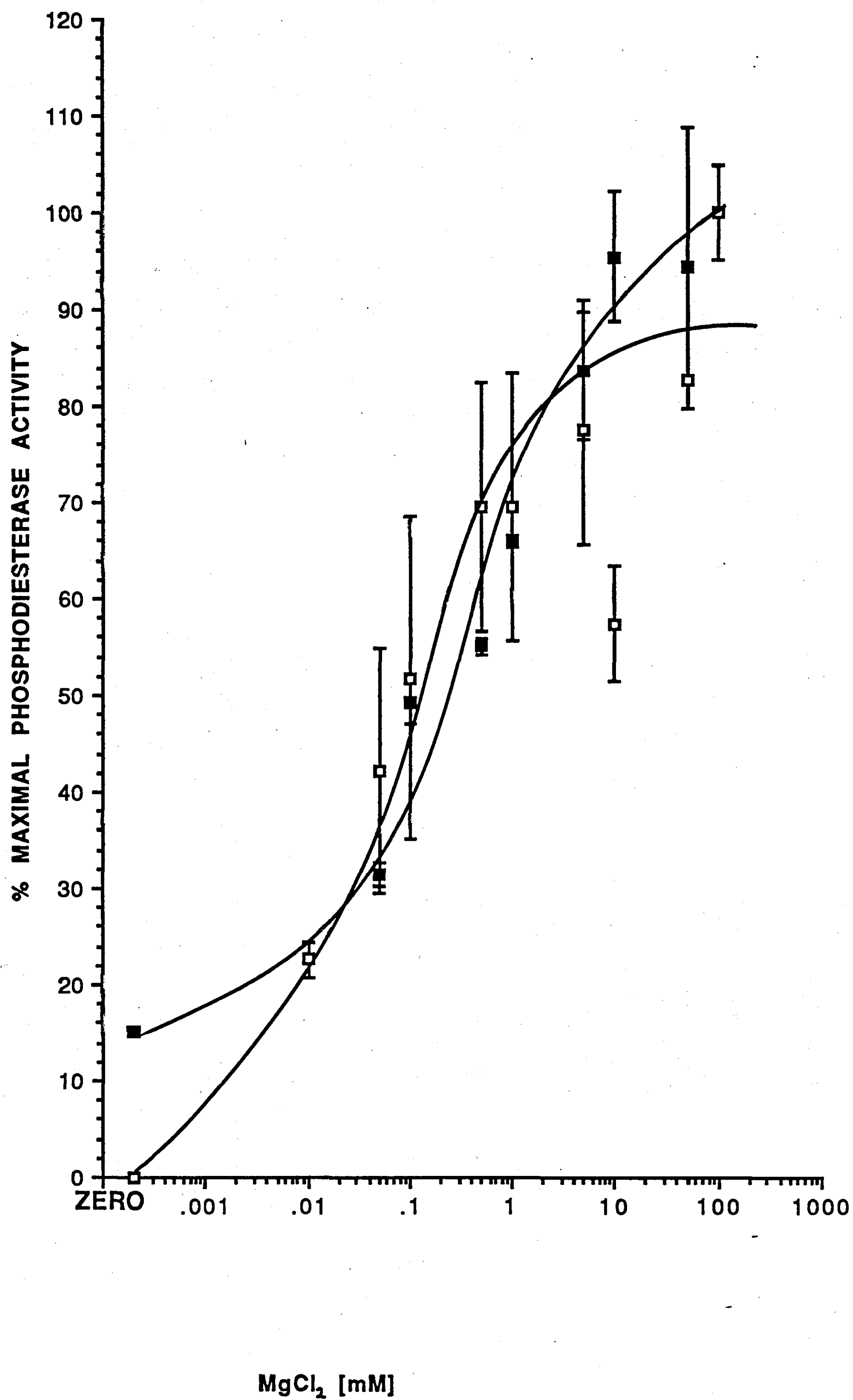


FIGURE 3.41

EFFECT OF MAGNESIUM ON PDE MQ-V CYCLIC AMP PHOSPHODIESTERASE ACTIVITY.

The cAMP phosphodiesterase activity of PDE MQ-V was assayed at 1μM substrate in the absence and presence of increasing concentrations of magnesium. The basal activity was 7.9 pmol/min/ml. Results are expressed as the % of maximal phosphodiesterase activity observed ± SD for triplicates.

FIGURE 3.41

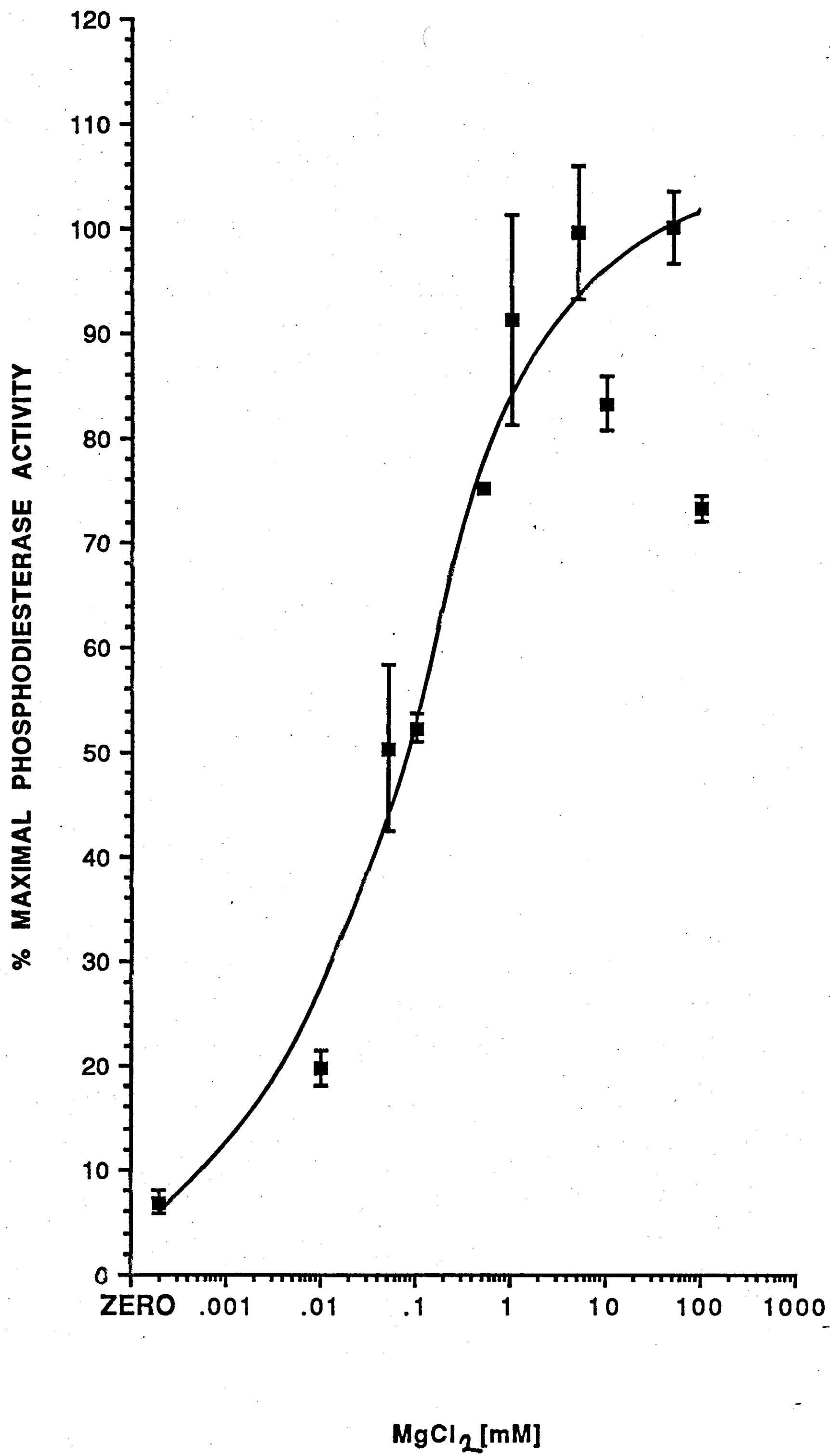


FIGURE 3.42:

INHIBITION OF RAT HEPATOCYTE PDE MQ-I BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-I was assessed in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.42:

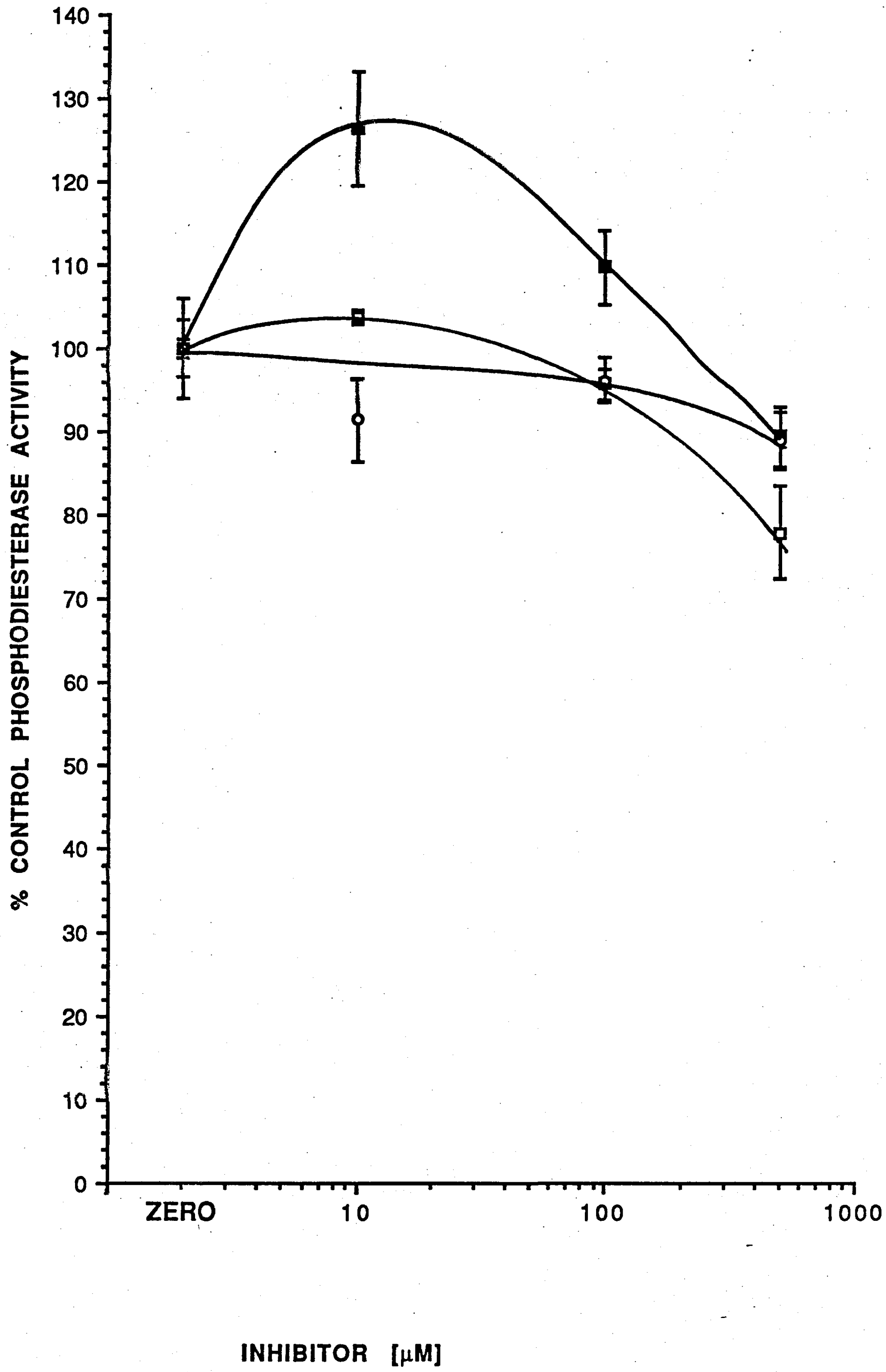


FIGURE 3.43:

INHIBITION OF RAT HEPATOCYTE PDE MQ-I BY ZAPRINAST, ROLIPRAM AND ICI 118233.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-I was assessed in the absence and presence of the indicated concentrations of the inhibitors Zaprinast, Rolipram and ICI 118233. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

Zaprinast (■).

Rolipram (□).

ICI 118233 (o).

FIGURE 3.43:

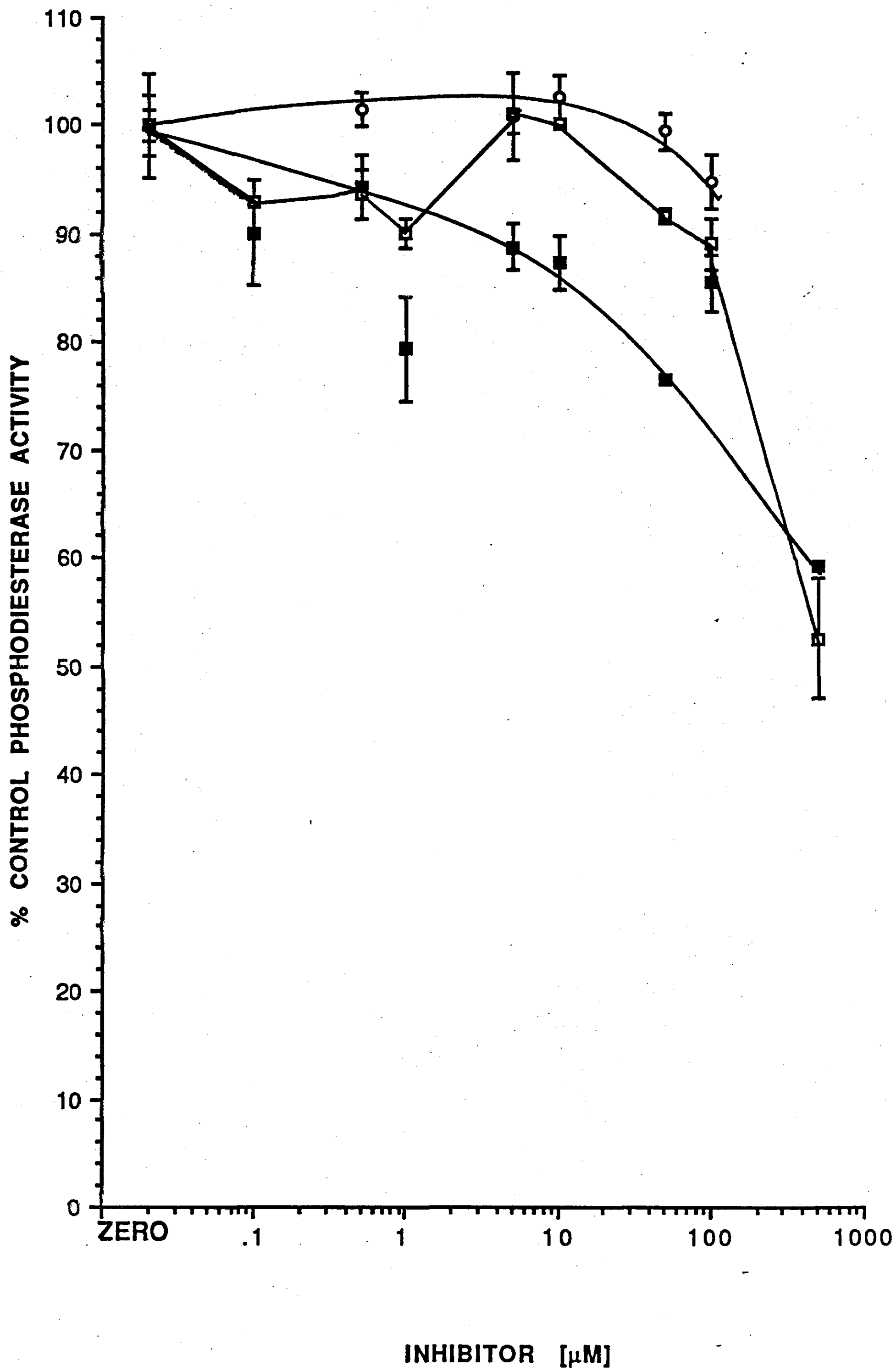


FIGURE 3.44:

INHIBITION OF RAT HEPATOCYTE PDE MQ-II BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-II was assessed in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.44:

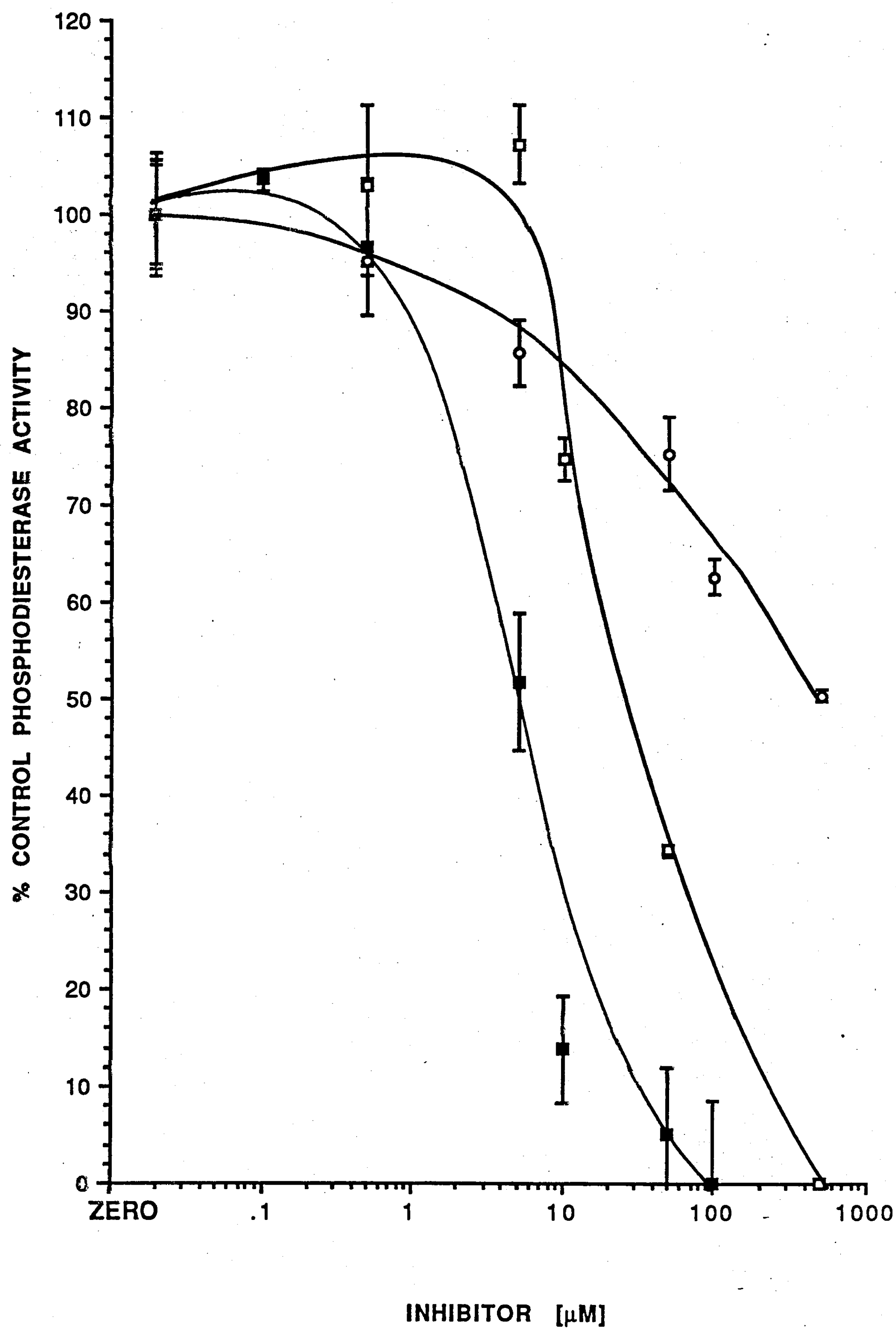


FIGURE 3.45:

INHIBITION OF RAT HEPATOCYTE PDE MQ-II BY ZAPRINAST, ROLIPRAM AND ICI 118233.

The cGMP phosphodiesterase activity of hepatocyte-derived PDE MQ-II was assessed in the absence and presence of the indicated concentrations of the inhibitors Zaprinast, Rolipram and ICI 118233. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

Zaprinast (■).

Rolipram (□).

ICI 118233 (o).

FIGURE 3.45:

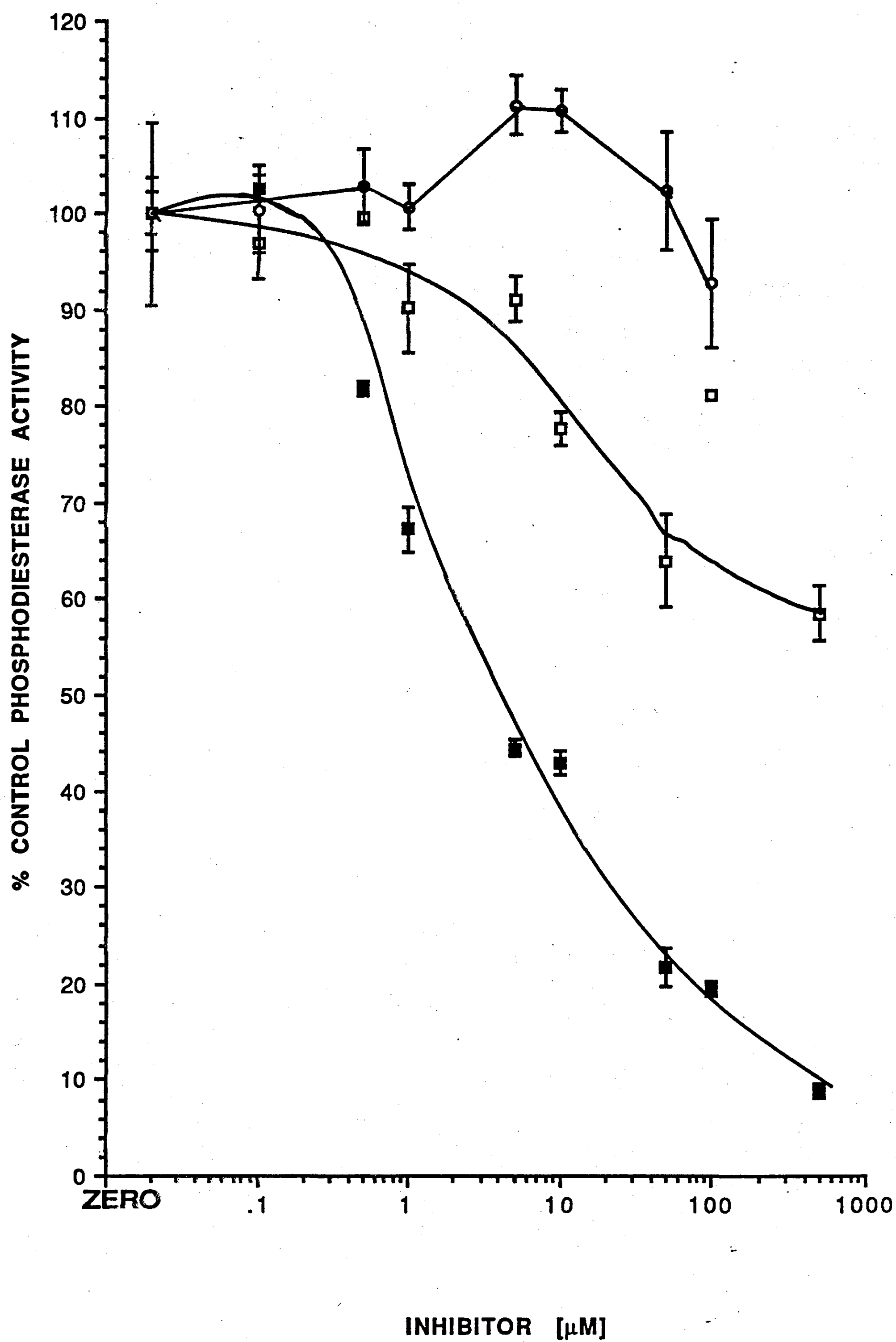


FIGURE 3.46:

INHIBITION OF RAT HEPATOCYTE PDE MQ-III BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-III was assessed in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.46:

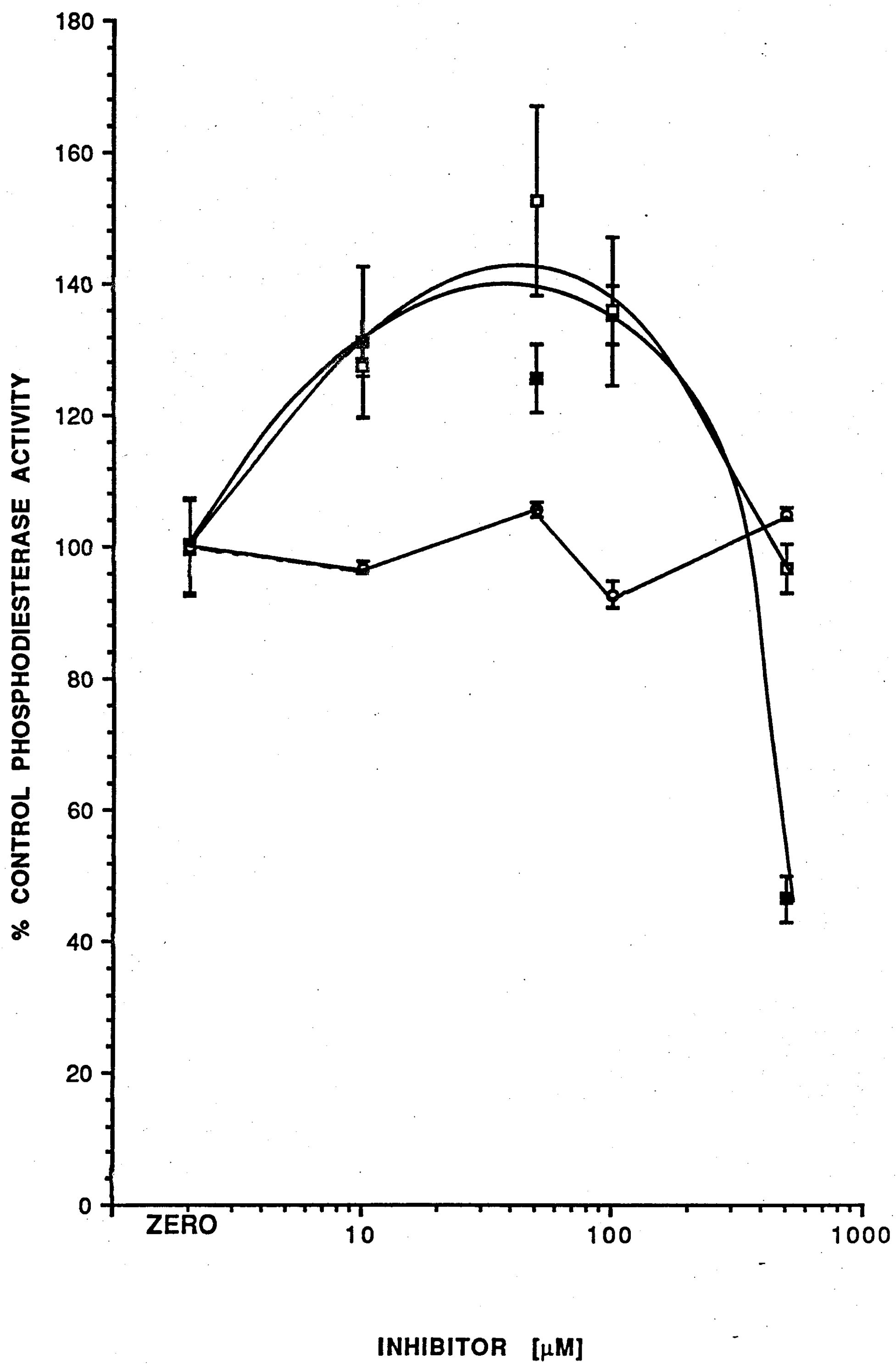


FIGURE 3.47:

INHIBITION OF RAT HEPATOCYTE PDE MQ-III BY ZAPRINAST, ROLIPRAM AND ICI 118233.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-III was assessed in the absence and presence of the indicated concentrations of the inhibitors, Zaprinast, Rolipram and ICI 118233. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

Zaprinast (■).

Rolipram (□).

ICI 118233 (o).

FIGURE 3.47:

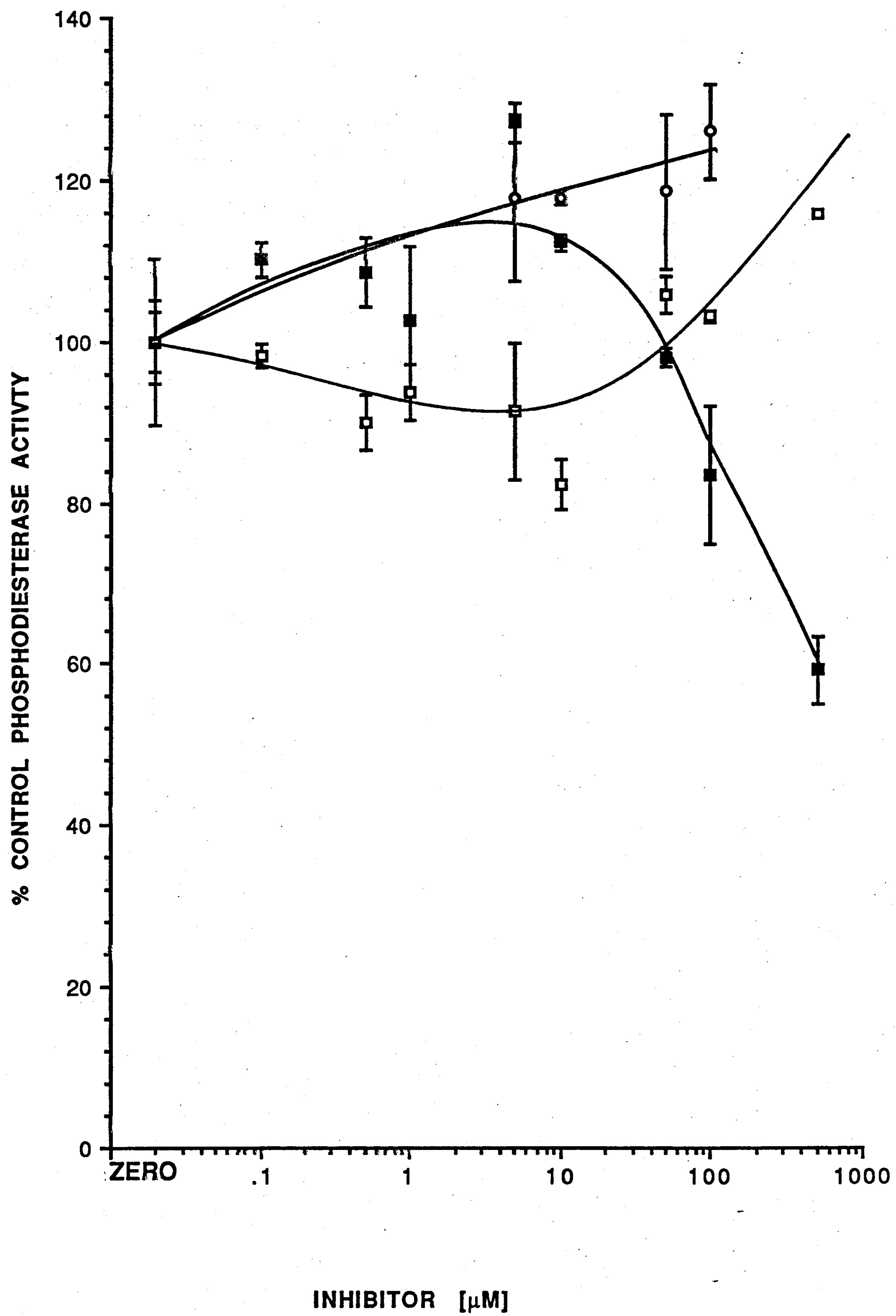


FIGURE 3.48:

INHIBITION OF RAT HEPATOCYTE PDE MQ-IV BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-IV was assessed in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.48:

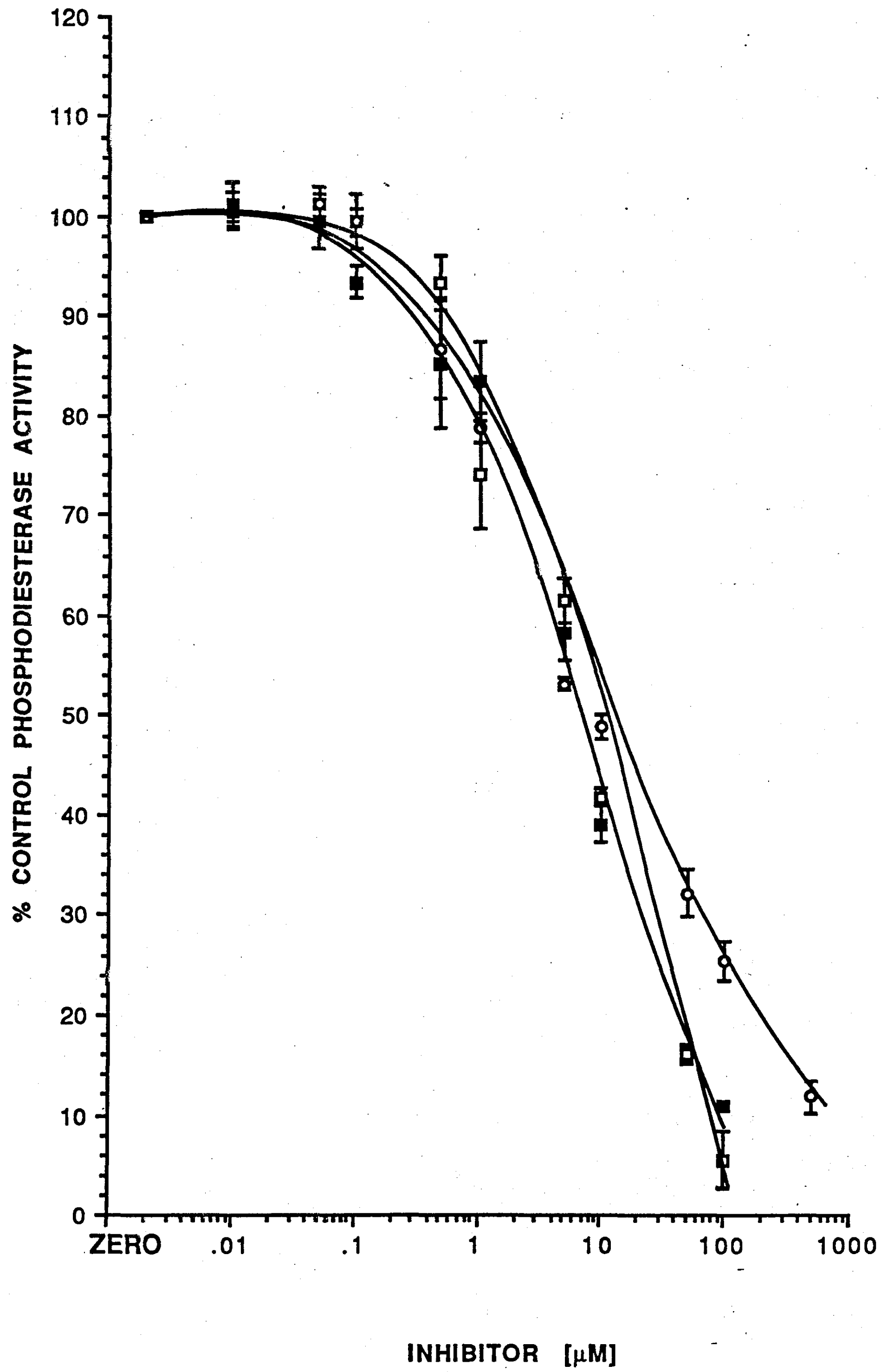


FIGURE 3.49:

INHIBITION OF RAT HEPATOCYTE PDE MQ-IV BY ZAPRINAST, ROLIPRAM AND ICI 118233.

The cAMP phosphodiesterase activity of hepatocyte derived PDE MQ-IV was assessed in the absence and presence of the indicated concentrations of the inhibitors Zaprinast, Rolipram and ICI 118233. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

Zaprinast (■).

Rolipram (□).

ICI 118233 (○).

FIGURE 3.49:

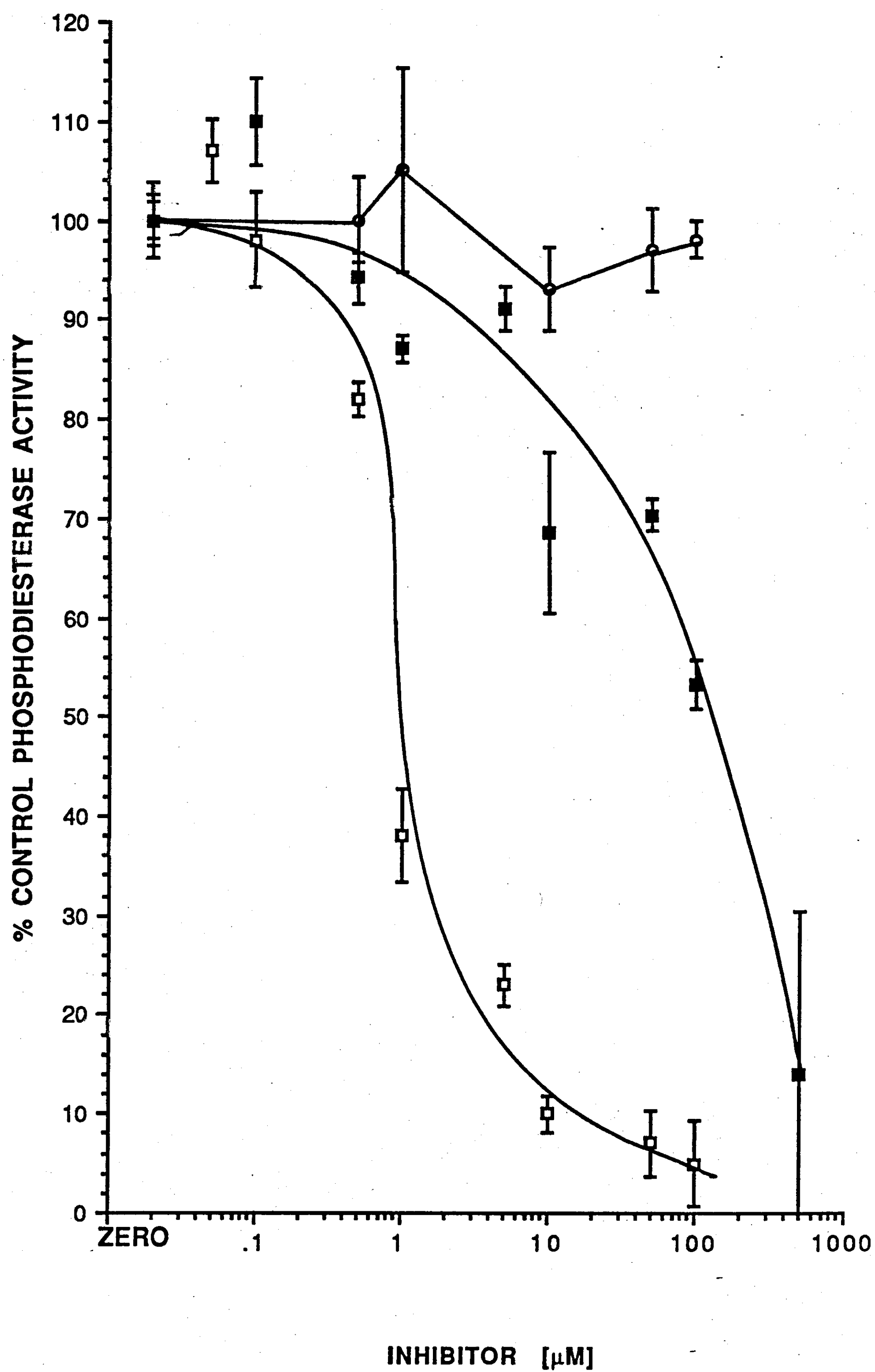


FIGURE 3.50:

INHIBITION OF RAT HEPATOCYTE PDE MQ-V BY IBMX, MILRINONE AND Ro-20-1724.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-V was assessed in the absence and presence of the indicated concentrations of the inhibitors IBMX, Milrinone and Ro-20-1724. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

IBMX (■).

Milrinone (□).

Ro-20-1724 (○).

FIGURE 3.50:

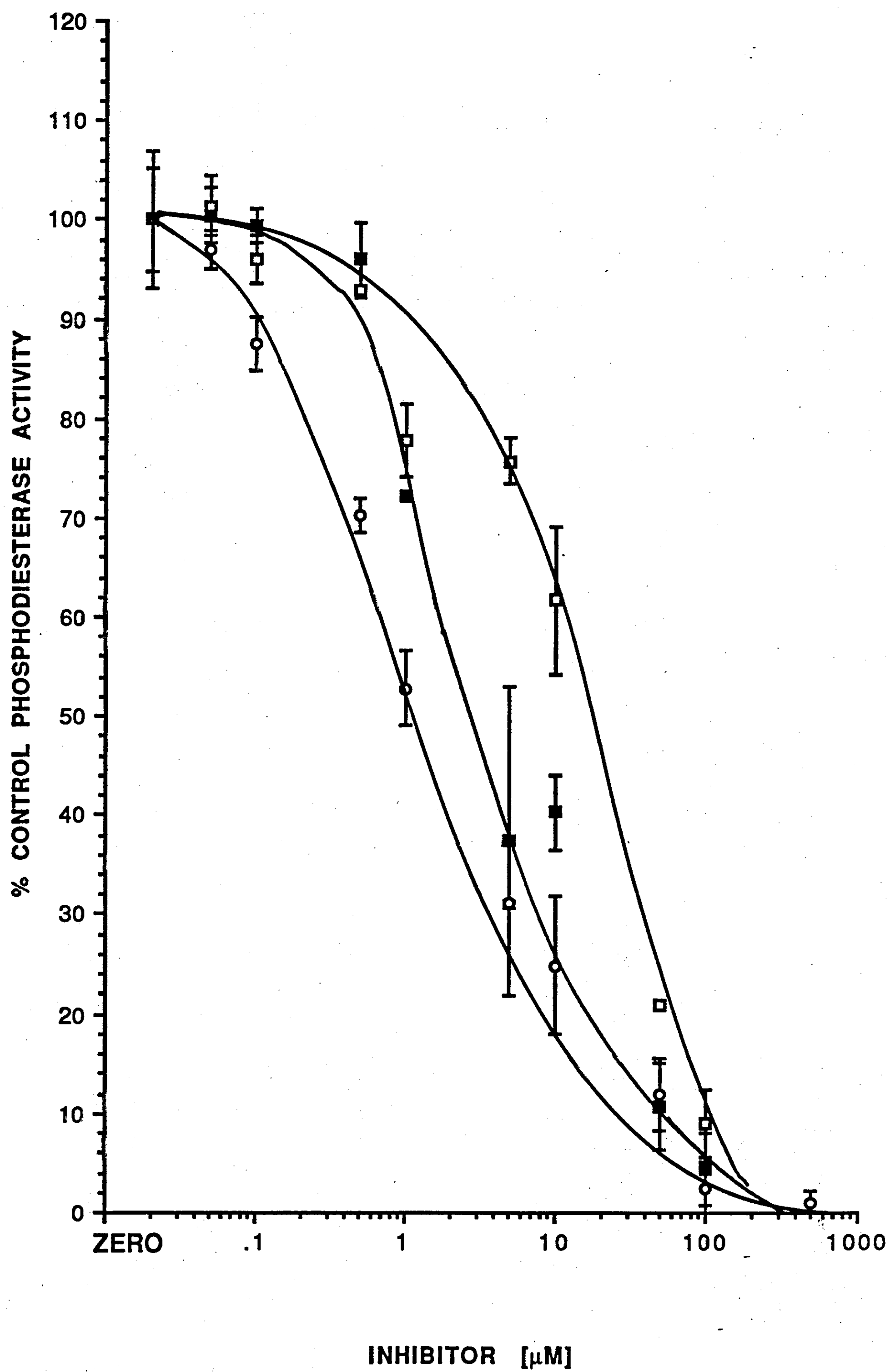


FIGURE 3.51:

INHIBITION OF RAT HEPATOCYTE PDE MQ-V BY ZAPRINAST, ROLIPRAM AND ICI 118233.

The cAMP phosphodiesterase activity of hepatocyte-derived PDE MQ-V was assessed in the absence and presence of the indicated concentrations of the inhibitors Zaprinast, Rolipram and ICI 118233. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-X). Results are expressed as the % of control activities (100%) ± SD for triplicate measurements.

Zaprinast (■).

Rolipram (□).

ICI 118233 (○).

FIGURE 3.51:

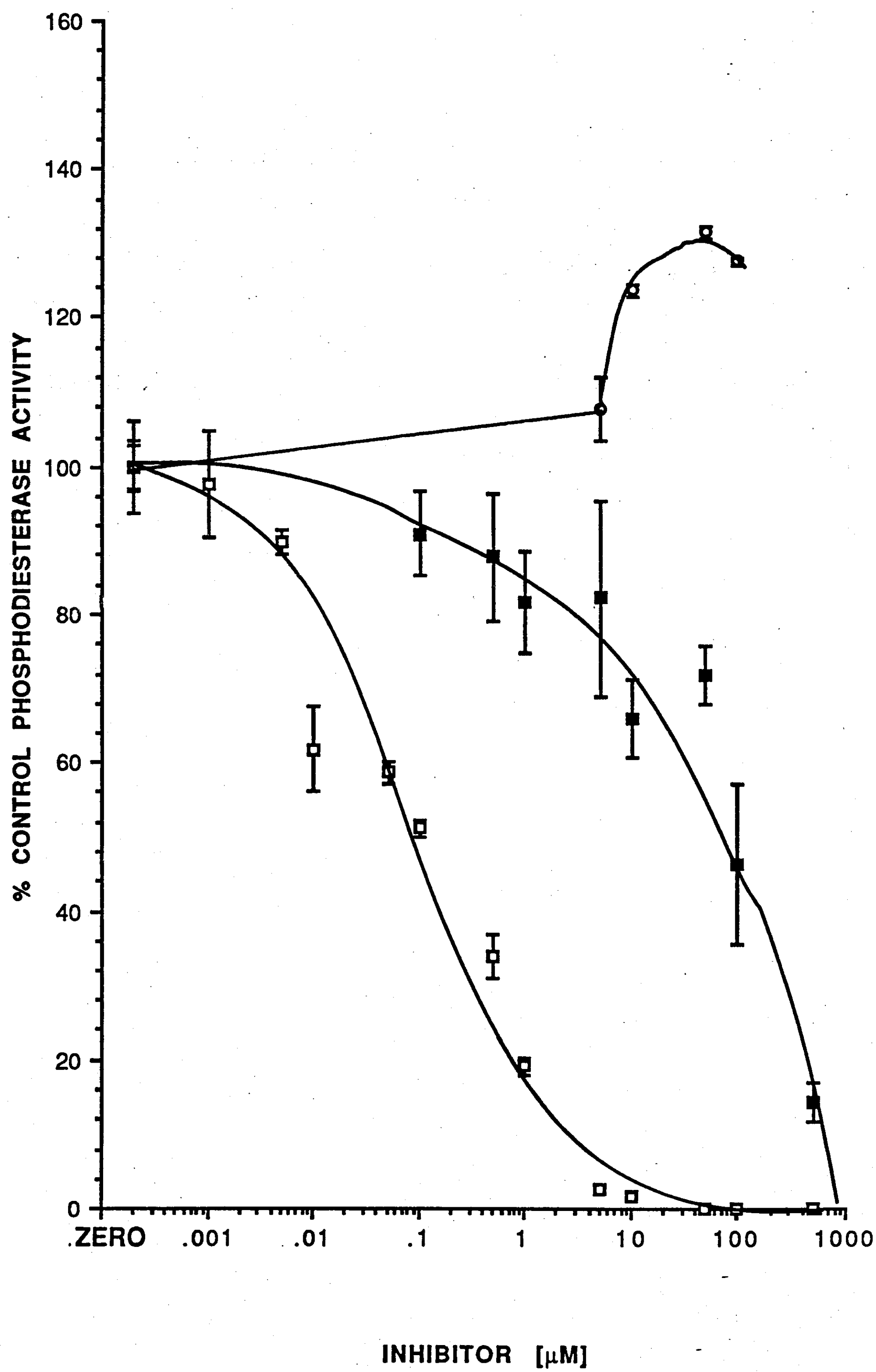


FIGURE 3.52:

INHIBITION OF RAT HEPATOCYTE-DERIVED PDE MQ-I, PDE MQ-II AND PDE MQ-III BY ICI 63197.

The phosphodiesterase activities of hepatocyte-derived PDE MQ-I, PDE MQ-II and PDE MQ-III were assessed in the absence and presence of the indicated concentrations of ICI 63197. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (VI-VIII). Results are expressed as the % of control activity (100%) ± SD for triplicate determinations.

PDE MQ-I-cAMP PDE activity (■).

PDE MQ-II-cGMP PDE activity (□).

PDE MQ-III-cAMP PDE activity (○).

FIGURE 3.52:

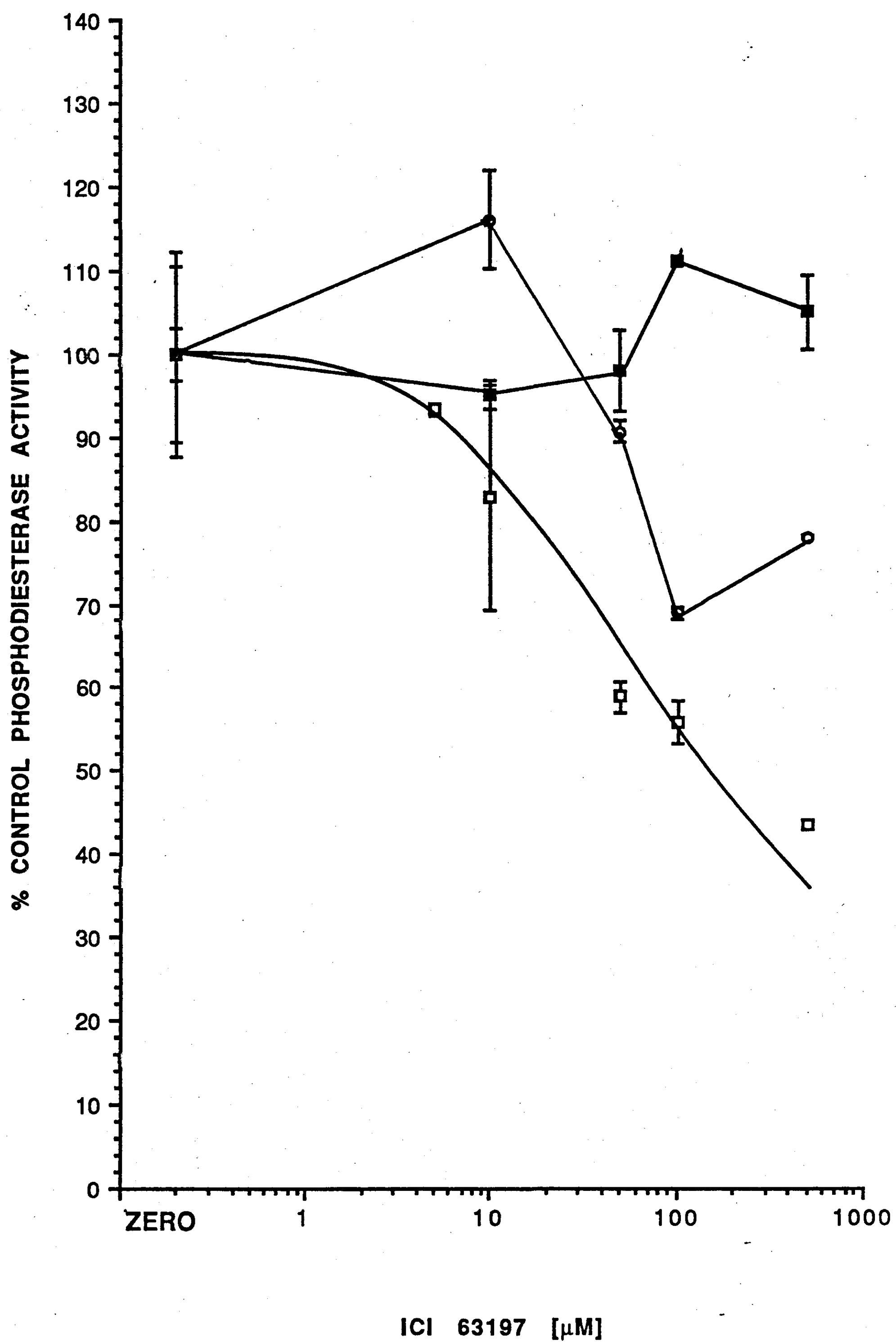


FIGURE 3.53:

INHIBITION OF RAT HEPATOCYTE-DERIVED PDE MQ-IV AND PDE MQ-V BY ICI 63197.

The phosphodiesterase activities of hepatocyte-derived PDE MQ-IV and PDE MQ-V were assessed in the absence and presence of the indicated concentrations of ICI 63197. The observed phosphodiesterase activities were corrected for the effect of solvent (DMSO) using the average correction curves shown in the Appendix (IX and X). Results are expressed as the % of control activity (100%) ± SD for triplicate determinations.

PDE MQ-IV-cAMP PDE activity (■).

PDE MQ-V-cAMP PDE activity (□).

FIGURE 3.53:

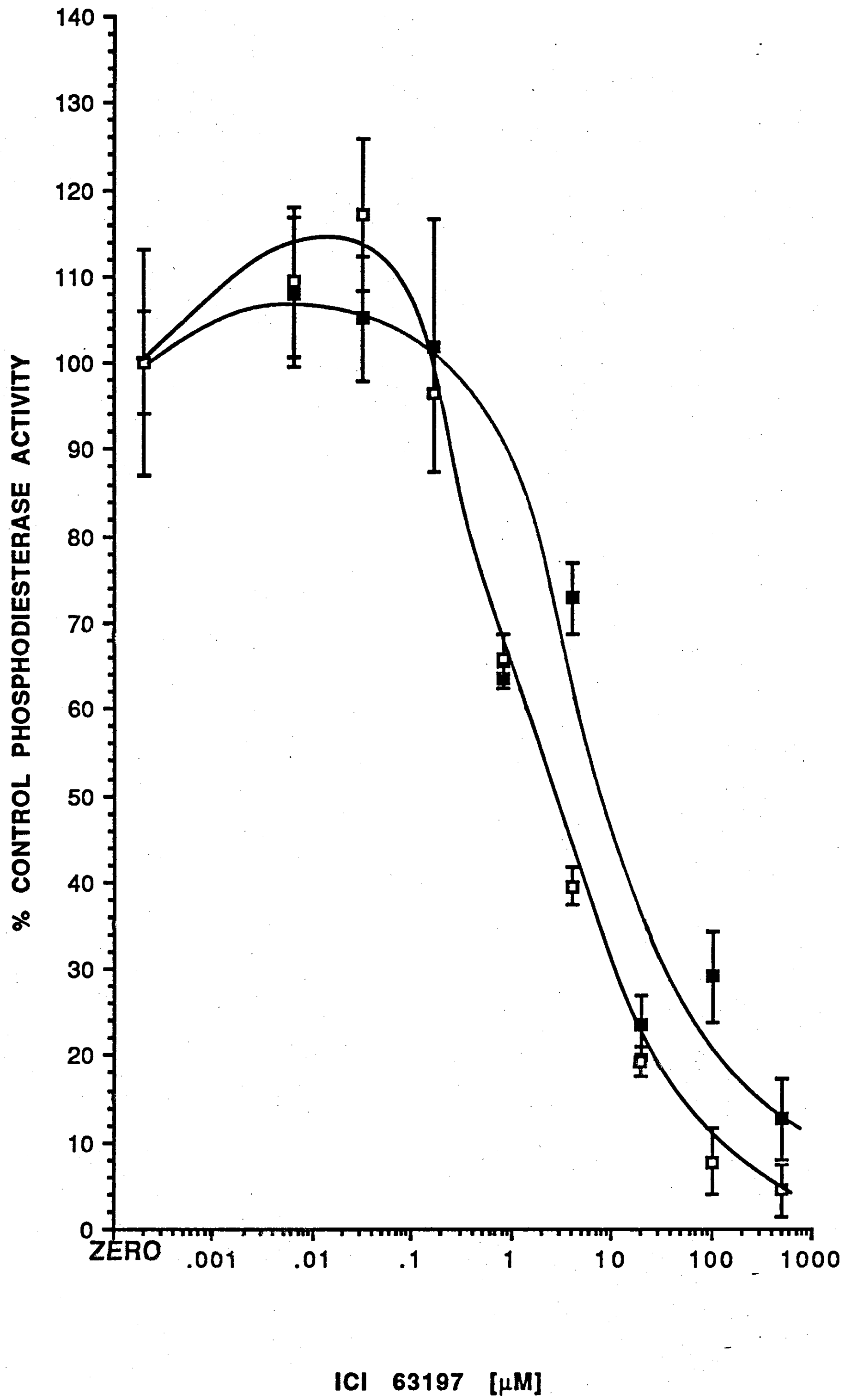


FIGURE 3.54:

CALIBRATION OF SEPHACRYL S-200 GEL FILTRATION COLUMN.

A column of Sephacryl S-200 (66cm x 1.6cm) was set up and equilibrated as in Section 2.17. The column was calibrated by applying 1-2mg of the following proteins; Ferritin (410,0000), Bovine Serum Albumin (66,000), Ovalbumin (45,000), Carbonic Anhydrase (29,000) and Cytochrome c (12,500). The elution volumes (Ve) of these proteins were determined. The inner volume (Vi) and void volume (Vo) of the column were calculated using the elution volumes of ε-DNP-Lysine and Blue-Dextran respectively. These were estimated to be 127 and 37mls respectively. The calibration curve was plotted as Ve/Vo against the log of the molecular weight. The plot yielded a straight line with a correlation coefficient of 0.96 over a molecular weight range of 12,500-66,000.

FIGURE 3.54:

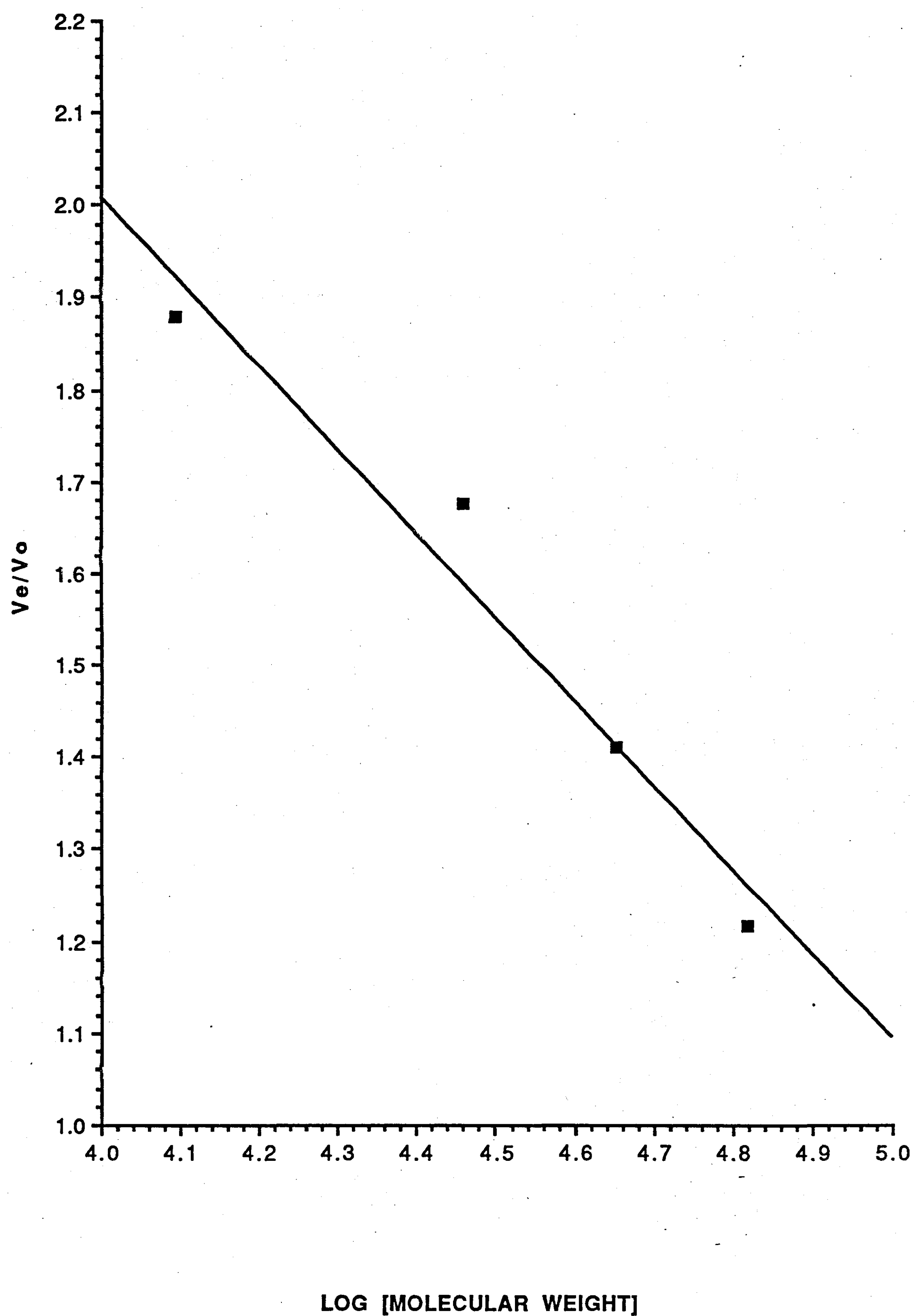


FIGURE 3.55:

SEPHACRYL S-200 GEL FILTRATION OF PDE MQ-I.

1 ml of rat hepatocyte-derived PDE MQ-I containing approximately 150 pmol/min of cAMP phosphodiesterase activity was applied to a column of Sephacryl S-200 as detailed in Section 2.17. Activity was eluted at 8ml/hr and 1.45 ml fractions were collected. cAMP phosphodiesterase activity was assayed using a 2 hour assay in place of the usual 10 minute assay. Results are expressed in pmol/min/ml. The value of the elution volume (Ve) was determined from the midpoint of the peak and using the calibration curve constructed for this column corresponded to a molecular weight of 27,000.

FIGURE 3.55:

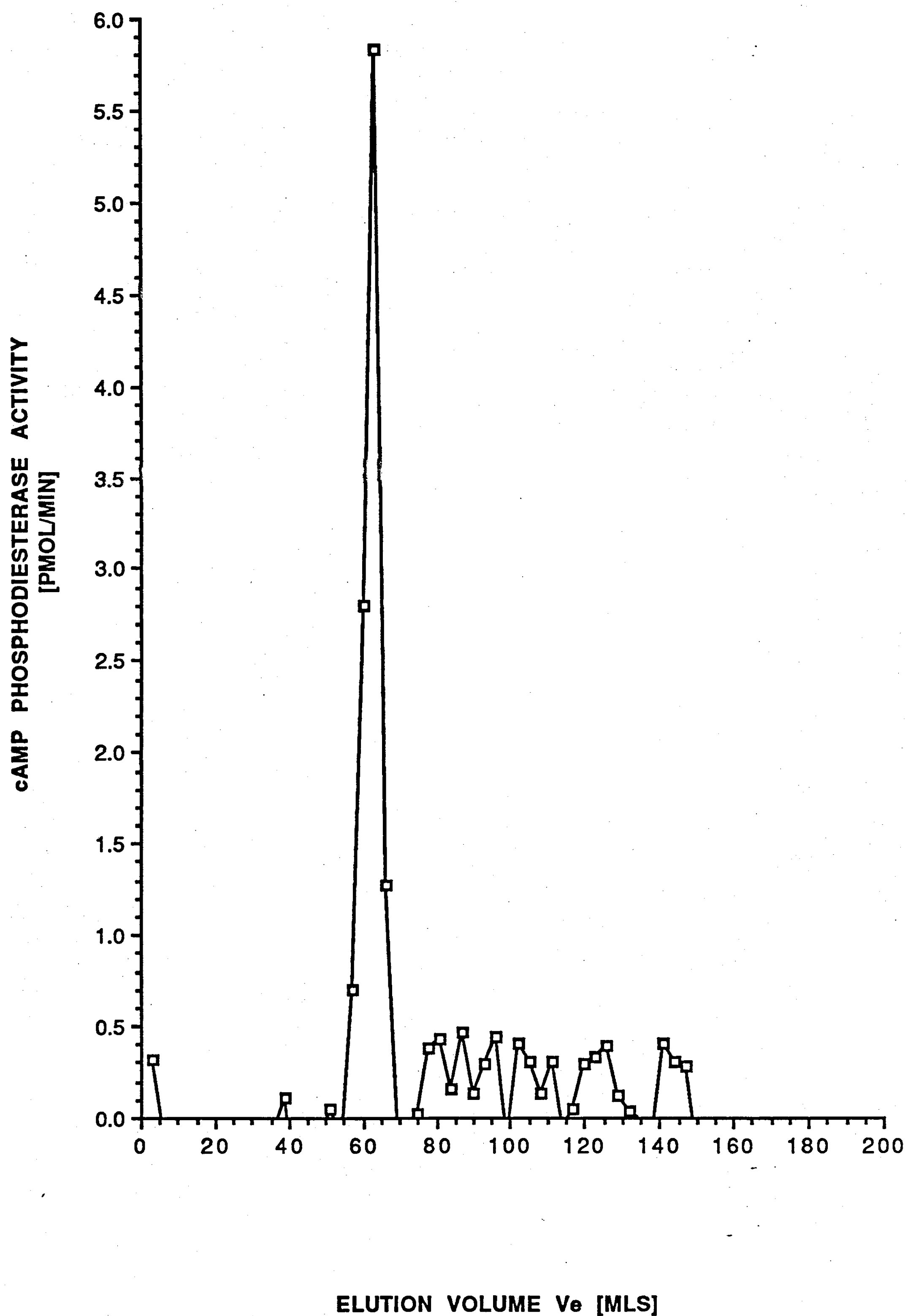


FIGURE 3.56:

EFFECT OF cGMP AND cCMP ON RAT HEPATOCYTE PDE MQ-1 cAMP PHOSPHODIESTERASE ACTIVITY

The cAMP phosphodiesterase activity of PDE MQ-I was assessed at 1μM substrate in the absence and presence of increasing concentrations of cyclic GMP (■) and cyclic CMP (□). Results are expressed as the % of control values (100%) ± SD of triplicate determinations.

The basal (control) activities were 31.86 pmol/min/ml and 27.1 pmol/min/ml for the two treatments respectively.

FIGURE 3.56:

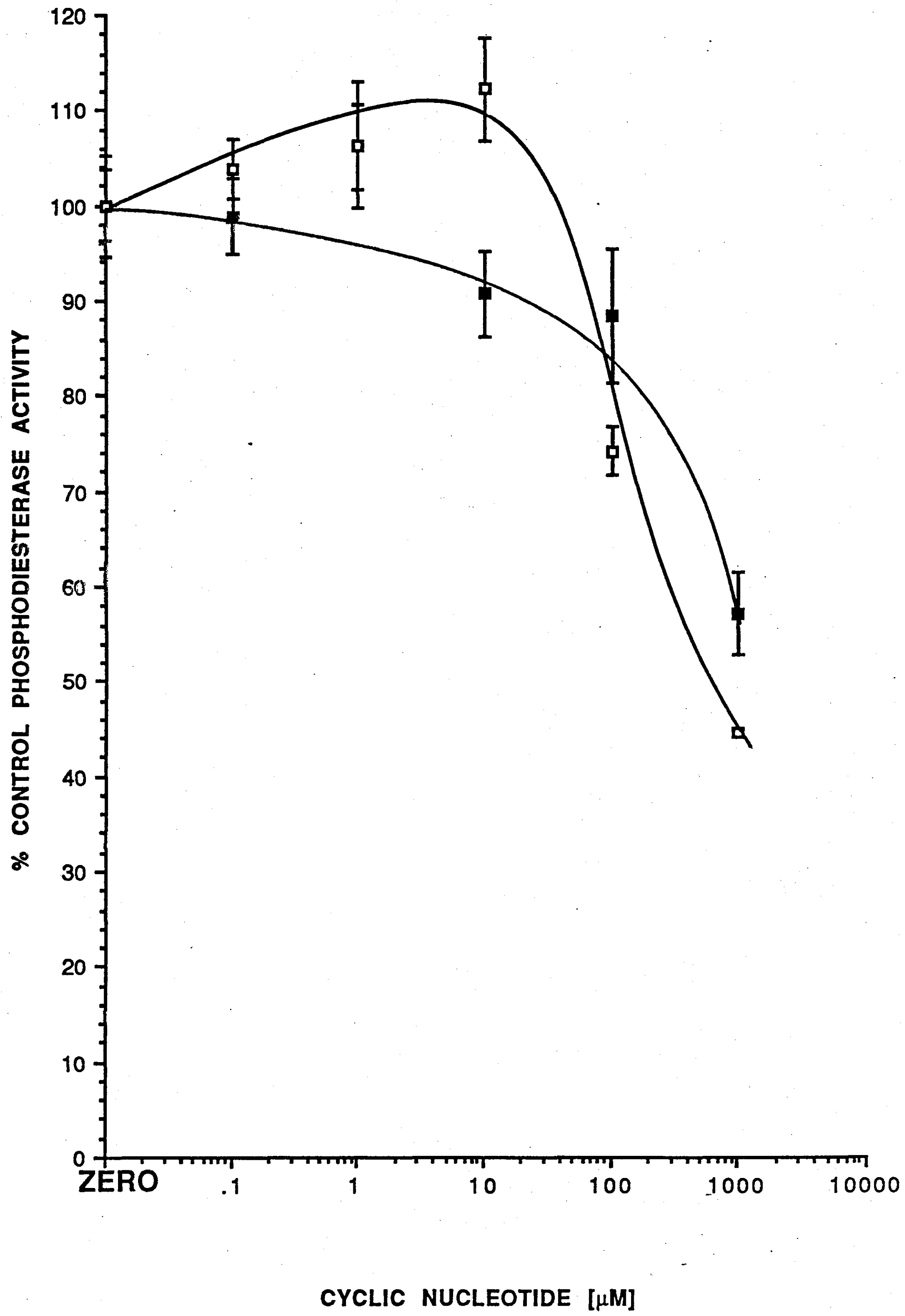


FIGURE 3.57:

CALIBRATION OF SEPHACRYL S-300 GEL FILTRATION COLUMN.

A column of Sephacryl S-300 (83cm x 1.6cm) was set up and equilibrated as in Section 2.17. The column was calibrated by applying 1-2mg of the following proteins; Ferritin (410,0000), Catalase (232,000), Alchol Dehydrogenase (150,000), and Cytochrome c (12,500). The elution volumes (Ve) of these proteins were determined. The inner volume (Vi) and void volume (Vo) of the column were calculated using the elution volumes of ε-DNP-Lysine and Blue-Dextran respectively. These were estimated to be 106mls and 50mls respectively. The calibration curve was plotted as Ve/Vo against the log of the molecular weight. The plot yielded a straight line with a correlation coefficient of 1.00 over a molecular weight range of 12,500 to 410,000.

FIGURE 3.57:

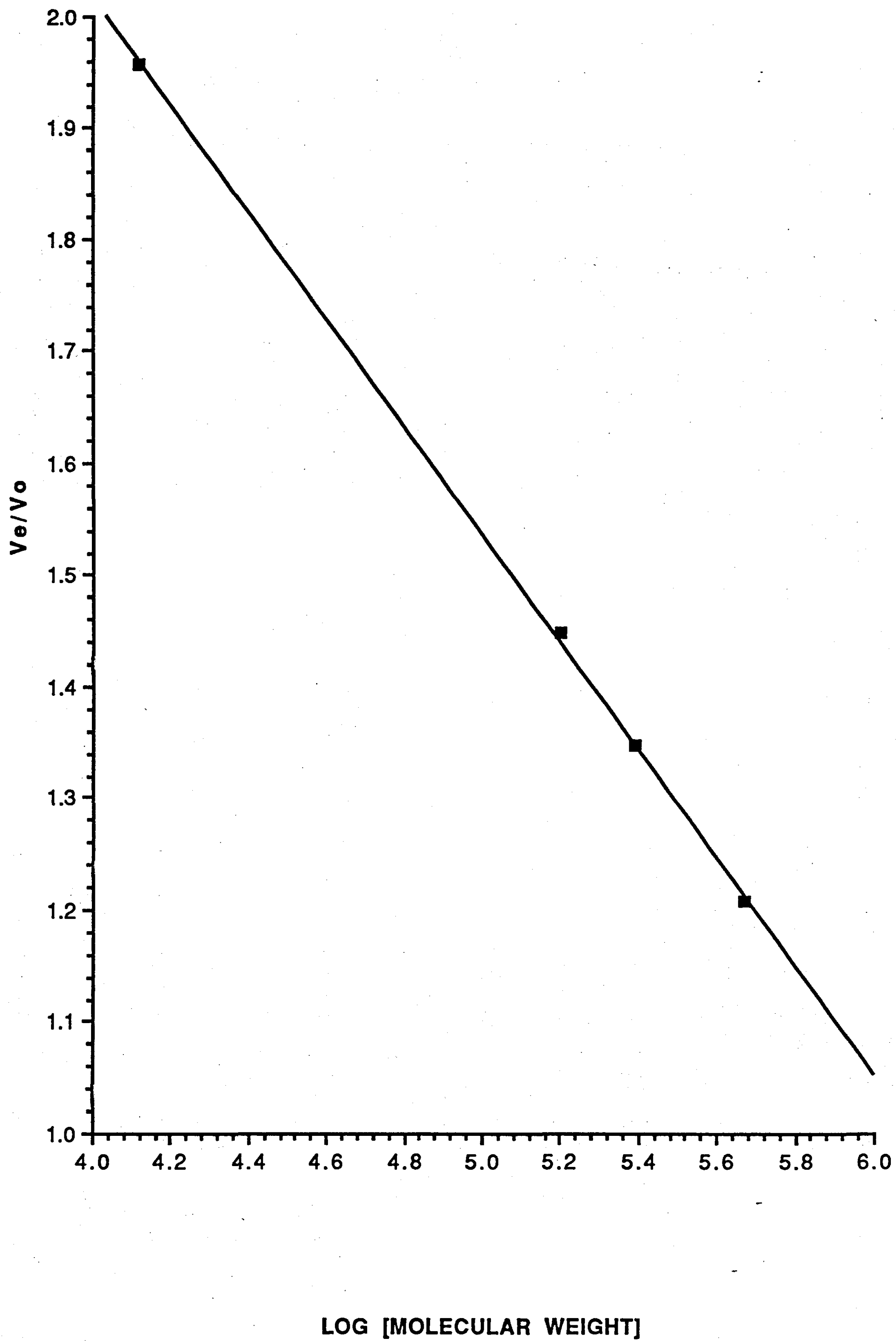


FIGURE 3.58:

SEPHACRYL S-300 GEL FILTRATION OF PDE MQ-II.

1.5 mls of rat hepatocyte-derived PDE MQ-II containing approximately 120 pmol/min of cGMP phosphodiesterase activity was applied to a column of Sephacryl S-200 as detailed in Section 2.17. Activity was eluted at 8ml/hr and 1.45 ml fractions were collected. cGMP phosphodiesterase activity was assayed using a 2 hour assay in place of the usual 10 minute assay. Results are expressed in pmol/min/ml. There appeared to be a shoulder observed on the peak of activity so two individual values of the elution volume (Ve) were recorded. Using the calibration curve for the S-300 column these corresponded to molecular weights of 220,000 and 183,000 respectively.

FIGURE 3.58:

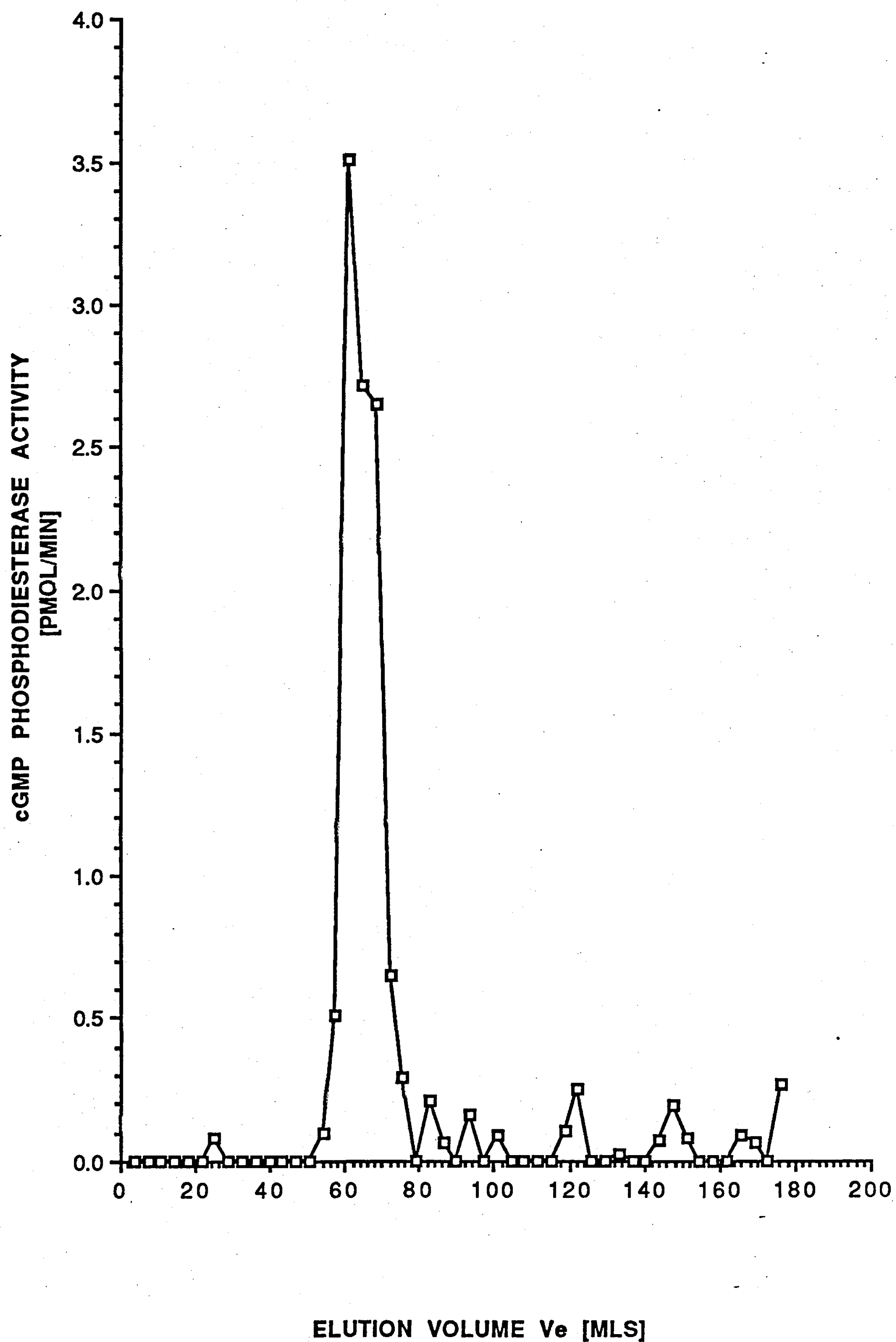


FIGURE 3.59

EFFECT OF THE CALMODULIN ANTAGONIST 5-IODO-C8 ON BASAL AND CALMODULIN STIMULATED PDE ACTIVITY.

The cAMP and cGMP phosphodiesterase activity of PDE MQ-II was assessed in the presence and absence of the calmodulin antagonist 5-Iodo-C8. Basal phosphodiesterase activity was assessed at 100μM $CaCl_2$, whilst calmodulin activated activity was determined in the presence of 200ng/ml of calmodulin plus 100μM $CaCl_2$. Results are expressed as the mean of triplicates ± SD.

Panel (a) shows the effect on basal (□) and calmodulin stimulated (■) cAMP PDE activity.

Panel (b) shows the effect on basal (□) and calmodulin stimulated (■) cGMP PDE activity.

FIGURE 3.59(a)

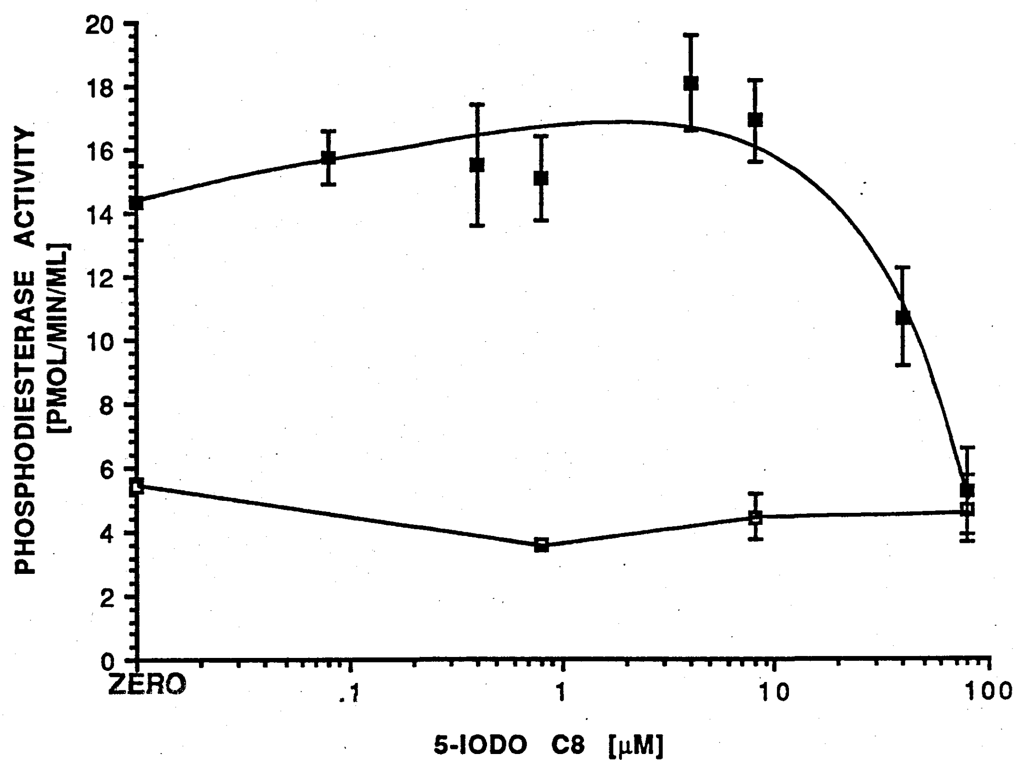


FIGURE 3.59(b):

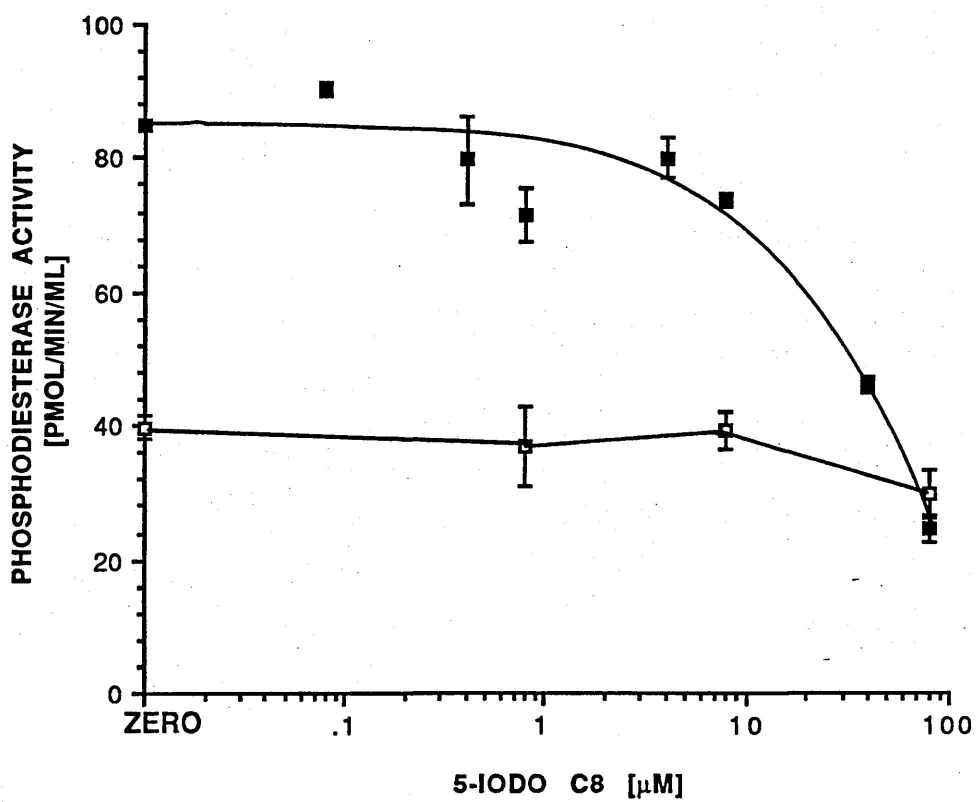


FIGURE 3.60:

MONO Q ANION-EXCHANGE PROFILE OF RAT ADIPOCYTE SOLUBLE PHOSPHODIESTERASE ACTIVITY.

A high speed supernatant was prepared from the epididymal adipose tissue isolated from five rats. A modified procedure of that detailed in Section 2.4 was used to prepare this supernatant. This involved the initial homogenisation being carried out at room temperature to prevent solidification of the fat. The supernatnat (40mls)was then applied to a Mono Q column at 1ml/min (see Section 2.11). Phosphodiesterase activity was eluted using a sodium chloride gradient at 1ml/min and 1ml fractions collected. Cyclic AMP (□) and cyclic GMP (■) phosphodiesterase activity was determined at 0.1μM substrate by assaying a 25μl aliquot of every alternate fraction. The phosphodiesterase activity is expressed in pmol/min.. An identical gradient was used as for the preparation of rat liver and hepatocyte soluble fractions (see Table 2.2)

FIGURE 3.60:

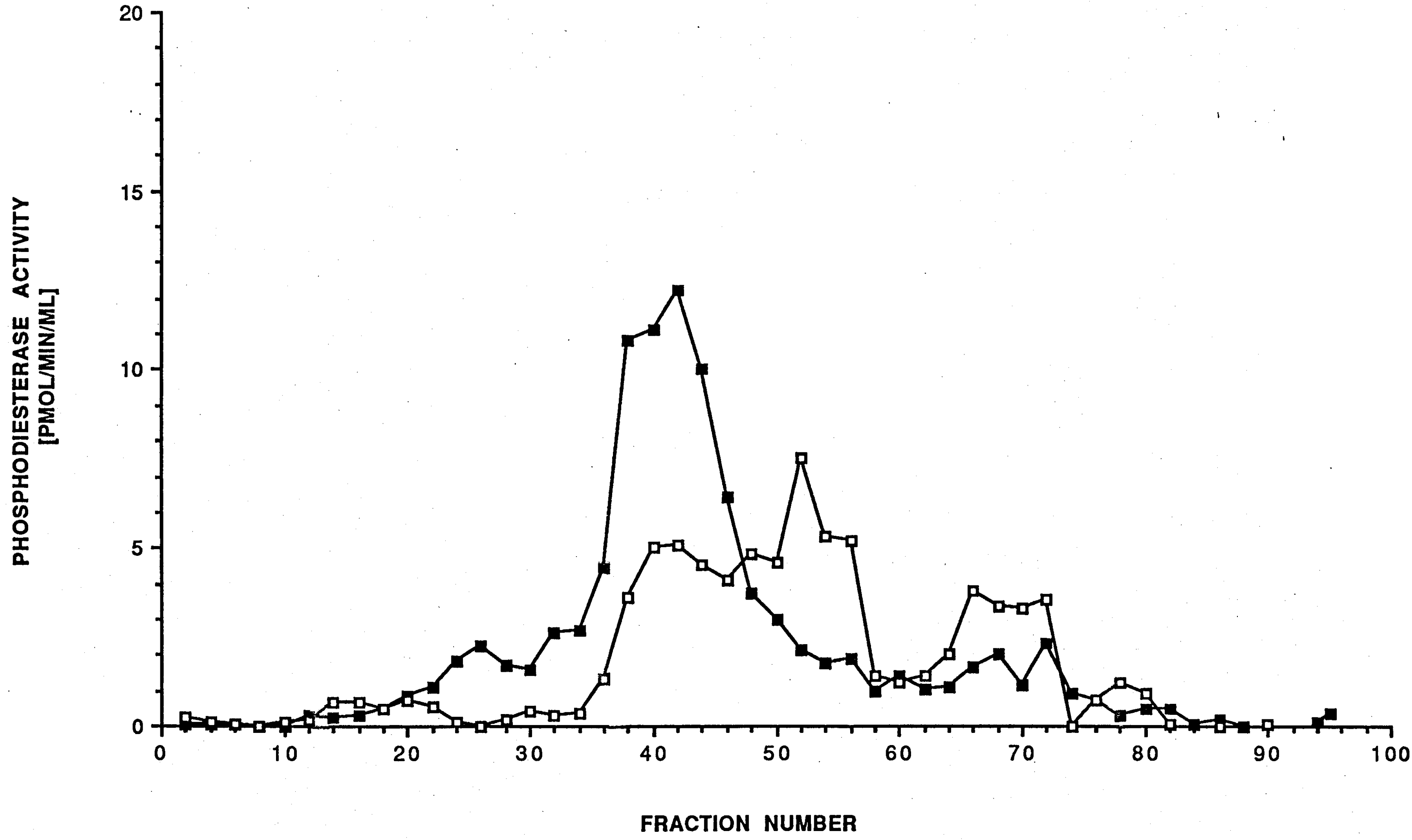


FIGURE 3.61:

MONO Q ANION-EXCHANGE PROFILE OF RAT BRAIN SOLUBLE PHOSPHODIESTERASE ACTIVITY.

A high speed supernatant was prepared from one rat brain exactly as in Section 2.4. This extract was then applied to a Mono Q column at 1ml/min (see Section 2.11). Phosphodiesterase activity was eluted using a sodium chloride gradient at 1ml/min and 1ml fractions collected. Cyclic AMP (□) and cyclic GMP (■) phosphodiesterase activities were determined at 1μM substrate by assaying a 25μl aliquot of every alternate fraction. The phosphodiesterase activity is expressed in pmol/min.

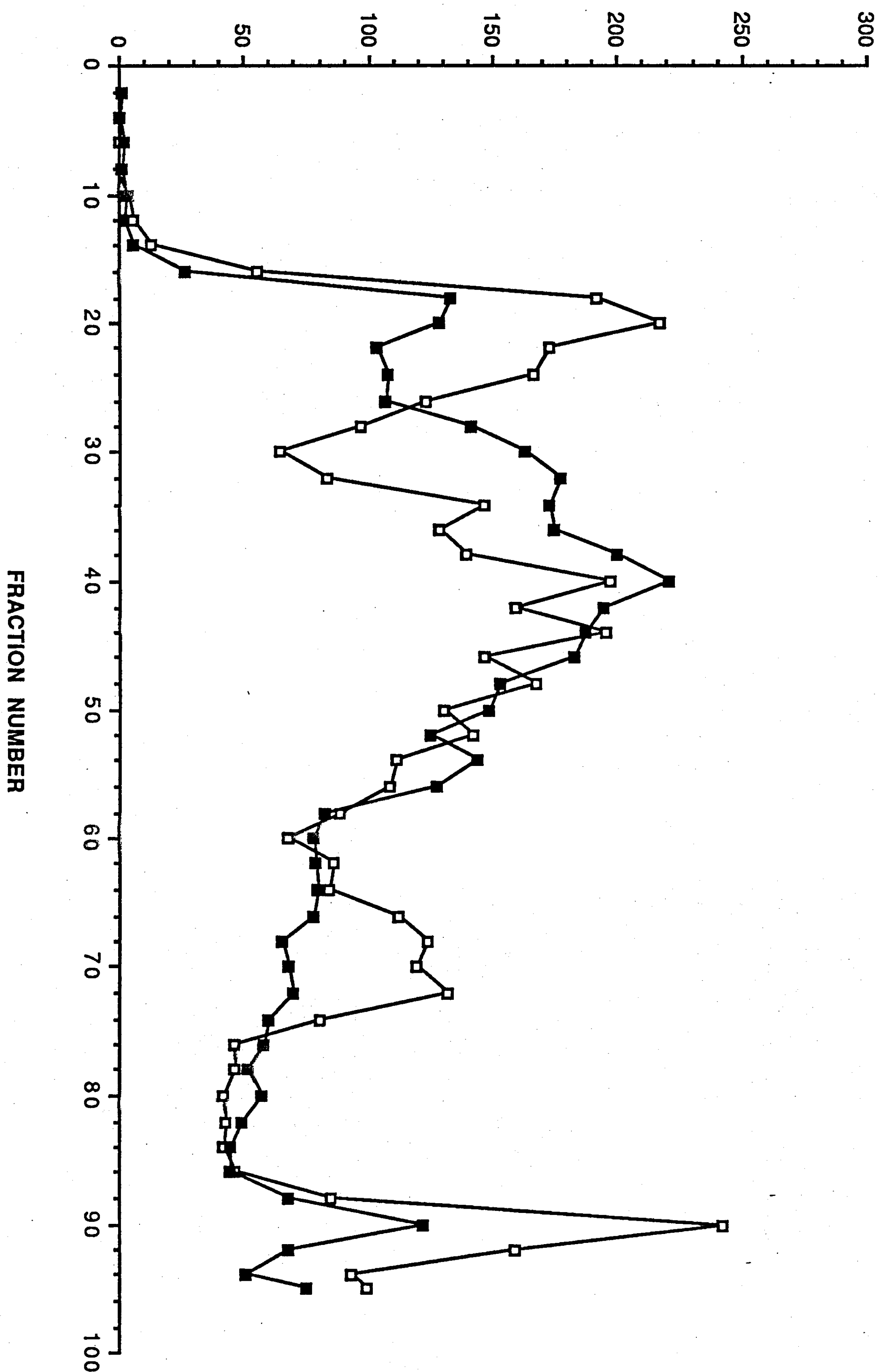


FIGURE 3.61:

FIGURE 3.62:

MONO Q ANION-EXCHANGE PROFILE OF RAT KIDNEY SOLUBLE PHOSPHODIESTERASE ACTIVITY.

A high speed supernatant was prepared from two rat kidneys exactly as described for the preparation of rat liver and rat hepatocyte soluble fractions (see Section 2.4). This extract (40mls) was applied to a Mono Q column at 1ml/min (see Section 2.11). Phosphodiesterase activity was eluted using a sodium chloride gradient at 1ml/min and 1ml fractions collected. Cyclic AMP (□) and cyclic GMP (■) phosphodiesterase activity was determined at 1μM substrate by assaying a 25μl aliquot of every alternate fraction. The phosphodiesterase activity is expressed in pmol/min.

FIGURE 3.62:

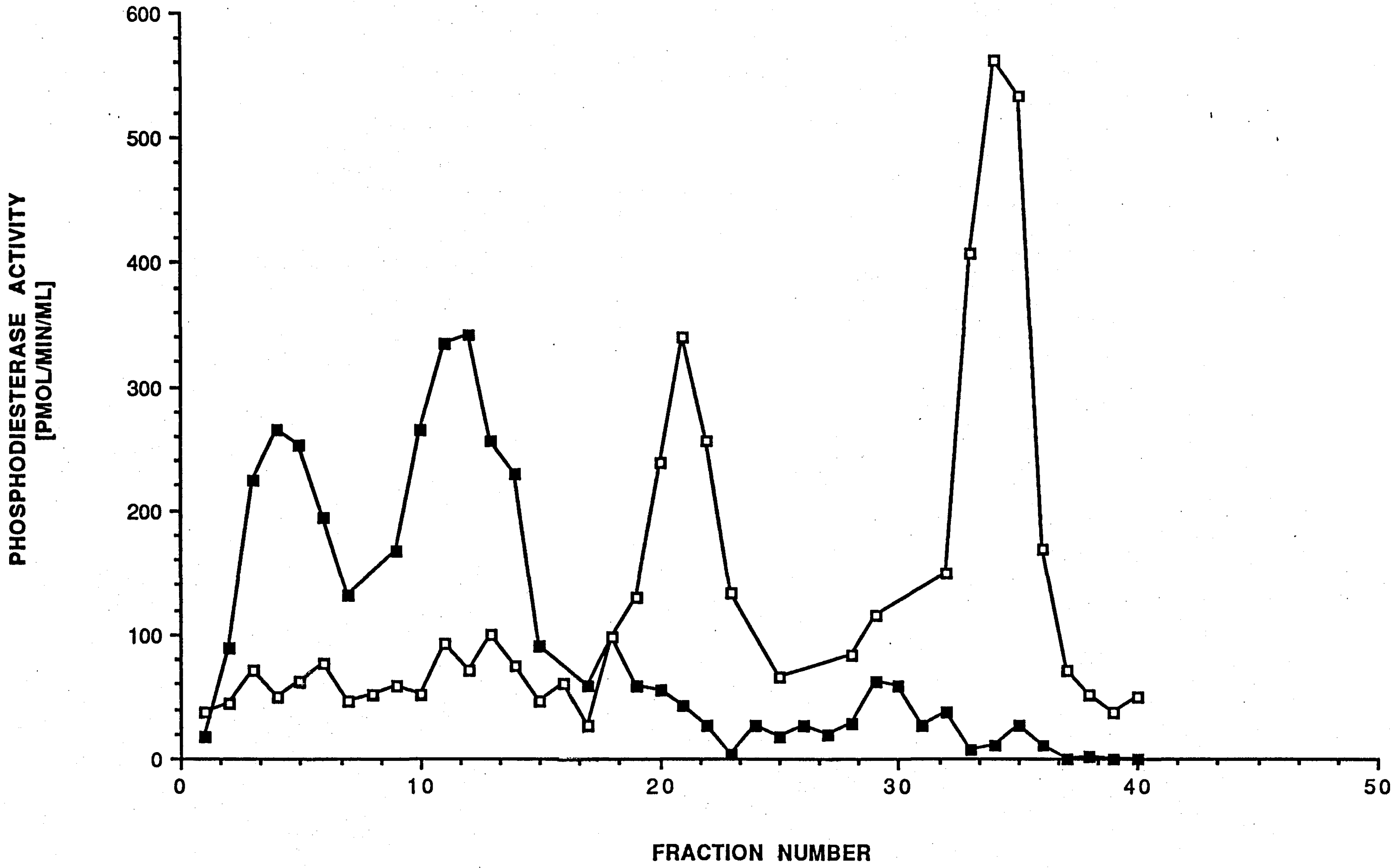


FIGURE 3.63:

INHIBITION OF CYCLIC AMP HYDROLYSIS OF RAT HEPATOCYTE-DERIVED PDE MQ-IV AND PDE MQ-V BY cGMP.

The cAMP phosphodiesterase activity of PDE MQ-IV and V were assessed at 1μM substrate in the absence and presence of increasing concentrations of cyclic GMP. Results are expressed as the % of control values (100%) ± SD of triplicate determinations.

The basal (control) activities were 48.3 pmol/min/ml and 57 pmol/min/ml for the two activities respectively.

(□) Cyclic AMP phosphodiesterase activity of PDE MQ-IV.

(■) Cyclic AMP phosphodiesterase activity of PDE MQ-V.

FIGURE 3.63:

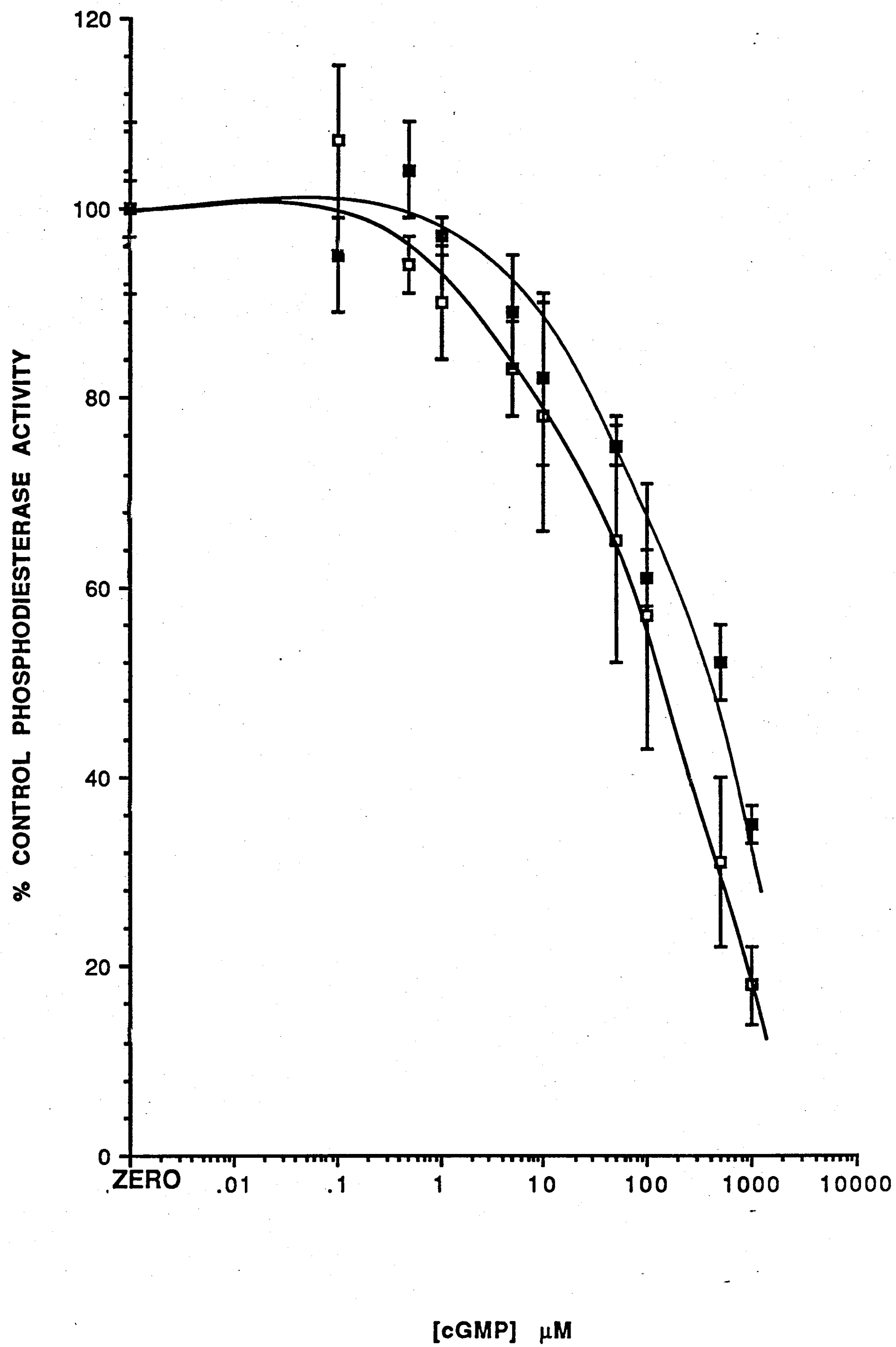


TABLE 3.1:

EFFECT OF AGENTS ON THE CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASE ACTIVITY OF DE-52 PEAKS.

The fold effects of Ca^{2+} (50µM), Ca^{2+}/Calmodulin (50µM Ca^{2+} 2µg/ml Calmodulin) and cyclic GMP (2µM) on cyclic AMP and cyclic GMP hydrolysis were determined at 1µM substrate. Values are expressed as the mean ± SEM. RATIO indicates the ratio of cAMP:cGMP hydrolysis determined at 1µM substrate and is an index of the substrate selectivity of the resolved peaks. N.A. not applicable. n is the number of determinations.

PEAK		Ca^{2+}	Ca^{2+}/CaM	cGMP	RATIO (cAMP:cGMP)
I	cAMP	0.96±0.05 (n=5)	1.32±0.22 (n=5)	1.18±0.1 (n=3)	1.07±0.09 (n=7)
	cGMP	1.10±0.12 (n=4)	2.1±0.4 (n=4)	N.A	
II	cAMP	1.17±0.14 (n=4)	1.25±0.2 (n=4)	6.88±2.58 (n=4)	0.35±.12 (n=3)
	cGMP	0.99±0.07 (n=3)	1.19±0.1 (n=3)	N.A.	
III	cAMP	1.10±0.14 (n=3)	1.07±0.07 (n=3)	1.13±0.17 (n=3)	4.16±1.74 (n=3).
	cGMP	1.05±0.07 (n=3)	1.36±0.40 (n=3)	N.A	

TABLE 3.2:

PROGRAMME USED FOR THE SEPARATION OF SOLUBLE PHOSPHODIESTERASE ACTIVITY BY MONO Q CHROMATOGRAPHY

This Table represents the parameters that were set to obtain the sodium chloride gradient used for separation of soluble phosphodiesterases from both rat liver and rat hepatocyte soluble fractions. CONC$^{\underline{N}}$ %B represents the % of Buffer B at a particular time in the gradient where 100% B represents a buffer containing 0.5M NaCl. ML/MIN represents the speed at which the column was run. CM/ML indicates the chart speed. PORT SET 6.1 and 6.0 are the instructions for turning the fraction collector on and off respectively.

TIME (MINS)	PARAMETER	SETTING
0.0	CONC$^{\underline{N}}$ %B	0.0
0.0	ML/MIN	1.0
0.0	CM/ML	0.5
0.0	PORT SET	6.1
35.0	CONC$^{\underline{N}}$ %B	40.0
85.0	CONC$^{\underline{N}}$ %B	80.0
88.0	CONC$^{\underline{N}}$ %B	100
95.0	CONC$^{\underline{N}}$ %B	100
95.0	PORT SET	6.0

TABLE 3.3:

YIELD OF RAT LIVER PDE MQ-PEAKS I-V

The average yield of cAMP and cGMP phosphodiesterase activity, assessed at 1μM substrate, was calculated for each of the individual Peaks prepared from approximately half a rat liver supernatant and is expressed as pmol/min of phosphodiesterase activity ± SEM. The % of total soluble phosphodiesterase activity was calculated assuming the resolved peaks represented 100% of the applied cyclic AMP and cyclic GMP PDE activities. RATIO is the ratio of cyclic AMP:cyclic GMP hydrolysis assessed at 1μM substrate. n is the number of determinations.

MQ-PDE	Substrate	Yield of PDE activity [pmol/min]	% Total soluble PDE activity	RATIO
I	cAMP	1570±174 (n=6)	22.5	5.4±0.61 (n=4)
	cGMP	273±95 (n=4)	3.5	
II	cAMP	162±26 (n=7)	2.3	0.126±0.007 (n=6)
	cGMP	1530±274 (n=5)	19.5	
III	cAMP	3103±562 (n=5)	44.6	0.46±0.06 (n=4)
	cGMP	5813±1480 (n=3)	74.2	
IV	cAMP	998±217 (n=5)	14.3	8.98±2.57 (n=4)
	cGMP	116±51 (n=3)	1.5	
V	cAMP	1126±269 (n=5)	16.2	10.1±2.7 (n=4)
	cGMP	110±66 (n=3)	1.4	

TABLE 3.4:

EFFECTS OF AGENTS ON LIVER AND HEPATOCYTE DERIVED PDE MQ PEAKS.

The phosphodiesterase activity of pooled individual peaks was assessed at 1µM substrate. Final assay concentrations of Ca^{2+} and Calmodulin were 50µM and 2µg/ml respectively, while the cyclic GMP concentration was 2µM. An asterix (*) indicates that the effect of the agent was significantly different from control, with $P < 0.05$. ND indicates that while enzyme activity was too low for an accurate detection of small effects, no gross effects of Ca^{2+} and Calmodulin were detectable. Results are expressed as the means ± SEM, with n being the number of experiments. Values are fold-effects relative to control in the absence of agents.

MQ-PDE	SOURCE	Ca^{2+}/Calmodulin on cAMP PDE	Cyclic GMP on cAMP PDE	Ca^{2+}/Calmodulin on cGMP PDE
I	Liver	1.13±0.06 (n=4)	0.90±0.09 (n=4)	1.72±0.16* (n=3)
	Hepatocyte	0.91±0.06 (n=3)	1.09±0.09 (n=3)	1.28±0.06* (n=4)
II	Liver	4.77±1.01* (n=5)	1.10±0.17 (n=5)	2.16±0.21* (n=4)
	Hepatocyte	3.83±0.35* (n=3)	0.93±0.09 (n=3)	2.26±0.20* (n=3)
III	Liver	1.09±0.15 (n=4)	6.32±1.86* (n=3)	1.04±0.07 (n=3)
	Hepatocyte	1.05±0.09 (n=3)	5.92±0.39* (n=3)	1.09±0.14 (n=3)
IV	Liver	1.20±0.13 (n=6)	0.90±0.08* (n=5)	ND
	Hepatocyte	1.23±0.13 (n=3)	0.81±0.09* (n=3)	ND
V	Liver	0.98±0.04 (n=5)	0.83±0.06* (n=5)	ND
	Hepatocyte	1.22±0.01 (n=3)	0.77±0.03* (n=3)	ND

TABLE 3.5:

ELUTION OF cGMP PDE ACTIVITY OF PDE MQ-II FROM AFFI-GEL BLUE.

0.75ml aliquots of PDE MQ-II (containing 46 pmol/min of cGMP PDE activity each) isolated from a rat liver soluble fraction was made up to 5mM $MgCl_2$ and incubated with 110μl aliquots of Affi-gel Blue prepared and equilibrated as in Section 2.12. Incubation was for 30 minutes at 4°c on a rotary mixer. Gels were washed in 2 x 1ml aliquots of equilibration buffer (see Section 2.12). Elution of cGMP PDE activity was attempted using 2 x 1ml aliquots of Buffer A (50mM Tris-HCl pH 7.4, 0.1mM EGTA, 5mM Benzamidine, 0.2mM PMSF, 0.1mM dithiothreitol, 2μM Leupeptin) containing the indicated additions. The Ca^{2+}/Calmodulin containing buffer contained 5μg/ml of Calmodulin and 500μM $CaCl_2$. The total applied activity was 46 pmol/min per 0.75ml aliquot. The washes contained 4.58 ± 1.13 pmol/min of cGMP PDE activity (10% of applied activity). The % binding of the cGMP PDE activity of PDE MQ-II to the matrix was 99.8%. Eluted activity is expressed both as pmol/min of cGMP PDE activity retrieved ± SD of triplicates and as a % of the total bound.

ELUTION CONDITION	ACTIVITY [PMOL/MIN]	% RECOVERED
0.5M NaCl/5mM $MgCl_2$	2.57±0.8	5.6%
1.0M NaCl/5mM $MgCl_2$	0	0%
2mM EDTA	1±0.16	2.22%
0.5M NaCl/2mM EDTA	2.17±0.24	4.7%
1.0M NaCl/2mM EDTA	3.86±3.9	8.4%
Ca2+/Calmodulin/2mM EDTA	3.88±0.21	8.4%

TABLE 3.6:

IC_{50} VALUES FOR LIVER AND HEPATOCYTE DERIVED PDE MQ-PEAKS

The concentration of drug causing 50% inhibition of basal cAMP or cGMP phosphodiesterase activity was determined at 1μM substrate (see Section 2.14). Results are means ± SEM with n being the number of determinations. All drug effects were corrected for the effect of solvent (DMSO) before calculation of IC_{50} values. N.I. not inhibitory at concentrations up to either 500μM* or 100μM^{+}. In instances where an IC_{50} value could not be determined then the % inhibition observed at the maximal drug dose is shown in brackets. cAMP was normally the substrate except for # which was cGMP.

DRUG	SOURCE	I	II	III	IV	V
IBMX	**Liver**	>500* (n=5)	5.6±1.4 (n=5)	97±22 (n=3)	14.4±5.3 (n=3)	10.9±1.1 (n=3)
	Hepatocytes	>500* (n=4)	6.7±1.5 (n=5)	323±69 (n=4)	5.2±0.9 (n=4)	9.8±5.2 (n=4)
Milrinone	**Liver**	>500* (n=5)	145±2 (n=4)	>500* (n=3)	10.3±5.3 (n=4)	17±2.6 (n=3)
	Hepatocytes	>500 (n=3)	61±25 (n=3)	>500* (n=3)	7.3±1.5 (n=3)	20±5 (n=3)
Ro-20-1724	**Liver**	>500* (n=3)	>500* (n=3)	>500* (n=3)	190±30 (n=3)	1.6±0.8 (n=3)
	Hepatocytes	>500* (n=3)	303±107 (n=3)	>500* (n=3)	13±4 (n=3)	1.5±0.8 (n=4)
ICI 118233	**Hepatocytes**	>500+ (n=3)	>500+# (n=3)	>500+ (n=3)	>500+ (n=3)	.500+ (n=3)
Zaprinast	**Hepatocytes**	>500* (n=3)	4.5±1.1*# (n=3)	>500* (n=3)	300±141 (n=3)	.500* (n=3)
Rolipram	**Hepatocytes**	>500* (n=3)	>500*# (n=3)	.500* (n=3)	0.5±0.07 (n=3)	0.095±0.005 (n=3)

TABLE 3.7:

YIELD OF RAT HEPATOCYTE PDE MQ-PEAKS I-V

The average yield of cAMP and cGMP phosphodiesterase activity, assessed at 1μM substrate, was calculated for each of the individual Peaks prepared from the total hepatocyte-derived supernatant and is expressed as pmol/min of phosphodiesterase activity ± SEM. The % of total soluble phosphodiesterase activity was calculated assuming the resolved peaks represented 100% of the applied cyclic AMP and cyclic GMP PDE activities. RATIO is the ratio of cyclic AMP:cyclic GMP hydrolysis assessed at 1μM substrate. n is the number of determinations.

MQ-PDE	Substrate	Yield of PDE activity [pmol/min]	% Total soluble PDE activity	RATIO
I	cAMP	907±101 (n=10)	39.6	5.07±0.14 (n=8)
	cGMP	174±61 (n=10)	5.7	
II	cAMP	64±20 (n=8)	2.8	0.08±0.013 (n=7)
	cGMP	1108±167 (n=7)	36.2	
III	cAMP	235±59 (n=9)	10.3	0.15±0.015 (n=7)
	cGMP	1739±402 (n=7)	56.8	
IV	cAMP	562±101 (n=5)	24.6	23.5±2.7 (n=3)
	cGMP	19±5 (n=3)	0.62	
V	cAMP	519±142 (n=5)	22.7	10.1±2.7 (n=3)
	cGMP	22±11 (n=3)	0.72	

TABLE 3.8:

KINETIC PROPERTIES OF RAT HEPATOCYTE-DERIVED PDE MQ-PEAKS.

Phosphodiesterase activities were resolved from rat hepatocyte high speed supernatants using a Mono Q column. All values quoted for PDE MQ-I were determined using an enzyme preparation which had been further chromatographed on Affi-gel Blue (see Section 2.16). K_m values and Hill coefficients (h) were determined from Hill plots or limiting K_m values (K_{m1} and K_{m2}) from Lineweaver-Burk plots. In instances where Lineweaver-Burk plots were downwardly curving, and thus indicative of apparent negative cooperativity, the limiting values for K_{m1} and K_{m2} were obtained from their linear extremae. In all instances, triplicate determinations of phosphodiesterase activity were performed at 19 substrate concentrations (see Legends for Figures 3.21 to 3.28 for actual range). N.A. Not applicable. N.D. Not determined.
All values are expressed as the mean ± SEM of at least three independent determinations.

MQ-PDE	SUBSTRATE	Constants derived from Hill plots.		Limiting K_m values.		
		K_m(μM)	Hill coefficient (h)	K_{m1}(μM)	K_{m2}(μM)	V_{max} ratio
I	cAMP	24.9±5.9 (n=3)	0.97±0.09 (n=3)	31.3±3.3 (n=3)	N.A.	1.07±0.14
	cGMP	237±56 (n=3)	1.04±0.04 (n=3)	246±19.8 (n=3)	N.A.	
II	cAMP	N.D.	N.D.	N.D.	N.D	
	cGMP	5.1±1.7 (n=3)	0.72±0.02 (n=3)	1.9±0.51 (n=3)	11±2.6 (n=3)	N.D.
III	cAMP	37.7±6.7 (n=4)	1.62±0.20 (n=4)	N.A.	N.A.	0.31±0.07
	cGMP	35.5±6.9 (n=4)	1.2±0.06 (n=4)	N.A.	N.A	
IV	cAMP	6.33±0.34 (n=3)	0.69±0.05 (n=3)	0.8±0.08 (n=3)	8.01±0.30 (n=3)	N.D.
	cGMP	N.D.	N.D.	ND	N.D.	
V	cAMP	0.62±0.07 (n=4)	1.01±0.13 (n=5)	N.A.	N.A.	N.D.
	cGMP	N.D.	N.D.	ND	N.D.	

TABLE 3.9:

EFFECT OF Mg^{2+} IONS AND CYCLIC GMP ON RAT HEPATOCTYE-DERIVED PDE MQ-PEAKS.

Phosphodiesterase activities were resolved from rat hepatocyte high speed supernatants using a Mono Q column. All values for PDE MQ-I were determined using an enzyme preparation which had been further chromatographed on Affi-gel Blue (as described in Section 2.16). EC_{50} values for Mg^{2+} effects were determined using either 1μM cyclic AMP (PDE MQ-I, PDE MQ-III, PDE MQ-IV and PDE MQ-V) or 1μM cyclic GMP (PDE MQ-II) as substrate. All effects of cyclic GMP were determined using 1μM cyclic AMP as substrate.
N.A. Not applicable. N.D. Not determined.
All values are quoted as the means of triplicate determinations ± SEM.

PEAK	EC_{50} Mg^{2+}	IC_{50} cGMP	EC_{50} cGMP	observations
I	N.A.	850±57	N.D.	3
II	20±2	N.D.	N.A.	3
III	193±18	43±9.8	0.466±0.098	4
IV	50±7	214±50	N.A.	3
V	40±6	570±87	N.A.	4

CHAPTER 3: DISCUSSION.

In most cells/tissues phosphodiesterase activity is observed in both the soluble and particulate fractions. A lot of attention has been focussed on particulate-derived phosphodiesterase isozymes, particularly in hormonally sensitive tissues such as liver and adipose tissue, due to the fact that these contain a number of hormone stimulated phosphodiesterase activities. In liver and hepatocytes, the two membrane bound species of phosphodiesterase that are stimulated by hormones such as insulin and glucagon, have been purified and characterised (Whitson and Appleman, 1982; Pyne, et al., 1987a; Boyes and Loten, 1988; Marchmont, et al., 1981b; Pyne. et al., 1986b; Houslay, 1988). These have been termed the 'dense-vesicle' and 'peripheral' phosphodiesterases respectively (Heyworth, et al., 1983d; Marchmont, et al., 1981b; Pyne, et al., 1987a; Pyne, et al., 1986b). These activities may play an important role both in the anti-lipolytic and anti-glycogenolytic actions of insulin as well as providing a negative feedback system to decrease cyclic AMP levels in hormonally stimulated cells (Heyworth, et al., 1983d; Beebe, et al., 1985; Corbin, et al., 1985; Gettys, et al., 1987). Whilst much is known about the number and type of particulate-derived phosphodiesterases in tissues such as the liver and adipose tissue, relatively little is known about the soluble phosphodiesterases in these tissues.

The soluble fraction has been noted to contribute a significant proportion of the total phosphodiesterase activity in tissues and cell types such as adipose tissue (Manganiello and Vaughan, 1973), liver (Whitson and Appleman, 1982; Heyworth, et al., 1983d), heart (Weishaar, et al., 1987a) and 3T3-L1 adipocytes (Elks and Manganiello, 1985). The aim of this study was to identify and characterise the soluble phosphodiesterase activities present in the soluble fraction of both rat liver and hepatoctyes.

Initially, the effects of two potential regulators of phosphodiesterase activity, namely Ca^{2+} and cyclic GMP, on the total cyclic AMP and cyclic GMP phosphodiesterase activities observed in a crude rat liver soluble fraction, were determined. This is because, *in vivo*, Ca^{2+} is a potential regulator of the calmodulin–sensitive phosphodiesterase, whilst cyclic GMP is a potential regulator of both the cyclic GMP–stimulated and cyclic GMP–inhibited phosphodiesterases. This was performed by assessing total phosphodiesterase activity of an isolated soluble fraction in the presence of saturating levels of Ca^{2+} or 2 μM cyclic GMP. This analysis revealed that Ca^{2+} could stimulate both the total cyclic AMP and the total cyclic GMP phosphodiesterase activity in this fraction, whilst cyclic GMP was also found to promote a marked stimulation of the cyclic AMP phosphodiesterase activity. These results indicated the presence of a Ca^{2+}–stimulated phosphodiesterase activity and a cyclic GMP–stimulated activity.

The fold stimulations of total cyclic AMP and cyclic GMP phosphodiesterase activities obtained with saturating levels of Ca^{2+} were, however, small in both cases. There were three possible explanations for the small effects observed. Firstly, that the Ca^{2+}/calmodulin–stimulated phosphodiesterase formed the majority of the cyclic nucleotide phosphodiesterase activity in the rat liver soluble fraction, but exhibited only a very small stimulation in the presence of Ca^{2+}. Secondly, that the levels of endogenous calmodulin in the preparation were too low to support maximal stimulation of phosphodiesterase activity, despite the presence of saturating Ca^{2+} concentrations. Lastly, that Ca^{2+} could stimulate the phosphodiesterase activity of this enzyme to a much greater degree than that observed in the crude supernatant but that the fold stimulation was masked due to the presence of a number of other Ca^{2+}–insensitive phosphodiesterases present in the supernatant.

The second possibility was discounted by the observation that the fold stimulations of the total cyclic AMP and cyclic GMP

phosphodiesterase activity obtained in the presence of saturating Ca^{2+} were almost identical to those obtained when saturating calmodulin was included in the assays. This indicated that calmodulin levels were already saturating in the rat liver soluble preparation.

In order to address the first and third possibilities, it was necessary to separate the individual phosphodiesterase activities present in the rat liver soluble fraction. A number of other groups have used anion-exchange chromatography on matrices such as DE-52 or DEAE-Sephacel (Thompson and Appleman, 1981; Russel, et al., 1973; Turnbull and Hickie, 1984; Weishaar, et al., 1986; Reeves, et al., 1987) to achieve this aim. In this study, I initially decided to use DE-52 to separate the individual activities. During the course of this study I consistenty used a substrate concentration of 1 μM to assess both cyclic AMP and cyclic GMP hydrolysis. For each of the individual resolved activities, I calculated a substrate selectivity ratio which expresses the ratio of cyclic AMP phosphodiesterase activity to cyclic GMP phosphodiesterase activity at 1 μM. This index gives an indication of the substrate selectivity of any individual activity but will only be appropriate at this concentration of cyclic nucleotide.

Three separate peaks were obtained when phosphodiesterase activity was eluted from the DE-52 column using a linear sodium chloride gradient (termed Peaks I to III). Peak I was found to hydrolyse both cyclic AMP and cyclic GMP equally well, Peak II was more selective for cyclic GMP, whilst Peak III was more selective for cyclic AMP. The individual properties of these Peaks were assessed by monitoring their responsiveness to saturating levels of Ca^{2+}, Ca^{2+}/calmodulin and to 2 μM cyclic GMP.

Ca^{2+}, by itself, was not found to stimulate significantly the cyclic AMP or cyclic GMP phosphodiesterase activities of any of these three Peaks of activity. This implied that, under the conditions that the separation was performed (i.e. in the presence of EGTA), the calmodulin moiety did not associate with phosphodiesterase activity.

When assays of calmodulin activity were performed, it was shown that on DE-52 chromatography calmodulin did indeed elute as an entity separate from phosphodiesterase. This is consistent with the observations of other workers, who showed that anion-exchange chromatography in the presence of EGTA is sufficient to prevent association of calmodulin with the Ca^{2+}/calmodulin-stimulated phosphodiesterase (Ho, et al., 1977; Strewler, et al., 1983).

Using Ca^{2+} and calmodulin together, it was shown that Peak I contained a Ca^{2+}/calmodulin-stimulated cyclic nuclotide phosphodiesterase. The degree of stimulation of the cyclic GMP phosphodiesterase activity of Peak I by Ca^{2+}/calmodulin was found to be about 2 fold, which was much greater than the fold stimulation of cyclic GMP phosphodiesterase activity observed when Ca^{2+} was added to the crude supernatant. This observation, and the ocurrence of two other peaks of activity that were insensitive to Ca^{2+}/calmodulin, therefore explains the low 'fold-stimulations' of phosphodiesterase activity observed when Ca^{2+} was added to a crude soluble fraction of rat liver.

In tissues such as bovine brain, where the Ca^{2+}/calmodulin-stimulated activity contributes a significant proportion of the total soluble phosphodiesterase activity, the addition of Ca^{2+} elicits a much greater increase in the total cyclic nucleotide phosphodiesterase activity than that observed here for the rat liver soluble fraction, presumably as a consequence of the preponderance of this activity in this tissue (Sharma, et al., 1980; Beavo, 1988).

Low (μM) concentrations of cyclic GMP have been used by a number of investigators as an activator/inhibitor of cyclic AMP hydrolysis (Beavo, et al., 1971; Weber and Appleman, 1982; Yamamoto, et al., 1984a; Harrison, et al., 1986b; MacPhee, et al., 1987). In this study I routinely used 2 μM cyclic GMP, a concentration that has been shown to be capable of eliciting a marked stimulation of the cyclic AMP phosphodiesterase activity of purified

preparations of the cyclic GMP-stimulated phosphodiesterase (Martins, et al., 1982; Yamamoto, et al., 1983a) including the rat liver soluble form (Pyne, et al., 1986b). This concentration of cyclic GMP will also cause a significant inhibition of cyclic AMP hydrolysis of purified and partially purified preparations of the cyclic GMP-inhibited phosphodiesterase (Grant and Colman, 1984; Yamamoto, et al., 1984a; Degerman, et al., 1987) including the rat liver 'dense-vesicle' phosphodiesterase (Pyne, et al., 1987a; Boyes and Loten, et al., 1988). Use of this concentration of cyclic GMP should thus allow detection of both the cyclic GMP-stimulated and cyclic GMP-inhibited phosphodiesterases. This analysis revealed that Peak II contained the cyclic GMP-stimulated phosphodiesterase and Peak III a cyclic AMP-specific activity that was insensitive to cyclic GMP. The presence of these three types of activity have also been observed by other workers in a soluble rat liver fraction (Russel, et al., 1973; Turnbull and Hickie, 1984).

On closer examination it was noted that, despite the fact that saturating levels of Ca^{2+}/calmodulin stimulated markedly the cyclic GMP hydrolysis of Peak I, these agents caused a much smaller stimulation of cyclic AMP hydrolysis. Thermal inactivation studies revealed that the cyclic AMP and cyclic GMP activities in this peak decayed with kinetics indicative of different active sites. Whilst this observation provided an indication that the cyclic AMP and cyclic GMP phosphodiesterase activities contained within this peak were associated with different enzymes, it was also possible that these two activities were associated with the same enzyme, with hydrolysis occurring at different active sites within either the same polypeptide or different subunits of the putative enzyme. This possibility was discounted by the observation that the cyclic AMP and cyclic GMP phosphodiesterase activities of Peak I could be separated from each other by chromatography on the dye-ligand affinity matrix Affi-gel Blue. In this instance, it was observed that the majority of the cyclic AMP phosphodiesterase activity of Peak I did not bind to this matrix, whilst

the majority of the cyclic GMP phosphodiesterase activity did. This implies that there were indeed two separate enzymic activities contained within this peak; one predominantly hydrolysing cyclic AMP and the other hydrolysing cyclic GMP. It was also observed that Ca^{2+}/calmodulin-stimulated phosphodiesterase activity was capable of binding to the Affi-gel Blue matrix. This was taken to imply that it was the species which preferentially hydrolysed cyclic GMP which was the Ca^{2+}/calmodulin-stimulated enzyme.

In order to assess this by an independent methodology, I used chromatographic separation on a high resolution anion-exchange column (Mono Q). When the rat liver soluble fraction was separated on this column this allowed for the separation of five peaks of phosphodiesterase activity (termed PDE MQ-I to PDE MQ-V). These were shown to differ both in their substrate selectivity and in their responses to effectors such as Ca^{2+}/calmodulin and cyclic GMP. These analyses revealed the presence of an a cyclic AMP-selective, IBMX-insensitive phosphodiesterase (PDE MQ-I), a Ca^{2+}/calmodulin-stimulated species (PDE MQ-II), a cyclic GMP-stimulated phosphodiesterase (PDE MQ-III) and two cyclic AMP-specific activities (PDE MQ IV and PDE MQ-V) in the rat liver soluble fraction. This was the first clear indication that the number and type of phosphodiesterase activities contained in the rat liver soluble fraction was greater than the three types observed previously in my studies and by other workers (Russel, et al., 1973; Turnbull and Hickie, 1984; Moss, et al., 1983).

PDE MQ-I was found to predominantly hydrolyse cyclic AMP and bound very poorly to Affi-gel Blue. In contrast, PDE MQ-II, which preferentially hydrolysed cyclic GMP, bound to the Affi-gel Blue column and was stimulated by Ca^{2+}/calmodulin. The behaviour of these two Mono Q-resolved Peaks was analagous to the behaviour of the cyclic AMP and cyclic GMP phosphodiesterase activities of DE-52 Peak I during Affi-gel Blue chromatography. This behaviour

strongly suggested that DE-52 Peak I was indeed comprised of two separate activities.

Attempts to elute the cyclic GMP phosphodiesterase activity from the Affi-gel Blue matrix were unsuccessful. This was the case when the source of the cyclic GMP phosphodiesterase activity was DE-52 Peak I or PDE MQ-II (from the Mono Q column). Although binding to Affi-gel Blue was usually performed in the presence of 5 mM $MgCl_2$, it was observed that when 5 mM $MgCl_2$ was replaced with 2 mM EDTA, PDE MQ-II could still bind to Affi-gel Blue. This observation suggested that binding of this activity to Affi-gel Blue could occur in the absence of added Mg^{2+} ions. When EDTA was used to elute activity that had been bound in the presence of $MgCl_2$ no substantial recovery of cyclic GMP phosphodiesterase activity was noted. Although the precise site(s) at which Affi-gel Blue binds to the enzyme(s) are unknown, it has been suggested that this matrix will bind proteins that possess a tertiary structure known as the 'dinucleotide fold'. This structure has been observed in all nucleotide binding proteins for which a three dimensional structure is known (Thompson, et al., 1975). Morril, et al., (1979) suggested that the substrate binding site of the Ca^{2+}/calmodulin-stimulated phosphodiesterase is constructed by a NAD-binding domain similar to that known to form the NAD- and ATP-binding sites in several dehydrogenases and kinases.

The dye ligand Cibacron Blue 3GA, which acts as the binding ligand in Affi-gel Blue, has been shown to be a competitive inhibitor of the Ca^{2+}/calmodulin-stimulated phosphodiesterase isolated from bovine brain (Morril, et al., 1979). On this basis, PDE MQ-II, which contains a Ca^{2+}/calmodulin-stimulated phosphodiesterase activity, may also bind to this matrix at or near to it's active site. Whilst no phosphodiesterase activity could be detected on matrix containing bound cyclic GMP phosphodiesterase activity, no activity could be eluted using high substrate concentrations (either cyclic AMP or cyclic GMP) which would be expected to compete with the Cibacron Blue

3GA for the nucleotide binding site and thus allow elution of the enzyme activity. One possible explanation for this behaviour is that Cibacron Blue 3GA may act as a non-competitive inhibitor of the enzyme by binding at a site distinct from the nucleotide binding site. Alternatively, the affinity of the Affi-gel Blue binding site on the enzyme may be so high as to preclude elution by substrate competition.

The properties of this activity seemed then to be different to those of the bovine brain form of the enzyme and this may be related to the observations that distinct isozymic forms of the Ca^{2+}/calmodulin-stimulated phosphodiesterase enzyme exist both within tissues and between species (Purvis, et al., 1981; Vandermeers, et al., 1983; Kincaid, et al., 1984; Sharma, et al., 1984; Sharma and Wang, 1986b; Sharma and Wang, 1990).

The observation that PDE MQ-I, unlike most other phosphodiesterase activities (Morril, et al., 1979; Marchmont, et al., 1981b; Pyne, et al., 1986b; Pyne, et al., 1987a), is unable to bind to Affi-gel Blue, even in the presence of Mg^{2+}, implied that this enzyme lacks either the appropriate dinucleotide fold or an appropriate divalent cation binding site. In a comparison of the inhibitor sensitivities of the rat liver-derived PDE MQ-Peaks, this activity was also found to differ from the other soluble activities and indeed other classes of phosphodiesterase (Weishaar, et al., 1985b; Hidaka and Endo, 1984). It was found to be insensitive to the reference phosphodiesterase inhibitors IBMX, Ro-20-1724 and milrinone. Whilst PDE MQ-III was similar in showing an insensitivity to these phosphodiesterase inhibitors, although it did show a marginal sensitivity to IBMX, it could easily be distinguished from PDE MQ-I by the ability of low (μM) concentrations of cyclic GMP to cause a marked stimulation of cyclic AMP hydrolysis, a property characteristic of the cyclic GMP stimulated class of phosphodiesterase which was not shared with PDE MQ-I. On this basis I propose that PDE MQ-I is a novel activity that had not previously been observed in the rat liver soluble fraction due to it's

coelution with the Ca^{2+}/calmodulin-stimulated cyclic GMP phosphodiesterase.

Rat liver contains a number of phosphodiesterase activities that are located in the particulate fraction derived from homogenates. At least one of these species, the 'dense-vesicle' phosphodiesterase can be released from it's membrane location by the action of endogenous proteases (Loten, et al., 1978; Loten, et al., 1980; Pyne, et al., 1987a). Hypotonic conditions have been shown to cause the release of the membrane bound 'dense-vesicle' phosphodiesterase due to lysis of lysosomes and the subsequent release of endogenous proteases (Loten, et al., 1978). This may also be true of other phosphodiesterase activities including the insulin-stimulated 'peripheral' plasma membrane phosphodiesterase.

If particulate phosphodiesterases are released from their membrane location either by the action of proteases or by other mechanisms (such as the loss of a divalent cation necessary for binding of a peripheral protein to a membrane) they will accumulate in the isolated soluble fraction. Consequently, the observed activities will not represent true soluble proteins. I was concerned that some of the multiplicity of phosphodiesterases that I observed in the soluble fraction derived from a rat liver homogenate may have arisen as a result of release of species of phosphodiesterases derived from a particulate source. The precautions that I took to prevent this occurence included the preparation of the homogenate under isotonic conditions and the presence of protease inhibitors during homogenisation and all subsequent steps. EGTA was also present during all steps to chelate endogenous calcium and so prevent activation of the Ca^{2+}-activated neutral protease, an activity that has been shown to be present in rat liver (Takai, et al., 1977; DeMartino, 1981).

EGTA has long been known to cause the release of peripheral proteins from membrane fractions, presumably as a result of it's ability

to chelate divalent cations such as Ca^{2+}. Because the soluble fractions were always prepared in the presence of EGTA this may have caused the release of peripherally localised phosphodiesterases such as the 'peripheral' plasma membrane phosphodiesterase. Such an artefactual release may be a possible explanation for the presence of the novel IBMX-insensitive phosphodiesterase in the soluble fraction. When the soluble fraction was prepared from rat liver in the presence of 0.1 mM $CaCl_2$ in place of EGTA there appeared to be no noticeable differences in the number and the substrate selectivities of the phosphodiesterases separated by Mono Q chromatography, indicating that these activities had not been released from membranes as the result of a Ca^{2+} chelation event.

From an analysis of the responses of the cyclic AMP hydrolysis catalysed by the individual PDE MQ-Peaks to the addition of 2 μM cyclic GMP, it was also apparent that no activity resembling the membrane bound 'dense-vesicle' phosphodiesterase (CGI-PDE) could be identified. If the presence of this activity in the soluble fraction is used as an index of protease-mediated release of phosphodiesterase activity, then it was apparent that no such event had taken place. Because it was only possible to assess a small number of potential events, the possiblity still remains, however, that some of the activities observed in the soluble fraction may represent solubilised particulate species. This is especially true of the two cyclic AMP-specific activities PDE MQ-IV and PDE MQ-V. The inhibitor sensitivities of these two activities bore a significant resemblance to the 'peripheral' plasma membrane phosphodiesterase, including insensitivity to cyclic GMP and sensitivity to the inhibitor ICI 63197 (Pyne, et al., 1986b; Houslay, 1988). Either or both of these two activities may represent a solubilised form of this enzyme. However, due to the lack of a suitable anti-'peripheral' PDE antisera at the time this study was carried out, this possiblity was not tested.

If it is assumed that each of the individual PDE MQ-Peaks represents only a single type of phosphodiesterase activity, then it was

apparent that the cyclic AMP and cyclic GMP phosphodiesterase activity of the rat liver soluble fraction is dominated by the cyclic GMP–stimulated phosphodiesterase (PDE MQ–III). The novel IBMX–insensitive phosphodiesterase represented about 20% of the total cyclic AMP phosphodiesterase activity, which was almost equal to the proportion contributed by the two cyclic AMP–specific phosphodiesterases, which together formed about 30% of the total rat liver–derived cyclic AMP phosphodiesterase activity.

The Ca^{2+}/calmodulin–stimulated phosphodiesterase (PDE MQ–II) contributed about 20% of the total cyclic GMP hydrolysis, whereas the most of the remainder of the cyclic GMP phosphodiesterase activity was provided by PDE MQ–III. PDE MQ–II contributed only about 2% of the total cyclic AMP phosphodiesterase activity. Although five fold stimulation of cyclic AMP phosphodiesterase activity was obtained in the presence of Ca^{2+}/calmodulin, it can be estimated that these agents would be unlikely to increase the total cyclic AMP hydrolysis by much more than 12% whilst, in contrast, cyclic GMP hydrolysis would be increased by about 24%. These estimated increases are very similar to the actual measured increases in total cyclic AMP and cyclic GMP phosphodiesterase activity elicited by saturating levels of Ca^{2+}, which were 19% and 27% respectively.

In a similar manner, one can estimate the fold–effect that would be expected if the concentration of cyclic GMP reached 2 μM in the rat liver–derived soluble fraction. Such a calculation, assuming a fold stimulation of cyclic AMP hydrolysis of 6.3, predicts that this level of cyclic GMP will increase the total cyclic AMP hydrolysis by about 3.3 fold This is similar to the level that was observed when 2 μM cyclic GMP was used to stimulate the total cyclic AMP phosphodiesterase activity of the rat liver–derived soluble fraction, which was 2.7 fold. These data would indicate that increases in cellular Ca^{2+} probably only mediate a small effect on the total rat liver soluble cyclic nucleotide phosphodiesterases, whilst increases in the

levels of cyclic GMP are more likely to have a profound effect on the total cyclic AMP phosphodiesterase activity.

Rat liver will contain a number of cell types, such as Kupffer cells, in addition to hepatocytes. Also, freshly excised liver will contain large amounts of trapped blood and, as such, homogenates of rat liver will presumably contain phosphodiesterases derived from these other cell types. I was concerned that the multiplicity of phosphodiesterase isozymes observed in the soluble rat liver fraction may, in part, be explained by a contribution from cell types other than hepatocytes. In order to address this possibility I decided to prepare hepatocytes by a collagenase digestion method. This approach allowed me to assess the number and type of phosphodiesterases in soluble fractions prepared from homogenates of freshly prepared hepatocytes. It should be noted that in all instances the hepatocytes were used without further incubation.

When the hepatocyte-derived soluble phosphodiesterases were fractionated on a Mono Q column, under identical conditions to those employed for the separation of soluble phosphodiesterases from the soluble rat liver fraction, a broadly similar elution profile was observed. The five peaks of activity possessed very similar properties to those of the rat liver-derived peaks in terms of their sensitivities to the effectors Ca^{2+}/calmodulin and cyclic GMP.

One noticeable difference between the two preparations occurred in the substrate selectivity of PDE-MQ-III at 1 μM substrate. Whilst, in liver, this activity was marginally more selective for cyclic GMP over cyclic AMP, this selectivity was more pronounced in the hepatocyte-derived form. Despite this difference, however, there was no noticeable change in the fold stimulation of cyclic AMP hydrolysis elicited by 2 μM cyclic GMP. It may be that this difference arises as a result of the presence in the rat liver-derived soluble fraction, of a slightly different isozyme of the cyclic GMP-stimulated

phosphodiesterase derived from a non-hepatocyte source which has a different substrate selectivity.

Whilst it is known that there are isozymes of the cyclic GMP-stimulated phosphodiesterase in rat liver, these appear to differ primarily in their subcellular location (soluble versus particulate) and sensitivity to effectors such as fatty acids rather than in their kinetic characteristics (Pyne, et al., 1986a). It is also possible that this difference may arise as a result of different modifications to the cyclic GMP-stimulated phosphodiesterase that occured during preparation either of the rat liver or hepatocyte soluble fractions. Such events may have included partial proteolysis or the reversal of a phosphorylation event. It is known, for example, that partial proteolysis of this species in canine heart will alter it's sensitivity to various inhibitors (Price, et al., 1987). Phosphorylation of this class of phosphodiesterase has not, however, been demonstrated to date. The answers to these questions remain then to be determined.

It was also noted that the small peak of activity that was occasionally found to partially coelute with rat liver-derived PDE MQ-III was never present in the Mono Q elution profiles obtained when the hepatocyte souble fractions were the source,

For these reasons the hepatocyte-derived activities were characterised in much greater detail than the rat liver species. In addition, no other workers have reported any work on the soluble phosphodiesterases of rat hepatocytes to date. In this respect, it was thus decided to extend the range of inhibitors used in the inhibition characteristics, determine the kinetic characteristics of the individual PDE MQ-Peaks, as well as characterise a number of other parameters for these activities. Included in this latter category were the Mg^{2+}-dependency of the various activities, as well as the Ca^{2+} and calmodulin dependencies of PDE MQ-II and the cyclic GMP dependency for stimulation of cyclic AMP hydrolysis catalysed by PDE MQ-III.

PDE MQ-I hydrolysed both cyclic nucleotides with linear Michaelis-Menten kinetics, implying that there was only one enzyme present in this peak of phosphdodiesterase activity. This was substantiated by the observation that only a single peak of phosphodiesterase activity was observed on gel filtration analysis, corresponding to a protein of molecular weight 33-kDa. The K_m value for cyclic AMP hydrolysis (25 μM) was higher than reported values for cyclic AMP-specific species such as the cyclic GMP-inhibited and cyclic GMP-insensitive subclasses of cyclic AMP-specific phosphodiesterases, which are usually in the range 0.1 μM to 1 μM (Harrison, et al., 1986b; Grant, et al., 1988; Degerman, et al., 1987; Pyne, et al., 1987a; Boyes and Loten, et al., 1988; Marchmont, et al., 1981b; Thompson, et al., 1988). In this respect, the K_m for cyclic AMP hydrolysis was more comparable to that of the cyclic GMP-stimulated phosphodiesterases, although these two activities were clearly very different in properties. The latter would, for example, demonstrate cyclic GMP stimulated cyclic AMP hydrolysis, exhibit positively cooperative kinetics for both cyclic AMP and cyclic GMP hydrolysis, as well as exhibiting comparable K_ms for hydrolysis of both cyclic nucleotides (Martins, et al., 1982; Yamamoto, et al., 1983a). In comparison, cyclic GMP did not stimulate the cyclic AMP hydrolysis of PDE MQ-I, whilst it's K_m for cyclic GMP hydrolysis was about an order of magnitude larger than that for cyclic AMP.

When the Mg^{2+} dependency of the phosphodiesterase activity of PDE MQ-I was assessed it was found that this activity expressed nearly full phosphodiesterase activity in the absence of added Mg^{2+} ions, although a small stimulation of activity was observed with increasing Mg^{2+} concentrations. An excess of EDTA (10 mM) was used in the assays of PDE MQ-I phosphodiesterase activity in an attempt to chelate any trace amounts of Mg^{2+} that could potentially support cyclic AMP hydrolysis. When this was performed, PDE MQ-I was still capable of hydrolysing cyclic AMP at nearly it's full capacity. In comparison, the other four hepatocyte-derived activities were found

to be nearly totally dependent on the addition of added Mg^{2+} to support hydrolysis of cyclic nucleotides. This difference in behaviour of PDE MQ-I implied that this activity was fundamentally different to the other soluble phosphodiesterases.

There are two possible explanations to account for this property of PDE MQ-I. The first is that, whilst other phosphodiesterases may require Mg^{2+} for binding and/or hydrolysis of cyclic nucleotides, PDE MQ-I can bind and hydrolyse cyclic nucleotides in the absence of added metal ions such as Mg^{2+}. The second explanation is that PDE MQ-I is a metallo-enzyme that already contains a tightly bound metal cofactor. The observation that high concentrations of EDTA do not cause inhibition of cyclic AMP phosphodiesterase activity, does not exclude the possibility that this putative metal ion may be Mg^{2+}. It is quite conceivable that the metal ion is bound to the protein in such a way as to exclude possible chelation by agents such as EDTA. Alternatively, the putative metal ion may be another metal ion such as Zn^{2+}.

There are well documented examples of phosphodiesterases that exist as metallo-enzymes. The high K_m cyclic nucleotide phosphodiesterase of the yeast *Saccharomyces cerevisiae* contains 2.4 atoms of zinc/43-kDa subunit (Londesborough and Suoranta, 1983). The rat liver 'peripheral' plasma membrane phosphodiesterase has also been shown to be a metallo-enzyme (Londesborough, 1985). It is of interest to note that when the amino acid sequence of the yeast Zn^{2+}-containing enzyme is compared with those of several other phosphodiesterases, including the *Drosphila* cyclic AMP phosphodiesterase and the bovine Ca^{2+}/calmodulin-stimulated and cyclic GMP-stimulated phosphodiesterases, it bears little or no sequence homology with these enzymes (Charbonneau, et al., 1986; Nikawa, et al., 1987; Colicelli, et al., 1989; Swinnen, et al., 1989; Davis, et al., 1989). It is tempting to speculate that, given the large number of differences in properties between the PDE MQ-I species and the other soluble phosphodiesterases, this activity may be more

related to the yeast high K_m enzyme than to any of the other classes of phosphodiesterase.

It has been shown by numerous other workers that several classes of phosphodiesterase are sensitive to proteolysis and that this event can lead to the production of catalytically active species that possess different properties to those of the 'parent' activity. This has been suggested to occur, for example, after trypsinisation of a soluble uterine phosphodiesterase (Strada, et al., 1981). The best documented example of this is the bovine brain Ca^{2+}/calmodulin – stimulated phosphodiesterase. Partial proteolysis of this activity produces a species that is insensitive to Ca^{2+}/calmodulin and which has altered kinetic parameters that are similar to those of the Ca^{2+}/calmodulin – stimulated enzyme (as opposed to the basal (non – stimulated) enzyme) (Tucker, et al., 1981; Kincaid, et al., 1985).

Given that the monomeric size of PDE MQ – I (33 – kDa) is much smaller than that of other purified phosphodiesterases such as the Ca^{2+}/calmodulin – stimulated PDE (LaPorte, et al., 1979; Kincaid, et al., 1981b; Sharma, et al., 1984), the cyclic GMP – stimulated phosphodiesterase (Martins, et al., 1982; Yamamoto, et al., 1983a; Pyne, et al., 1986a) and the cyclic GMP – inhibited phosphodiesterases (Harrison, et al., 1986b; Pyne, et al., 1987a; Boyes and Loten, 1988; Degerman, et al., 1987; Grant, et al., 1988; MacPhee, et al., 1986), it is conceivable that PDE MQ – I represents a proteolysed product of another type of rat liver phosphodiesterase rather than an activity in it's own right. Molecular cloning analyses have revealed that most of the known phosphodiesterase sequences contain a conserved region of approximately 270 amino acids (Charbonneau, et al., 1986; Swinnen, et al., 1989; Colicelli, et al., 1989; Davis, et al., 1989). Given the similarity of this region between the known phosphodiesterase sequences it has been suggested that this region contains the active site where cyclic nucleotide hydrolysis takes place (Charbonneau, et al., 1986). Furthermore, the number of amino acids in this sequence is more than sufficient to encode a protein of molecular weight 33 – kDa. It is

possible then to envisage a scenario where proteolysis of a larger phosphodiesterase produces a molecule that essentially contains only the 'active site' of the parent molecule, thus accouting for the presence of PDE MQ-I in the soluble fraction.

I suggest that, on the basis of the known characteristics of PDE MQ-I, this is not the case and that PDE MQ-I is an independent activity. Furthermore, I took precautions to avoid the effect of proteolysis by adding protease inhibitors during the preparation of the PDE MQ-Peaks. In addition, PDE MQ-I is eluted very rapidly from the Mono Q column (within 20 minutes) so it is rapidly separated from the majority of other soluble proteins. It seems extrememly unlikely that proteolysis could produce a catalytic activity that is independent of added Mg^{2+} from a 'parent' molecule that is dependent on the presence of this divalent metal ion for catalysis. Furthermore, it's marked inhibitor insensitivity shows that the catalytic activity of PDE MQ-I differs significantly from all of the other phosphodiesterase species examined in this study and is therefore unlikely to be derived from any of them. Likewise it's inability to bind to Affi-gel Blue suggests strongly that this enzyme is different in properties compared to other phosphodiesterases.

In support of this proposition, an enzyme that has bears a striking resemblance to PDE MQ-I has been purified from pig liver by Helfman and his colleagues (Helfman, et al., 1981). This enzyme was a multifunctional phosphodiesterase capable of hydrolysing both purine and pyrimidine cyclic nucleotides. The purified enzyme behaved as a monomer of molecular weight 31,000 to 37,000 under native conditions. It lacked absolute substrate specificity hydrolysing both cyclic AMP and cyclic CMP and to a lesser extent cyclic GMP. The apparent K_m for cyclic AMP (25 µM) was lower than the apparent K_m for cyclic CMP (182 µM) but hydrolyses cyclic CMP as efficiently as cyclic AMP because of a higher V_{max} for this pyrimidine cyclic nucleotide (Helfman, et al., 1981). This activity was also found to be insensitive to phosphodiesterase inhibitors such as IBMX and theophylline, but was

inhibited by increased phosphate concentrations (Kuo, et al., 1978). The enzyme from pig liver eluted from DEAE-cellulose columns at low ionic strengths, as PDE MQ-I does, and was found to be unable to bind to Affi-gel Blue. When the enzyme was assayed in the absence of added Mg^{2+} ions it was still found to be capable of hydrolysing nucleotides. The addition of Mg^{2+} stimulated the enzyme slightly, whilst the inclusion of EDTA as high as 20 mM had no effect on the activity (Helfman, et al., 1981). All these properties are identical to the properties of PDE MQ-I that I measured.

Whilst I did not assess the ability of PDE MQ-I to hydrolyse cyclic CMP, I did show that cyclic CMP could act as inhibitor of cyclic AMP hydrolysis catalysed by PDE MQ-I. If it was assumed that cyclic CMP acted as a competitive inhibitor, the observed IC_{50} for it's action would be consistent with a K_m above 100 μM. It seems almost certain then that these two activities are one and the same enzyme.

Cyclic CMP, a pyrimidine cyclic nucleotide, has been implicated in playing a biological role in the control of proliferation of leukaemia cells. Cech and Ignarro, (1977) have shown that an enzyme system, cytidylate cyclase, capable of synthesising cyclic CMP from CTP, is present in normal mouse liver. Further to this, these workers demonstrated that cyclic CMP formation proceeds more rapidly in regenerating normal liver than in controls. The highest levels of cytidylate cyclase activity were observed in liver with lowest levels observed in lung (Cech and Ignarro, 1977). Whilst these observations indicate that one particular function of PDE MQ-I in hepatocytes may be to degrade cyclic CMP that has been produced by the cytidylate cyclase activity, it is also apparent that this activity forms a large proportion of the total soluble cyclic AMP phosphodiesterase activity in both hepatocytes and in whole liver and this may be an equally important function.

Kinetic analysis of cyclic GMP phosphodiesterase activity catalysed by PDE MQ-II indicated that this activity hydrolysed this

cyclic nucleotide with apparently negatively cooperative kinetics. This observation could be interpreted in two ways. Either there was one activity present in this peak which behaves with negatively cooperative kinetics or there were two activities which behaved with linear kinetics. Some support for the latter possibility was obtained when PDE MQ-II was applied to a gel filtration column. In all instances, there was evidence of two closely eluting activities which would be consistent with there being two separate activities present in this Peak.

A Ca^{2+}-insensitive cyclic GMP phosphodiesterase activity has been proposed to be present in the soluble fraction of rat liver (Strewler, et al., 1983). This activity coelutes with the Ca^{2+}/calmodulin-stimulated cyclic GMP phosphodiesterase on DE-52 chromatography when this is performed in the presence of EGTA. If the separation is performed in the presence of Ca^{2+}, in place of EGTA, these workers observed that the Ca^{2+}/calmodulin-stimulated phosphodiesterase activity eluted at higher ionic strenghts so that it now partially coeluted with the Peak II activity. Ho, et al., (1977) were the first to show that addition of Ca^{2+} and calmodulin to crude preparations of soluble calmodulin-sensitive phosphodiesterase from bovine heart altered the chromatographic behaviour of the enzyme, presumaly because of a Ca^{2+}-dependent association with calmodulin, a highly acidic protein. These workers observed that this alteration in the chromatographic properties of the Ca^{2+}/calmodulin-stimulated enzyme revealed the presence of a Ca^{2+}-insensitive activity. Furthermore, these workers showed that this activity did not bind to calmodulin-Sepaharose, whilst the Ca^{2+}/calmodulin-sensitive activity did.

When the kinetics of these two cyclic GMP phosphodiesterases were examined by these workers (Strewler, et al., 1983), the Ca^{2+}/calmodulin stimulated activity was found to exhibit linear kinetics for cyclic GMP hydrolysis with a K_m for cyclic GMP hydrolysis of 6.5 μM whilst the Ca^{2+}-insensitive activity exhibited anomalous kinetics with evidence of negative cooperativity (K_m values of 1.2 μM

and 27 μM. PDE MQ-II has a low K_m value of 3 μM and a high K_m value of 13 μM for cyclic GMP hydrolysis. It is thus conceivable, based on this evidence, that the anomalous kinetics obtained for cyclic GMP hydrolysis catalysed by PDE MQ-II may be explained by the presence of these two activities.

It has long been known that partial proteolysis of the Ca^{2+}/calmodulin-stimulated phosphodiesterase will produce a species that is no longer sensitive to Ca^{2+}/calmodulin (Tucker, et al., 1981; Kincaid, et al., 1985). This observation may explain the presence of this Ca^{2+}-insensitive activity in the soluble fraction of rat liver. However, Strewler, et al., (1983) argued that the existence of this Ca^{2+}-insensitive activity was not due to proteolysis of the Ca^{2+}/calmodulin-stimulated enzyme since they included Leupeptin in all their buffers. This peptide acts a protease inhibitor and has been shown to inhibit proteolytic activation of a phosphodiesterase in rat liver homogenates (Strewler and Manganiello, 1979). On this basis, and the observation that the apparent molecular weight of the Ca^{2+}-insensitive activity (325,000) was greater than the apparent molecular weight of the Ca^{2+}/calmodulin-stimulated activity (150,000), they considered that this activity was not derived from the Ca^{2+}/calmodulin-stimulated activity. If this is the case, then it may be that this activity is related to the cyclic GMP-binding cyclic GMP-specific phosphodiesterases that have been observed in rat lung and platelets (Hamet and Coquil, et al., 1978; Coquil, et al., 1980; Francis, et al., 1980; Coquil, et al., 1985), although no work has been done in this direction.

These same workers were also able to show that the Ca^{2+}/calmodulin-stimulated activity was much more selective for cyclic GMP than for cyclic AMP (Strewler, et al., 1983), an observation that I noted for the PDE MQ-II activity. The Ca^{2+}-insensitive cyclic GMP phosphodiesterase was, however, not found to be selective for cyclic GMP since it hydrolysed cyclic AMP with nearly equal efficiency at 0.5 μM substrate. It exhibited anomalous kinetics for

both cyclic AMP and cyclic GMP hydrolysis, the apparent K_ms for cyclic AMP hydrolysis being 15 μM and 302 μM. Since these workers used DE−52 to separate these activities, it would be expected that the IBMX−insensitive activity would co−elute with the Peak I activity and since it is insensitive to Ca^{2+}/calmodulin would not be expected to show any alteration in chromatographic properties in the presence of Ca^{2+}. It is my contention that the cyclic AMP phosphodiesterase activity of the Ca^{2+}−insensitive species that these workers observed is due to the presence of the IBMX−insensitive activity (PDE MQ−I). In support of this, the low K_m value for cyclic AMP hydrolysis (15 μM) is very similar to the K_m for cyclic AMP hydrolysis catalysed by PDE MQ−I (25 μM). This explanation would also explain the anomalous kinetics for cyclic GMP hydrolysis of this Ca^{2+}−insensitive activity.

An analysis of the Ca^{2+} dependency for stimulation of cyclic GMP hydrolysis by PDE MQ−II revealed that the EC_{50} value was 20 μM. This was the total concentration of calcium added to the assays and does not represent the actual free concentration. There were, however, intrinsic difficulties in estimating the actual level of free Ca^{2+} in the assays due to the presence of EGTA, a Ca^{2+} chelator. The ability of EGTA to chelate Ca^{2+} is critically influenced by a number of factors such as ionic strength, pH and the 'basal' level of Ca^{2+} contributed by contamination from buffer constituents and the water used to prepare these buffers. Further to this, EGTA is a very poor buffer of Ca^{2+} concentrations above 0.5 μM, which arises as a consequence of the relative binding affinities of the four binding sites for Ca^{2+} (Miller and Smith, 1984). The consequence of this is that small alterations in the total added Ca^{2+} can produce large changes in the actual free concentration of Ca^{2+}. All these problems mean that the value for the estimated EC_{50} for Ca^{2+}−stimulation of PDE MQ−II can only be used as a rough indication of the EC_{50} rather than an absolute value. A further complication is that Ca^{2+} and calmodulin show synergistic interactions in the activation of phosphodiesterase. An

increase in calmodulin or Ca^{2+} concentration results in an increase in the apparent enzyme affinity for towards Ca^{2+} or calmodulin respectively (Brostrom and Wolff, 1974). The implication of this is that, depending on the level of calmodulin used to determine the EC_{50} for Ca^{2+} stimulation of PDE MQ-II phosphodiesterase activity, this will alter the value of the measured EC_{50}.

The measured EC_{50} for calmodulin stimulation of PDE MQ-II, in the presence of a saturating Ca^{2+} concentration (500 μM), was 30 ng/ml which corresponds to 1.7 nM for a monomeric protein of 17,000 molecular weight. This value compares with an affinity for the bovine brain 61-kDa and 63-kDa isozymes of the Ca^{2+}/calmodulin-stimulated enzyme of 1 nM (Hansen and Beavo, 1986). In contrast, the bovine heart form has an affinity constant, which is about 10 fold lower at 0.1 nM (Hansen and Beavo, 1986). It would thus appear that the Ca^{2+}/calmodulin-stimulated isozyme in rat liver is very similar to the bovine brain enzymes with respect to it's activation by calmodulin. When the calmodulin anatagonist 5-Iodo-C8 was used to inhibit the phosphodiesterase activity of PDE MQ-II, it was found that it had virtually no inhibitory action against the basal cyclic GMP and cyclic AMP phosphodiesterase activity of PDE MQ-II, whilst it was capable of inhibiting the 'Ca^{2+}/calmodulin-stimulated' portion of the phosphodiesterase activity. The observed IC_{50} for it's action in the presence of saturating Ca^{2+} (100 μM) and calmodulin (0.12 μM) was observed to be 13 μM when cyclic GMP was the substrate and 16 μM when cyclic AMP was the substrate. These values are slightly greater than the IC_{50} reported for it's action against partially purified preparations of the bovine heart Ca^{2+}/calmodulin-stimulated isozyme, which was 3 μM (MacNeil, et al., 1988). Whether this small difference is related to the occurence of isozymes of the Ca^{2+}/calmodulin-stimulated phosphodiesterase is uncertain, but it is known that the bovine heart isozyme has a higher affinity for calmodulin than the bovine brain form (Hansen and Beavo, 1986).

When the kinetic properties of the PDE MQ-III species were examined, it was found that the activity displayed anomalous positively cooperative kinetics for the hydrolysis of both cyclic nucleotides. On this basis it seemed unlikely that there were other species of phosphodiesterase present in this peak of activity. The estimated K_m values for cyclic GMP and cyclic AMP were found to be very similar to each other (36 μM and 38 μM respectively), with cyclic AMP displaying a more marked positively cooperative behaviour than cyclic GMP (a larger h_{app}). The hepatocyte-derived activity was more selective for cyclic GMP than for cyclic AMP. This selectivity for cyclic GMP can be explained by the much larger V_{max} for cyclic GMP than for cyclic AMP.

When the properties of this rat hepatocyte-derived activity were compared to those of the purified rat liver soluble and membrane-bound forms of the enzyme, it was apparent that a number of differences existed. Firstly, the purified rat liver soluble form of the enzyme, whilst exhibiting similar K_m values to PDE MQ-III for cyclic AMP and cyclic GMP (40 μM and 25 μM respectively), was found to exhibit linear kinetics for the hydrolysis of cyclic GMP (Pyne, et al., 1986a). This has been attributed to the ability of cyclic GMP to induce a loss of positively cooperative kinetics for cyclic AMP hydrolysis. As such, these workers suggested that the reason why the hydrolysis of cyclic GMP by the rat liver enzyme obeys Michaelis-Menten kinetics is that under the conditions that kinetic determinations are carried out, both the regulatory and substrate sites would be expected to be occupied by cyclic GMP. This did not appear to be the case for PDE MQ-III, which clearly displayed positively cooperative kinetics for hydrolysis of cyclic GMP. It should be noted that enzymes purified from bovine tissues have very similar K_m values for hydrolysis of cyclic AMP (30 μM to 33 μM) (Martins, et al., 1982; Yamamoto, et al., 1983a) to PDE MQ-III. The same is true of the Hill coefficient (h_{app}), being 1.8 to 1.9 for the bovine enzymes, whilst PDE-MQ-III was found to have a Hill coefficient of 1.62. These

bovine-derived activities also exhibited positively cooperative kinetics for cyclic GMP hydrolysis, as PDE MQ-III does.

The second point of difference was that the purified soluble rat liver enzyme was inhibited by the non-selective phosphodiesterase inhibitor IBMX with an IC_{50} of 44 µM (Pyne, et al., 1986). Whilst I did observe a similar IC_{50} for inhibition of the rat liver-derived activity (97 µM), the IC_{50} for inhibition of the hepatocyte-derived activity was much larger (323 µM). The reasons for these differences is not immediately apparent, but it may be related to the existence of distinct isozymes of the cyclic GMP-stimulated phosphodiesterase in cell types other than hepatocytes.

IBMX is a well establised non-selective inhibitor of cyclic AMP phosphodiesterases (Weishaar, et al., 1985b; Wong and Ooi, 1985; Wells and Miller, 1988). However, it has been shown to interact with both substrate and regulatory sites of cyclic GMP-stimulated phosphodiesterases. Indeed, low concentrations of IBMX have been noted as causing activation of cyclic AMP hydrolysis for the bovine liver enzyme (Yamamoto, et al., 1983b) and crude and highly purified preparations from rat liver (Erneux, et al., 1982; Pyne, et al., 1986a). Higher concentrations of IBMX are inhibitory. These workers have shown that IBMX could stimulate cyclic AMP hydrolysis about 200 to 300% at a maximal concentration of 50 µM, with inhibition of cyclic AMP hydrolysis observed at higher concentrations of this inhibitor. Whilst I did observe some stimulation of cyclic AMP hydrolysis in the presence of IBMX at concentrations below 100 µM, the extent of this stimulation was much smaller with only effects up to about 30% being observed. The reasons for this difference in properties is not apparent.

The degree of stimulation of cyclic AMP hydrolysis of hepatocyte-derived PDE MQ-III elicited by cyclic GMP was of a very similar magnitude to that observed for the bovine heart enzyme and the purified rat liver soluble and particulate enzymes (Martins, et al., 1982; Pyne, et al., 1986a). It was, however, considerably less than the

32–fold stimulatory effect seen with the bovine liver enzyme (Yamamoto, et al., 1983a). Nevertheless, the K_a for activation of the bovine liver enzyme (0.5 μM) and the purified rat liver enzymes (0.23 μM to 0.28 μM) were very similar to that observed for the PDE MQ–III activity (0.47 μM).

Using the higher resolving power of Mono Q anion–exchange chromatography over that of the more conventional DE–52 chromatography, it was apparent that the one peak of cyclic AMP–spe cific activity observed on the latter could be separated into two peaks on Mono Q chromatography. These two cyclic AMP–specific activities were similar in a number of their properties including their insensitivity to cyclic GMP and Ca^{2+}/calmodulin. Although a small and statistically significant stimulation of the cyclic AMP phosphodiesterase activity of PDE MQ–V was observed when hepatocytes was the source, the stimulation was only of the order of 20% which was much smaller than that observed for PDE MQ–II when cyclic AMP was the substrate (fold stimulation of 4.77). Further to this, it was not investigated if this activation was a direct effect of calmodulin or was mediated by some other mechanism such as the activation of a contaminating Ca^{2+}–dependent protease.

When the inhibitor sensitivities of the two cyclic AMP–specific activities were compared, they displayed very similar sensitivities. They were both shown to be sensitive to the inhibitors IBMX, milrinone, ICI 63197 and Rolipram. PDE MQ–V, however, showed a slightly greater susceptibility to inhibition by Rolipram than PDE MQ–IV. Moreover, these two activities were shown to be the only soluble activities that were sensitive to rolipram. The inhibitor ICI 118233 was not found to be inhibitory for either of these two activities or indeed any of the other activities. The cyclic GMP phosphodiesterase inhibitor, Zaprinast (M&B 22,948), was found to exert only a very weak inhibitory effect on PDE MQ–IV and an even weaker effect on PDE MQ–V; in contrast this inhibitor selectively inhibited the cyclic GMP phosphodiesterase activity of PDE MQ–II, which contains the Ca^{2+}/calmodulin–

stimulated phosphodiesterase. PDE MQ-IV and PDE-MQ-V differed however in their sensitivity to the cyclic AMP-specific PDE inhibitor Ro-20-1724. PDE MQ-V was found to be sensitive to inhibition by Ro-20-1724, whilst PDE MQ-IV was much less sensitive. This difference was most apparent when rat liver was the source, although it was still noted with hepatocyte-derived activities.

It has been observed by a number of workers that there are subclasses of the cyclic AMP-specific phosphodiesterase. These have been broadly classified as cyclic GMP-inhibited (Yamamoto, et al,, 1984a; Pyne, et al., 1987a; Whitson and Appleman, 1982; Weber and Appleman, 1982; Weishaar, et al., 1987a; Weishaar, et al., 1987b; Weishaar, et al., 1987c; Weishaar, et al., 1987d; Manganiello, et al., 1990b) or cyclic GMP-insensitive (or Rolipram-sensitive) (Yamamoto, et al., 1984a; Weishaar, et al., 1987a; Weishaar, et al., 1987b; Reeves, et al., 1987; Whitson and Appleman, 1982; Pyne, et al., 1987b; Conti and Swinnen, 1990). The cyclic GMP-inhiibited activities are characterised by the ability of low (μM) levels of cyclic GMP to elicit a potent inhibition of cyclic AMP hydrolysis. The cyclic GMP-insensitive activities possess cyclic AMP hydrolytic activities that are much less sensitive to inhibition by cyclic GMP. These two subclasses of phosphodiesterase can be further distinguished from each other on the basis of sensitivity to drugs such as cilostamide or rolipram (Degerman, et al., 1987; Umekawa, et al., 1984; Schneider, et al., 1987; Weishaar, et al., 1987a; Livi, et al., 1989). From the characteristics of PDE MQ-IV and PDE MQ-V it would seem that these activities belong to the cyclic GMP-insensitive subclass of phosphodiesterase.

It has been suggested that, in addition to sensitivity to cyclic GMP, the inhibitor Ro-20-1724 can be used to distinguish this class of phosphodiesterase from the cyclic GMP-inhibited subclass of phosphodiesterase (Elks and Manganiello, 1985; Elks and Manganiello, 1984a; Weishaar, et al., 1987a). The former class would exhibit sensitivity to Ro-20-1724, whilst the latter would be less sensitive. This would appear to be an oversimplification since PDE MQ-IV and

PDE MQ – V, although they are both clearly insensitive to inhibition by cyclic GMP, also show differential sensitivity to Ro – 20 – 1724 The simplest interpretation of these results is that there are multiple forms of this type of phosphodiesterase present in hepatocytes which can be distinguished on the basis of their sensitivity to Ro – 20 – 1724.

In support of this notion, recent cloning analysis has revealed the presence of multiple cDNA clones for cyclic AMP – specific phosphodiesterases. Davis, et al., (1989) isolated three cDNA clones from rat brain (termed RD1 to RD3) having a large degree of sequence homology to each other that appeared to arise as a result of differential splicing. A second clone has also been isolated from a rat brain cDNA lilbrary that differs from the RD1 clone (Colicelli, et al., 1989). When this clone (DPD) was expressed in the yeast *Saccharomyces cerevisiae*, the enzyme encoded was shown to possess very similar properties to those of PDE MQ – IV and PDE MQ – V. DPD encoded a high – affinity phosphodiesterase activity (K_m of 3.5 μM) that was insensitive to Ca^{2+}/calmodulin, insensitive to cyclic GMP inhibition and did not hydrolyse cyclic GMP. RD1 was also shown to encode a phosphodiesterase with very similar properties to the DPD and PDE MQ – IV and PDE MQ – V activities. Both RD1 and DPD were found to be similarly inhibited by Ro – 20 – 1724 and Rolipram (Henkel – Tigges and Davis, 1990).

Swinnen, et al., (1989) also isolated a number of cDNA clones from rat sertoli and rat testis libraries. They termed these clones ratPDE1 to ratPDE4 and, on the basis that they have significant homology with the cyclic AMP – specific phosphodiesterase from *Drosophila melanogaster* (*dunce* phosphodiesterase), have hypothesised that these will also encode high – affinity cyclic AMP – specific phosphodiesterases. However, these individual clones have not yet been expressed to determine the characteristics of the phosphodiesterases that they encode. These four clones appeared to be independent because, although very similar to each other, they differed in sequence both

within the 'highly conserved' region and in particular at their amino and carboxyl terminii.

Together, these results would suggest that the number of cyclic AMP-specific phosphodiesterases may be far greater than originally suspected from a biochemical analysis. The relationship between the hepatocyte-derived PDE MQ-IV and PDE MQ-V and these cloned species is however unclear at the present time but remains to be established.

Whilst two cyclic AMP-specific phosphodiesterases have been identified in the rat hepatocyte fraction, by separation on a Mono Q anion-exchange column, the actual number of activities in the soluble fraction may be larger than this. When the kinetics of cyclic AMP hydrolysis of PDE MQ-IV and PDE MQ-V were examined, it was apparent that PDE MQ-IV hydrolysed cyclic AMP with anomalous kinetics indicative of negative cooperativity. As for the PDE MQ-II activity, which also displayed kinetics indicative of negative cooperativity, this may also be explained by the presence of multiple cyclic AMP phosphodiesterase activities in PDE MQ-IV. One supporting piece of evidence for this notion comes from the observation that PDE MQ-IV elutes from the Mono Q column as a much larger peak of activity than any of the other activities. Such a broad elution profile may be indicative of multiple species of cyclic AMP-specific phosphodiesterases which differ in their surface electronegativities (and presumably their primary sequence). Alternatively, the 'multiplicity' suggested by the broad elution profile may have arisen as a result of proteolysis of a single 'parent' molecule producing species which maintain their specificity for cyclic AMP as substrate but which have slightly different surface electronegativities due to removal of different portions of the polypeptide chain. At present it is not possible to assess which of these possibilities is correct.

Two high affinity, membrane-bound cyclic AMP phosphodiesterases have been identified in rat liver and rat hepatocytes.

These are the 'dense-vesicle' and 'peripheral' plasma membrane phosphodiesterases (Marchmont, et al., 1981b; Pyne, et al., 1987b; Loten, et al., 1978; Pyne, et al., 1987a; Pyne, et al., 1986b). In all the properties of PDE MQ-IV and PDE MQ-V that I examined these activities did not appear to be related to the 'dense-vesicle' phosphodiesterase. Whilst cyclic GMP can exert a potent inhibitory effect on the hydrolysis of cyclic AMP by the particulate rat liver 'dense-vesicle' cyclic AMP-specific phosphodiesterase, the cyclic AMP phosphodiesterase activities of PDE MQ-IV and PDE MQ-V were relatively insensitive to inhibition by cyclic GMP. Furthermore, the 'dense-vesicle' enzyme is also very potently inhibited by milrinone, whereas PDE MQ-IV and PDE MQ-V have IC_{50} values which are some 10-fold lower.

The sensitivity of PDE MQ-IV and PDE MQ-V to the inhibitor ICI 63197 may suggest that these two activities are related to the 'peripheral' plasma membrane phosphodiesterase which is potently inhibited by this compound. It may be that these two activities represent soluble homologues of this membrane bound activity. It is also conceivable that either or both of these two activities represent solubilised form(s) of the 'peripheral' plasma membrane phosphodiesterase. Due to the lack of an available antisera to the 'peripheral' plasma membrane phosphodiesterase, it was not possible to assess this possibility directly.

In summary, I have identified five soluble phosphodiesterase activities that are present in both the soluble fraction of rat liver and hepatocytes. In particular, I have identified a novel IBMX-insensitive phosphodiesterase (PDE MQ-I) that forms a substantial proportion of the total soluble cyclic AMP phosphodiesterase activity. This has the unusual property of being highly insensitive to a number of phosphodiesterase inhibitors including the non-selective inhibitor IBMX. This suggests that caution should be applied in interpreting whole cell cyclic AMP accumulation studies where such a compound is routinely used to block the hydrolysis of cyclic AMP. Indeed it has

been shown, in hepatocytes, that even at high concentrations of IBMX, glucagon-mediated increases in intracellular cyclic AMP can still be decreased (Heyworth, et al., 1983d).

When the soluble fractions were prepared from a number of other rat tissues including brain, kidney and adipocytes it was apparent that different elution profiles were obtained from those of rat liver. The kidney soluble fraction was shown to possess four activities, two of which were selective for cyclic GMP and two which were selective for cyclic AMP. The two cyclic GMP phosphodiesterases were shown to be stimulated by Ca^{2+}/calmodulin and cyclic GMP respectively. However, these effects were not characterised by me any further. Recently work on these activities has been carried out in this laboratory by other workers (Hoey and Houslay, 1990). The two cyclic AMP-specific peaks were not susceptible to inhibition by cyclic GMP, and like PDE MQ-IV and PDE MQ-V were susceptible to inhibition by ICI 63197. These two activities were very similar to PDE MQ-IV and PDE MQ-V in other respects; although both sensitive to inhibition by Ro-20-1724 the latter activity was found to be more sensitive to this compound than the former. Importantly, such analyses showed that there was no evidence for the presence of an activity corresponding to PDE MQ-I, which would be expected to elute at low ionic strength from the Mono Q column. This implies that this activity may show a very limited tissue distribution.

The adipocyte soluble fraction was similar to that of the kidney profile in that again there was no evidence of the PDE MQ-I species. The major cyclic GMP hydrolysing activity, although eluting at a position similar to that of the cyclic GMP-stimulated activity, was shown to contain the Ca^{2+}/calmodulin-stimulated phosphodiesterase. The reason for this difference in elution properties is unknown, but it may suggest that this activity is a distinct member of the Ca^{2+}/calmodulin-stimulated family of enzymes, a number of which have been identified and purified (for review see Wang and Sharma, 1990). There were also two cyclic AMP-selective activities, one of

which partially co-eluted with the Ca^{2+}/calmodulin-stimulated activity. The position of this activity was different to that of the PDE MQ-IV and PDE MQ-V activities that were observed in rat liver and hepatocyte fractions. Whilst no detailed characterisation of these activities was carried out, this difference in elution properties may suggest that these are distinct members of the cyclic AMP-specific phosphodiesterases.

The soluble fraction of rat brain contained at least four phosphodiesterase activities. Because of a lack of time these activities were not characterised but once again there was no evidence of an activity that resembled PDE MQ-I. All these observations support the hypothesis that this activity is restricted to a limited number of tissue/cell types perhaps even as far as only being expressed in hepatocytes but this suggestion will need further analysis.

One of the interest of the laboratory that this work was carried out in is to identify and characterise insulin-stimulated phosphodiesterases. Two such membrane bound activities have been identified and purified to date and these are the insulin and glucagon stimulated 'dense-vesicle' phosphodiesterase and the insulin-stimulated 'peripheral' plasma membrane phosphodiesterase. Whilst these activities may play a role in decreasing the intracellular levels of cyclic AMP under certain defined conditions, it was shown by Heyworth, et al., (1984a) that when insulin-induced activation of these two enzymes was blocked, then insulin could still promote a decrease in hepatocyte intracellular levels that have previously been increased with glucagon. The ability of insulin to decrease the glucagon-elevated intracellular cyclic AMP concentration was, however, still blocked by the cyclic AMP phosphodiesterase inhibitor IBMX (Heyworth, et al., 1984a). It seems therefore that the effect of insulin is mediated by one or other of the multiple cyclic AMP phosphodiesterase species found in the hepatocyte (Thompson and Strada, 1978; Beavo, et al., 1982; Houslay, 1986; Beavo, 1988). Using Percoll gradient analysis, Heyworth, et al., (1983d) have shown that insulin can stimulate the activity of only two

phosphodiesterase and these are the membrane bound 'dense-vesicle' and 'peripheral' phosphodiesterases, they did not, however, observe stimulation of any other phosphodiesterases under these conditions.

If a 'third' insulin-stimulated activity is present, insulin's stimulation of the activity may either be readily reversible or extremely labile in nature. Two obvious candidates for such a reversibly activated cyclic AMP phosphodiesterase would be the Ca^{2+}/calmodulin-stimulated phosphodiesterase or the cyclic GMP stimulated phosphodiesterase since these are regulated in a reversible manner by Ca^{2+} and cyclic GMP respectively. In this chapter, the rat hepatocyte Ca^{2+}/calmodulin-stimulated phosphodiesterase has been shown to be soluble, as it is in other tissues, and it can be stimulated Ca^{2+} in the presence of calmodulin. It has been proposed that increases in intracellular Ca^{2+} may be a key element in the mechanism of action of insulin (Clausen, 1975; Kissebah, et al., 1975), particularly in adipocytes and muscle. This hypothesis, whilst having the strength that it may explain the activation of mitochondrial pyruvate dehydrogenase (pyruvate dehydrogenase phosphate phosphatase is activated by Ca^{2+}) (Denton, et al., 1972; Denton, 1981), has the disadvantage that a rise in cytoplasmic Ca^{2+} could bring about any of the cytoplasmic effects of insulin. Indeed, it has been pointed out that such a rise would be expected to bring about a range of effects that are not characteristic of insulin. This would include an increase in glycogen breakdown in liver due to the activation of phosphorylase kinase (Denton, 1981). Further, in rat liver, insulin opposes the effects of phenylephrine on glycogen breakdown and gluconeogenesis; effects which are widely considered to be brought about by increases in intracellular Ca^{2+} (Exton, 1980; Blackmore, et al., 1979; Hems, 1980). Consequently, insulin-induced activation of this activity, at least by an increase in cytoplasmic Ca^{2+} in hepatocytes, would seem to be unlikely. Further to this, in hepatocytes, this activity has been shown to be more selective for cyclic GMP as substrate, than cyclic AMP and even under fully activated

conditions, Ca^{2+} would be likely to increase soluble cyclic AMP phosphodiesterase activity only very slightly.

Another possibility is that the purported small increase in cyclic GMP concentration that occurs on exposure of hepatocytes to insulin (Czech, 1977), might activate the cyclic GMP-stimulated phosphodiesterase that occurs both in the cytosol and particulate fraction of hepatocytes (Pyne, et al., 1986a). This would appear to be a more likely prospect given the preponderance of this activity in both the soluble and particulate fractions of rat liver (Pyne, et al., 1986a). Although this activity was not found to be as predominant in the hepatocyte soluble fraction as it was in the rat liver soluble fraction, cyclic GMP can still produce a very marked increase in total soluble cyclic AMP phosphodiesterase activity. Whilst insulin can increase cyclic GMP levels in hepatocytes (Czech, 1977), it is not certain if cyclic GMP can attain concentrations that are sufficient to stimulate the activity of this enzyme following insulin challenge. It is thus conceivable that insulin may activate this putative third insulin-sensitive phosphodiesterase by means of a mechanism other than an increase in cyclic GMP.

Reports from a number of laboratories have suggested that insulin acts on target cells to potentiate the release from plasma membranes of soluble 'mediators' within the target cell (Larner, et al., 1979; Parker, et al., 1982; Saltiel, et al., 1981). Such species mimic some of the effects of insulin on target cells such as the activation of pyruvate dehydrogenase (Saltiel, et al., 1981; Parker, et al., 1982; Larner, et al., 1979) and 'low-K_m' cyclic AMP phosphodiesterase (Parker, et al., 1982; Saltiel and Cuatrecasas, 1986a; Saltiel, et al., 1986b). The chemical nature of this mediator is not well defined but it may actually consist of a number of different compounds that may be peptide in nature (Larner, et al., 1979) or consist of a complex carbohydrate-phosphate substances containing inositol and glucosamine (Saltiel and Cuatrecasas, 1986a; Saltiel, et al., 1986b). An insulin mediator mimics some of the effects of the short term effects of

insulin, particularly with respect to activation of mitochondrial pyruvate dehydrogenase (Larner, et al., 1979; Saltiel, et al., 1981; Parker, et al., 1982) and a high affinity 'low-K_m' cyclic AMP phosphodiesterase (Parker, et al., 1982; Saltiel and Cuatrecasas, et al., 1986a; Saltiel, et al., 1986b). The mediator substance isolated by Saltiel and Cuatrecasas actually consists of two complex carbohydrate substances that are produced on exposure of isolated hepatic membranes to insulin (Saltiel and Cuatrecasas, 1986a). Intriguingly, these workers showed that they could mimic the release of the 'mediator' by treating liver plasma membranes with a highly purified preparation of phosphatidylinositol-specific phospholipase C. In order to assay 'mediator' activity, these workers used a crude adipocyte membrane preparation containing a high-affinity cyclic AMP phosphodiesterase. The exact nature and mechanism of activation of this 'mediator'-sensitive phosphodiesterase is unclear. However, the mechanism by which insulin activates the cyclic GMP-inhibited ('dense-vesicle') PDE appears to involve an event that is persistent in nature (Loten, et al., 1978; Loten, et al., 1980), which is inconsistent with a simple interaction of the mediator with this phosphodiesterase.

The possibility thus exists that, if a third insulin-sensitive phosphodiesterase is present in hepatocytes, it may be activated by this mediator preparation by means of a direct interaction between the 'mediator' and the phosphodiesterase. Such a direct interaction between the soluble mediator and the PDE would thus be expected to be diluted out during Percoll fractionation explaining the lack of evidence of a third insulin-sensitive phosphodiesterase by this technique (Hetworth, et al., 1983d).

This hypothesis, allows one to speculate that any of the phosphodiesterase species present in hepatocytes may be activated by this 'mediator'. In support of this, Pyne and Houslay (1988) have shown that a 'mediator' preparation from rat hepatocytes achieved a reversible and dose-dependent activation of a purified preparation of the cyclic GMP-stimulated phosphodiesterase from both the soluble

and particulate fraction. In contrast, the 'mediator' preparation had no effect on the activity of apparently homogenous preparations of the 'dense-vesicle' PDE and the 'peripheral' plasma membrane PDE (Pyne and Houslay, 1988).

Whilst this evidence may suggest that the third insulin sensitive phosphodiesterase is the cyclic GMP-stimulated phosphodiesterase, it does not preclude the possibility that *in vivo* other phosphodiesterases may be the target. Two good candidates for such a species are the soluble PDE MQ-IV and PDE MQ-V. These contribute a significant proportion of the total soluble cyclic AMP phosphodiesterase in hepatocytes and in addition are specific for cyclic AMP as substrate. This is important since it has been shown that insulin can increase cyclic GMP levels in hepatocytes rather than decrease them. Activation of a cyclic GMP phosphodiesterase such as the Ca^{2+}/calmodulin-stimulated phosphodiesterase or the cyclic GMP-stimulated phosphodiesterase by this 'mediator' preparation would thus be expected to decrease cyclic GMP levels following insulin challenge rather than increase them. This is especially true of the cyclic GMP-stimulated phosphodiesterase which has been shown in this study to form a very large proportion of the total soluble cyclic GMP phosphodiesterase activity as well as a large proportion of the membrane bound cyclic GMP phosphodiesterase activity (Pyne, et al., 1986a).

Given the substrate specificity of the Ca^{2+}/calmodulin-stimulated phosphodiesterase in hepatocytes it would seem unlikely that this activity could act as an insulin-stimulated cyclic AMP phosphodiesterase. This also discounts the possibility that Ca^{2+} could conceivably act as an insulin 'mediator' in this action of insulin. Whilst PDE MQ-I has the necessary capacity to hydrolyse cyclic AMP, and also forms a significant proportion of the total soluble cyclic AMP phosphodiesterase activity, it is also unlikely to be a target for insulin stimulation in hepatocytes. This is based on the observation that the ability of insulin to decrease cyclic AMP levels is inhibited by the phosphodiesterase inhibitor IBMX (Heyworth, et al., 1983d), implying

that this insulin stimulated phosphodiesterase is sensitive to IBMX, which PDE MQ-I is not.

CHAPTER 4.

THE HORMONE-SENSITIVE 'PERIPHERAL AND 'DENSE-VESICLE' PHOSPHODIESTERASES.

Challenge of cells with either insulin or hormones which activate adenylate cyclase has been shown to enhance cyclic AMP phosphodiesterase activity (Appleman, et al., 1973; Thompson, et al., 1973; Houslay, 1986; Kono, et al., 1975). In hepatocytes, there appear to be at least three specific high-affinity cyclic AMP phosphodiesterases, whose activities are under rapid reversible regulation by hormones (Heyworth, et al., 1983d; Houslay, 1986). Two of these activities have been purified from rat liver. These enzymes, the 'dense-vesicle' phosphodiesterase and the 'peripheral' plasma membrane phosphodiesterase have been shown to differ in their physical properties, thermal stability, kinetics of degradation of cyclic AMP and cyclic GMP, inhibitor sensitivities, ^{125}I-labelled tryptic peptide maps and in the ability of cyclic GMP to inhibit the hydrolysis of cyclic AMP (Marchmont, et al., 1981b; Pyne, et al., 1987a, Pyne, et al., 1986b). The 'peripheral' plasma membrane phosphodiesterase can be stimulated by insulin through a process that involves phosphorylation (Marchmont and Houslay, 1980b; Marchmont and Houslay, 1981a; Pyne, et al., 1989). Activation of this enzyme by insulin can be blocked by pre-treatment of hepatocytes with glucagon (Heyworth, et al., 1983d; Heyworth, et al., 1984a).

The other insulin activated enzyme is the 'dense-vesicle' phosphodiesterase (Heyworth, et al., 1983d; Pyne, et al., 1987a), whose activity is rapidly increased when hepatocytes are treated with either insulin or glucagon (Loten, et al., 1978; Loten, et al., 1980; Heyworth,l et al., 1983d, Pyne, et al., 1987a). Glucagon has been suggested to exert it's effect on this enzyme through a mechanism involving cyclic AMP-dependent phosphorylation (Heyworth, et al., 1983d, Houslay, 1986; Kilgour, et al., 1989).. This mechanism seems to be quite distinct from the mechanism, as yet unidentified, by which insulin activates this enzyme (Heyworth, et al., 1984a).

These different methods of regulation may imply that these enzymes have specific functional roles in hepatocytes. It was of interest then to determine if these activities were specifically expressed

in liver/hepatocytes or if they exhibited a wider tissue distribution. The aim of this section of work was to assess for the presence of these two hormonally regulated enzymes in tissues other than liver. Specific anti-sera were availabe that had previously been raised to purified preparations of the 'dense-vesicle' phosphodiesterase (Pyne, et al., 1987a) and the 'peripheral' phosphodiesterase (Pyne, et al., 1986b). These were termed DV-1 and PM-1 respectively.

The amount of the antisera DV-1 and PM-1 were limited at the time this study was initiated so an attempt was made to purify the 'dense-vesicle' phosphodiesterase in order to allow for the production of more anti-sera to this enzyme.

Some portions of this work were carried out in collaboration with Dr. Nigel Pyne and Dr. Neil Anderson who were responsible for the production of the antisera DV1.

4.1: THE ANTISERA DV-1 AND PM-1.

The polyclonal antisera DV-1 and PM-1 have been raised against purified preparations of the 'dense-vesicle' and 'peripheral' plasma membrane phosphodiesterases respectively (DV-PDE and PM-PDE) in previous work in this laboratory (Pyne, et al., 1987a; Houslay, et al., 1988).

Using Western blotting, DV-1 could recognise the 57-kDa subunit of the purified protein. Immunoprecipitation of a hypotonic extract of rat liver membranes using the antisera DV-1 identifies a 57-kDa protein. A larger form of the enzyme could be identified from immunoprecipitates of sodium cholate extracts of liver membranes that has a molecular weight of 62/63-kDa. Hypotonic extraction of rat liver membranes has been shown to proteolytically release the 'dense-vesicle' PDE from the membrane due to the action of endogenous proteases (Loten, et al., 1978; Loten, et al., 1980; Pyne, et al., 1987a). This release is inhibited by a variety of protease inhibitors, including Benzamidine (Loten, et al., 1978). Cholate extraction in the presence of the protease inhibitors Benzamidine and PMSF, should therefore, solublise but prevent proteolytic cleavage of the enzyme.

Based on these observations, the 57-kDa species presumably reflects the proteolysed form of the enzyme whilst the 62-kDa protein is probably the native subunit molecular weight. The 63-kDa protein has been proposed to be a precursor of the enzyme although the evidence for this is lacking (Pyne, et al., 1987a). The antisera PM-1 recognises a 52-kDa protein on Western Blots of liver membranes (Houslay, et al., 1988).

In order to confirm that the DV-1 antibody could recognise 'dense-vesicle' phosphodiesterase activity, experiments were performed to assess if the antibody could remove cyclic AMP phosphodiesterase activity from a hypotonic extract prepared from rat liver. An antibody affinity column (DV-1-Sepharose) was prepared by coupling DV-1 to

Cyanogen bromide–activated Sepharose. Figure 4.1 shows that the column could remove cyclic AMP phosphodiesterse activity from a hypotonic extract. The activity appeared to be bound specifically to the matrix since washing in high salt removed only a small amount of the retained activity (5.3%–8.2% range).

The maximal phosphodiesterase activity removed from the hypotonic extract by DV–1–Sepharose was approximately 3.5 pmol/min/33μl of gel matrix corresponding to a column capacity of 107 pmol/min/ml of gel matrix. Removal of the cyclic AMP phosphodiesterase activity from the hypotonic extract caused a change in the response of the extract to cyclic GMP. The extract's cyclic AMP phosphodiesterase activity was inhibited approximately 55% by cyclic GMP. At incubation levels below the capacity of the column, removal of cyclic AMP phosphodiesterase activity produced an extract whose cyclic AMP phosphodiesterase activity was stimulated some 45% by cyclic GMP. As the amount of enzyme applied to the column increased towards, and then exceeded, the capacity of the column, the response to cyclic GMP of the cyclic AMP PDE activity remaining in the extract approached that of the hypotonic extract itself indicating that DV–PDE activity remained unbound to the affinity resin.

An attempt was made to remove the enzyme from the affinity matrix. Elution was assessed by iodination of the eluted proteins followed by fractionation on an SDS–PAGE gel. The interaction of the 'dense–vesicle' enzyme with the antibody appeared to be very strong since only under extreme conditions could activity be eluted. Figure 4.2 shows that only in the presence of 3.5M $MgCl_2$ or 8M urea could protein be removed from the affinity gel. Incubation in 1mM cyclic AMP/1mM cyclic GMP and 1% sodium cholate was ineffective. Two major polypeptides of 57–kDa and 51–kDa were identified, that corresponded to the molecular weights of the purified 'clipped' form of the 'dense–vesicle' phosphodiesterase (Pyne, et al., 1987a).

This data confirmed that the DV-1 antibody was capable of recognising the 'dense-vesicle' phosphodiesterase. A similar study using the antisera PM-1 was precluded due to the limited amount of the antisera available.

It was decided to assess the tissue distribution of these two hormonally regulated in phosphodiesterases using the specific anti-sera DV-1 and PM-1 to quantitate the enzyme in tissue homogenates.

4.2: TISSUE DISTRIBUTION OF THE HORMONE-SENSITIVE 'DENSE-VESICLE' AND PERIPHERAL PLASMA MEMBRANE PDEs.

The relative distribution of the 'dense-vesicle' and 'peripheral' phosphodiesterases was then assessed in several rat tissues namely, liver, heart, white adipose tissue and kidney.

4.2.1: Methods.

In order to address the problem of differential extraction and proteolysis it was decided to adopt a protocol whereby tissues were rapidly isolated from freshly sacrificed rats. All tissues were diced rapidly at 4°C; white adipose tissue, liver, and kidney were homogenised directly whilst heart tissue was further disrupted using a Polytron. Homogenates were prepared rapidly under isotonic conditions in the presence of the protease inhibitors Benzamidine, PMSF and Leupeptin, as detailed in Section 2.4 for the preparation of homogenates from rat liver. A given volume of homogenate was then solubilised directly in an equal volume of Laemmli sample buffer by boiling for 5 minutes (see Section 2.22.1). Total homogenate protein was then determined and the required volume of the solubilised extracts determined to provide 50µg of total protein calculated.

4.2.2: Immunoblotting of DV and PM PDEs in rat tissues.

The 'dense-vesicle' and 'peripheral' phosphodiesterases were expressed in all the tissues examined. Figure 4.3 shows that immunoblot analysis of homogenates of liver, heart, white adipose tissue

and kidney identified a single major band at 52-kDa with antiserum PM-1, corresponding to the molecular weight of the purified 'peripheral' plasma membrane phosphodiesterase (PM-PDE) and a 63-kDa band with antiserum DV-1 corresponding to the presumed molecular weight of the native 'dense-vesicle' phosphodiesterase (DV-PDE).

Using equal amounts of protein (50μg), it was apparent from the degree of staining from the peroxidase stained immunoblots, that the distribution of these two enzymes in the various tissues was not the same. It was thus decided to quantitate the amount of these two enzymes in each of these tissues by using the specific antibodies and ^{125}I-Protein-A.

Standard curves were constructed using purified protein previously prepared by the method of Pyne, et al., (1987a) for the 'dense-vesicle' phosphodiesterase and the method of Pyne, et al., (1986b) for the 'peripheral' phosphodiesterase. Blots were probed with antisera PM-1 or DV-1 and then with ^{125}I-Protein-A. The standard curves are shown in Figures 4.4 and 4.5. They were linear and exhibited slopes that were slightly greater than 1.

Table 4.1 shows that using this procedure, it was also apparent that the distribution of these enzymes in the tissues examined was not the same. It was found that whilst liver and kidney expressed approximately equal amounts of the DV-PDE (0.15 and 0.14 μg/mg of homogenate respectively), heart and white adipose tissue were found to express much higher levels (0.75 and 0.48 μg/mg of homogenate respectively). Using these results one can estimate the total amount of the 'dense-vesicle' phosphodiesterase in one rat liver. In a 250-275g rat, one liver has been shown to contain approximately 300 mg of protein in the homogenate (Pyne, et al., 1987a). This corresponds to a total amount of 'dense-vesicle' phosphodiesterase of 45μg per liver. In a purification of the 'dense-vesicle' phosphodiesterase the yield from

three rat livers was approximately 30μg of protein, which would indicate that these estimates of total enzyme levels are approximately correct.

The distribution of the PM-PDE amongst the tissues also differed but not as markedly as for the DV-PDE. Kidney, heart and white-adipose tissue all exhibited very similar levels of the PM-PDE (0.12, 0.11 and 0.11 μg/mg of homogenate), whilst liver contained slightly higher levels (0.15 μg/mg of homogenate).

This analysis indicated that there were distinct tissue distributions of the DV-PDE and the PM-PDE in rat tissues. However, it cannot be concluded that because a tissue expresses an enzyme, that enzyme is active. It is always possible that the enzyme may be inactivated or that it may be sequestered in a cellular compartment so that it's activity is latent. In order to address this question it was decided to estimate the total contribution of the 'dense-vesicle' phosphodiesterase to the total homogenate cyclic AMP phosphodiesterase activity.

4.3: USE OF ICI 118233 TO ASSESS TISSUE DISTRIBUTION OF THE 'DENSE-VESICLE' PHOSPHODIESTERASE.

Results presented in Chapter 3 and from previous work in this laboratory have shown that the phosphodiesterase inhibitor ICI 118233 is a specific and potent inhibitor of the 'dense-vesicle' phosphodiesterase. It has little or no inhibitory action against the 'peripheral' plama membrane phosphodiesterase, both the plasma membrane and cytosolic forms of the cyclic GMP-stimulated phosphodiesterase, the soluble Ca^{2+}/Calmodulin-stimulated phosphodiesterase, the soluble IBMX-insensitive phosphodiesterase, or the two soluble high affinity cyclic AMP-specific phosphodiesterases. Using this specific reagent, it was decided to assess the relative contribution of the 'dense-vesicle' PDE activity in total homogenates from liver, kidney, white adipose tissue and heart.

4.3.1: Characterisation of ICI 118233 inhibition of 'dense-vesicle' cyclic AMP phosphodiesterase activity.

Further characterisation of the action of ICI 118233 against the DV-PDE was carried out by analysis of the IC_{50} curves for inhibition of the DV-PDE cyclic AMP PDE activity, followed by a calculation of the K_i value for ICI 118233. When assayed over a highly extended concentration range, the 'dense-vesicle' enzyme displays evidence of apparent negative co-operativity (Pyne, et al., 1987a). Analysis of inhibition was thus performed at low substrate concentrations (0.1-1.0μM cyclic AMP), which allowed the 'high-affinity' component of it's activity to be followed.

To carry this out, a partially purified preparation of the DV-PDE was first prepared from hypotonic extracts by Mono Q chromatography (MQ-DV-PDE see Section 4.). Firstly, the IC_{50} for inhibition of cyclic AMP hydrolysis by cyclic GMP was determined at a substrate concentration of 0.1μM cyclic AMP. Figure 4.6 shows a typical inhibition curve for inhibition of cyclic AMP hydrolysis by cyclic GMP. From this, the estimated IC_{50} for cyclic GMP inhibition of cyclic AMP hydrolysis was 2.8μM +/- 0.7μM (+/- SEM, n=3). This figure compares well with the value for the IC_{50} for cyclic GMP inhibition of cyclic AMP hydrolysis obtained for the purified enzyme, which was 2μM at 1μM cyclic AMP substrate concentration (Pyne, et al., 1987a). The inhibition curve obtained was monophasic and almost complete inhibition was observed at high levels of cyclic GMP. Based on these observations it was concluded that the partially purified prepartion (MQ-DV-PDE) was sufficiently enriched in the 'dense-vesicle' PDE activity (CGI-PDE activity) to be of use in determining both the IC_{50} and the K_i for inhibition of the DV-PDE by ICI 118233.

Figure 4.7 shows a typical estimate of the IC_{50} for inhibition of cyclic AMP hydrolysis by ICI 118233 at 0.1μM cyclic AMP. Under

these conditions, the estimated IC_{50} for ICI 118233 action was 3.0µM +/- 0.6µM (+/- SEM, n=3).

Lineweaver-Burk plots of cyclic AMP hydrolysis for MQ-DV-PDE were constructed by analysing the kinetics of cyclic AMP hydrolysis between 0.1µM and 1µM. A typical analysis is shown in Figure 4.8 where apparently linear Lineweaver-Burk plots were obtained for the activity of the enzyme. Under these conditions the estimated K_m for cyclic AMP was 0.5µM +/- 0.17µM (+/- SEM, n=4). In the presence of increasing concentrations of ICI 118233, the Lineweaver-Burk plots remained linear and inhibition obtained was apparently competitive since the lines intercepted at the same point on the 1/v axis. Analysis of the ICI 118233 inhibition data by replotting the slope of the Lineweaver-Burk plot against ICI 118233 concentration (Figure 4.9) showed that the K_i value for inhibition of cyclic AMP hydrolysis was 3.8µM +/-1.2µM (+/- SEM, n=4). Since the estimated K_m for cyclic AMP hydrolysis for the 'high affinity' component of the enzyme (0.5µM) agreed well with the value for the purified enzyme (0.3µM) (Pyne, et al., 1987a), this further confirmed the validity of using a partially purified preparation of the 'dense-vesicle' phosphodiesterase for these analyses.

4.3.2: 'Dense-vesicle' PDE activity in rat tissues assessed using the selective inhibitor ICI 118233.

From the IC_{50} data it was decided to use a maximally effective dose of ICI 118233 (100µM) to allow for an assessment of the contribution this enzyme makes to total homogenate cyclic AMP hydrolysis. In these experiments, homogenates were prepared as for blotting experiments but were assayed immediately for cyclic AMP phosphodiesterase activity at 0.1µM cyclic AMP. From the % inhibition of homogenate PDE activity obtained with 100µM ICI 118233 it was possible to estimate the specific activity of the DV-PDE activity in each of the tissues. Table 4.2 shows the results expressed in pmol/min/mg of homogenate. Calculation of the tissue distribution

ratio (TD ratio) reveals that the pattern of expression corresponds well with that obtained using immunoblotting analysis. Heart and white adipose tissue once again expressed the highest relative amount of DV-PDE activity with lower levels of activity observed in liver and kidney.

Using both the values for the amount of DV-PDE protein and the activity of the DV-PDE an estimated specific activity of the DV-PDE in each of the tissues was calculated. The values were different in each of the tissues examined, the highest value obtained was 7857 pmol/min/mg in kidney and the lowest in heart, 3466 pmol/min/mg. The value for the liver enzyme was 5333 pmol/min/mg which is about 30% of the specific activity of the purified 'clipped' form of the enzyme (17,503 pmol/min/mg) (Pyne, et al., 1987a).

Although there are potent inhibitors of the 'peripheral' plasma membrane phosphodiesterase, e.g. ICI 63 197 (Houslay, et al., 1988), this compound is also an effective inhibitor of the 'dense-vesicle' phosphodiesterase (Pyne, et al., 1987a) and also of the two soluble high affinity cyclic AMP phosphodiesterases (see Chapter 3). Consequently, a comparable study for the 'peripheral' plasma membrane phosphodiesterase was not possible.

4.4: PURIFICATION OF RAT LIVER 'DENSE-VESICLE' PDE.

Because of the limited amount of the antibody DV-1, several attempts were made to purify the enzyme from rat liver using previously published methods developed by Pyne, et al., (1987a) with an aim to produce more antisera. Two methods were available to purify the hypotonically extracted enzyme, the Guanine-Sepharose method and the Affi-gel Blue method.

4.5: THE GUANINE – SEPHAROSE METHOD.

This involves the production of a hypotonic extract of liver membranes which contains the proteolytically solubilised enzyme (Pyne, et al., 1987a; Loten, et al., 1978; Loten, et al., 1980). The hypotonic extract is then applied to an anion – exchange column (ECTEOLA cellulose). Activity is batch eluted with high salt and the sample diluted and applied to a hydrophobic column (– Amino – pentyl Agarose). Activity is again batch eluted with high salt and the sample desalted by passage through a desalting column (Sephadex G – 25). The sample is finally applied to a column of Guanine – sepharose (Whitson and Appleman, 1982) and again eluted with salt. This procedure is referred to as the Guanine – Sepharose method.

4.5.1: Preparation of a hypotonic extract from rat liver.

A hyptonic extract was prepared from three rat livers by the method described in Pyne, et al., (1987a). In five different preparations, from three rat livers, the total yield of activity was some 31% +/− 1.35% (n=5 +/− SEM) of the minimum expected value quoted in Pyne, et al., (1987a). Whilst the cyclic AMP phosphodiesterase activity was very much lower than expected, the yields of protein were very similar (182mg +/− 12.5mg, n=5, +/− SEM). The cyclic AMP phosphodiesterase activity was inhibited by 10μM cyclic GMP some 28% +/− 4% (n=5, +/− SEM). A typical purification attempt is described below.

4.5.2: Purification steps for the Guanine – Sepharose method.

Application of the extract to the anion – exchanger ECTEOLA cellulose resulted in the binding of 97% of the cyclic AMP PDE activity. This activity remained bound during the washing procedure (6.2% of the cyclic AMP PDE activity eluted). Batch elution using 0.4M sodium chloride eluted some 38% of the cyclic AMP PDE activity as a single peak of activity that was inhibited some 55% by cyclic GMP (Figure 4.10). Passage of this activity over the matrix, ω – amino pentyl agarose, resulted in the binding of 98% of the activity

with less than 1% eluted during the washing procedure. Batch elution with 0.5M NaCl, resulted in the elution of a single peak of PDE activity that contained cyclic GMP-inhibited cyclic AMP PDE activity (67% inhibition) (Figure 4.11). Desalting of the extract on a Sephadex G-25 column eluted a single peak of activity that was inhibited some 80% by cyclic GMP (Figure 4.12). Guanine-Sepharose chromatography produced a single peak of activity that was inhibited some 75% by cyclic GMP (Figure 4.13). The final specific activity was 675 pmol/min/mg with a 0.8% yield and fold purification of 131. The results are summarised in Table 4.4. An analysis of the purified product by SDS-PAGE analysis revealed the presence of three major polypeptides at 68-kDa, 61-kDa and 55-kDa. The preparation was not, however, pure since a large number of other minor staining bands were present (Figure 4.14). The published procedure yields bands at 57-kDa and 51-kDa.

These results were typical of preparations prepared at least five times. The average yield was 1.31% +/- 0.41% (n=5, +/- SEM) and the average specific activity was 361 pmol/min/mg +/- 128 pmol/min/mg (n=5, +/- SEM). These results differed markedly from the published procedure which produced a preparation with a yield of 18% and a specific activity of 17,500 pmol/min/mg

A closer examination of the preparation procedure identified three specific areas that contributed to the low yield. The first of these was the low yield of cyclic AMP phosphodiesterase activity in the hypotonic extract which was some 31% of the expected value.

The second was the efficiency of the individual chromatographic steps. Analysis of the yields from the individual steps was performed by calculating the phosphodiesterase activity eluted from columns in the individual fractions prior to pooling of active fractions. The specific yields of the columns were calculated and are presented in Table 4.3. In five different preparations, the ECTEOLA cellulose and -amino-pentyl agarose columns routinely gave low yields of applied activity.

The Sephadex G-25 step gave very variable yields of activity (20%-100% recovery). The guanine-Sepharose step was highly efficient with yields of between 68 and 100%.

The third factor was the stability of the enzyme at the various stages. It was observed that during a 24 hour period at 4°C the total cyclic AMP phosphodiesterase activity was very stable during the first two steps but was found to be markedly unstable after the Sephadex G-25 and guanine-Sepharose steps (Table 4.3). Decay of activity after the final stage could be as marked as 70% within a 24 hour period at 4°C.

4.6: THE AFFI-GEL BLUE METHOD.

Given the low yields of activity produced from this procedure it was decided to attempt the purification by the alternative method using Affi-gel Blue. This method involves the application of the hypotonic extract to an Affi-gel Blue column in the presence of magnesium and elution using the magnesium chelator EDTA. The eluted activity is then applied to a second column of Affi-gel Blue and eluted by substrate elution with cyclic AMP which is then removed from the enzyme by chromatography on DEAE-Sepharose CL-6B. A typical purification attempt is described below.

4.6.1: Hypotonic extract production.

The hypotonic extract was prepared from five rat livers by a modification of the method described in Pyne, et al., (1987a) (see Section 2.31). The modifications introduced included homogenisation of the diced rat livers in a Waring Blender (to significantly improve on both the speed of preparation and the yield of activity) (see Section 2.31) and the introduction of a second high speed spin at 48,000*gav* after addition of of $MgCl_2$ to the hypotonic extract. This was introduced because it was routinely observed that addition of $MgCl_2$ caused the precipitation of protein from the hypotonic extract. When

assayed for phosphodiesterase activity this pellet contained less than 0.2% of the total cyclic AMP PDE activity in the hypotonic extract.

4.6.2: Characteristics of the rat liver hypotonic extract.

In order to assess if these modifications altered the properties of the hypotonic extract the characteristics of the preparation were examined. In a typical preparation from five rat livers the hypotonic extract contained 6,853 pmol/min of cyclic AMP PDE activity with a specific activity of 14.6 pmol/min/mg which agreed well with the published value of 18.9 – 28 pmol/min/mg (Pyne, et al., 1987a) and represented a significant improvement on the original extraction procedure. The extract demonstrated significant inhibition of cyclic AMP PDE activity in the presence of 10μM cyclic GMP; the activity was reduced some 54% to 3,833 pmol/min. Since rat liver contains a number of cyclic GMP – insensitive and cyclic GMP – stimulated cyclic AMP PDE activities it is often difficult to observe the activity of the 'dense – vesicle' PDE unless one has a significantly enriched preparation of the enzyme. Based on these observations it was concluded that the preparation of the hypotonic extract was significantly enriched in 'dense – vesicle' phosphodiesterase activity.

4.6.3: Chromatography of the hypotonic extract on Affi – gel Blue.

In contrast to previously published results, chromatography of the hypotonic extract on Affi – gel Blue in the presence of protease inhibitors resulted in little binding of total cyclic AMP PDE activity. Figure 4.15 shows that of the 6,853 pmol/min of total cyclic AMP PDE activity applied to an Affi – gel Blue column, 67% (4,558 pmol/min) eluted in the breakthrough fraction. A further 29% (1,995 pmol/min) eluted during the extended washing procedure. The cyclic AMP PDE activity of both the breakthrough fraction and the wash fraction were inhibited by cyclic GMP (61% and 46% respectively). Unexpectedly, phosphodiesterase activity was observed eluting in an EDTA dependent manner, as is shown in Figure 4.16. This represented 1,400 pmol/min of cyclic AMP PDE activity (20% of activity applied). However, this

activity was not inhibited by 10μM cyclic GMP implying the existence of a cyclic GMP-insensitive phosphodiesterase activity in the hypotonic extract.

This result was observed routinely using both the same batch of Affi-gel Blue and also other batches. Figure 4.17 shows the result of a typical experiment in which the ability of a variety of batches of Affi-gel Blue to bind DV-PDE activity was assessed. The conditions of the experiment were deliberately chosen so that the ratio of protein to gel matrix (4.3 mg/ml of gel matrix) was significantly greater than the ratio that would be found in the conventional purification procedure (about 50 mg/ml of gel matrix). This was done to bias the experiment in favour of binding. The results clearly showed that the enzyme activity did not bind significantly to any of the batches of Affi-gel Blue that were screened (less than 10% of total applied).

4.6.4: DE-52 chromatography of the Hypotonic extract.

In order to increase the specific activity of the 'dense-vesicle' preparation, before chromatography on Affi-gel Blue, it was decided to perform ion-exchange chromatography. A hypotonic extract was prepared from 5 rat livers and applied to a column of DE-52. This column was chosen in preference to ECTEOLA-cellulose since this had usually given low yields of activity. The column was developed using a linear NaCl gradient betweem 0 and 0.55M NaCl. The elution profile shown in Figure 4.18 indicated that there was a single major peak of cyclic AMP PDE activity eluting at approximately 0.275M NaCl. The responses of the ascending and descending limbs to cyclic GMP were however different. The ascending limb was clearly insensitive to cyclic GMP action whilst the descending limb was clearly inhibited by cyclic GMP. The implication of this was that there were two major cyclic AMP phosphodiesterases in the hypotonic extract that differed in their response to cyclic GMP, namely a cyclic GMP-insensitive activity and a cyclic GMP-inhibited activity (the 'dense-vesicle' phosphodiesterase).

No further activity was found to elute when the gradient was extended to 1.0M NaCl.

Fractions from the DE–52 column, containing the 'dense–vesicle' activity, when pooled, contained 1,880 pmol/min of cyclic AMP PDE activity. This represented a 39% yield of activity over the hypotonic extract. The specific activity of the preparation was increased nearly 7.5 fold from 27.7 pmol/min/mg to 204 pmol/min/mg. Cyclic GMP inhibited cyclic AMP PDE activity by nearly 75%, compared to 49% in the hypotonic extract, indicating further enrichment of the DV–PDE activity. Dialysis of the DV–PDE preparation against 3 x 2 litre changes of TM Buffer for 12 hours resulted in the loss of 15% of the activity with no change in the % inhibition by cyclic GMP.

4.6.5: Chromatography of DE–52 purified enzyme on Affi–gel Blue.

A small sample (2mls) of the dialysed extract was set aside to assess the stability properties of the enzyme. Application of the remainder of the extract (58mls) to a larger column of Affi–gel Blue (18mls) and at a much slower rate (0.2 ml/min) resulted in the binding of the PDE activity to Affi–gel Blue. 8% of the activity was found in the breakthrough fraction whilst less than 1% was found in the wash. However, application of the EDTA–containing elution buffer resulted in the elution of less than 2% of the activity applied, as is shown in Figure 4.19. The total yield of protein was approximately 200μg, giving a final specific activity of 200 pmol/min/mg. This was identical to the specific activity of the enzyme preparation at the DE–52 stage indicating that no significant purification had been obtained.

The possibility that the enzyme activity had decayed during this procedure was excluded by the experiment shown in Figure 4.20(a). Dialysed enzyme from the DE–52 step, when incubated in the presence and absence of 2mM β–mercaptoethanol, demonstrated a time dependent decay in activity over a 48 hour period. Enzyme incubated in the presence of β–Mercaptoethanol exhibited a loss of 55% of the

initial activity whilst that incubated in the absence of of β-mercaptoethanol showed only a loss of 33%. The time course of these effects were however different. The loss of activity for the enzyme without β-Mercaptoethanol was more rapid while the loss for the enzyme in β-Mercaptoethanol was much slower in onset. The % inhibition of the samples however remained high (60-80% range) Figure 4.20(b). However, these losses of activity were not sufficient to account for the apparent loss of activity on the Affi-gel Blue column.

4.7: TOWARDS AN ALTERNATIVE PURIFICATION PROTOCOL.

Given the problems I found working with such procedures, I decided to investigate the possibility that various compounds could be used as an alternative 'affinity' step. As a first step towards this, it was necessary to assess if interaction with the various compounds was reversible and that these compounds could be readily removed from the enzyme. The rationale behind this is that in order for an affinity step to be effective, ligand dependent elution needs to be achieved. If this is possible, then in order to assay the activity, the eluting ligand needs to be removed from the enzyme.

Hypotonic extracts can be prepared that demonstrate significant inhibition of cyclic AMP PDE activity in response to cyclic GMP. 'Dense-vesicle' PDE activity could be partially separated from a non cyclic GMP inhibited PDE activity on DE-52 but this procedure was rather slow and the resolution incomplete. It was thus decided to attempt to resolve the hypotonic extract phosphodiesterases on a Mono Q anion-exchange column.

4.7.1: Separation of hypotonic extract cyclic nucleotide PDE activity by Mono Q anion-exchange chromatography.

In a typical experiment, a hypotonic extract isolated from one rat liver contained 964 pmol/min of cyclic AMP PDE activity assessed at 0.1μM cyclic AMP. The cyclic AMP PDE activity of this extract was

inhibited 50% by 10μM cyclic GMP. Chromatography was performed on a Mono Q column using an identical gradient to that employed for the separation of soluble cyclic nucleotide phosphodiesterase (see Chapter 3: Table 3.2). Figure 4.21 shows the elution profile obtained when assaying for both cyclic AMP and cyclic GMP at 0.1μM substrate. In addition, cyclic AMP hydrolysis was assessed in the presence of 2μM cyclic GMP. This concentration of cyclic GMP was chosen so that both activation and inhibition of cyclic AMP hydrolysis could be followed more easily. At least 6 peaks of activity could be readily resolved by this technique.

Peaks I and II hydrolysed cyclic AMP preferentially, whilst Peak III hydrolysed cyclic GMP preferentially. The precise identity of these activities with respect to known activities was not determined. Peak IV clearly contained the cyclic GMP-stimulated phosphodiesterase activity. Peak V hydrolysed cyclic AMP almost exclusively but it's activity was not affected by cyclic GMP. Peak VI hydrolysed both cyclic AMP and cyclic GMP. Inhibition of cyclic AMP hydrolysis by cyclic GMP could be observed in fractions either side of the midpoint of the peak (fraction 91). No inhibition was observed in fraction 91 itself. Fraction 91 also exhibited a large amount of cyclic GMP hydrolytic activity. These results were observed in three instances where nearly identical profiles were obtained.

The activities quoted are calculated on the assumption of linearity of the assay. However, the assay has been shown to be linear up to 20% hydrolysis but after this limit it is difficult to accurately assess the actual hydrolytic rate. Based on this knowledge, the simplest interpretation of these results was that the 'dense-vesicle' phosphodiesterase is contained in Peak VI and had eluted from the Mono Q column in a concentrated form so that inhibition of cyclic AMP hydrolysis was not easily detected. This interpretation was supported by the fact that significant amounts of cyclic GMP PDE activity can be detected in the peak. This enzyme has been shown to

hydrolyse cyclic GMP (Whitson and Appleman, 1982; Pyne, et al., 1987a)

Although the cyclic GMP–insensitive and –inhibited forms of cyclic AMP PDE could be resolved by Mono Q chromatography using this gradient, a better separation could be achieved using an extended gradient between 0.3 and 0.5M NaCl. This is shown in Figure 4.22. This clearly showed the resolution of the cyclic GMP–insensitive form of cyclic AMP phosphodiesterase from the cyclic GMP–inhibited 'dense–vesicle' phosphodiesterase. The cyclic AMP phosphodiesterase activity of the DV–PDE was potently inhibited by 10μM cyclic GMP.

4.7.2 Characterisation of the Mono Q purified 'dense–vesicle' phosphodiesterase (MQ–DV–PDE).

The preparation was characterised in terms of kinetics of cyclic AMP hydrolysis, and inhibition by ICI 118233 and cyclic GMP. These results are shown in Figures 4.6–4.9. The results indicated that the preparation was sufficiently separated from other phosphodiesterases to be of use in screening for alternative affinity columns.

4.7.3: Reversibility of inhibition of the 'dense–vesicle' PDE by various inhibitors.

In order to assess this, MQ–DV–PDE was mixed with inhibitors at a final concentration of 100μM. Figure 4.23 shows the results of a typical experiment. Incubation of the DV–PDE preparation in 100μM of the inhibitors, cyclic GMP, IBMX, and ICI 118233, resulted in inhibition of the activity to 0.01%, 2.5% and 23% of control values respectively. Dialysis of control enzyme, incubated in TMB Buffer alone, caused the loss of approximately 30% of the activity. Dialysis caused the return of activity to 87%, 72% and 89% of control values for cyclic GMP, IBMX and ICI 118233 incubated enzymes respectively. The dialysed samples were all potently inhibited by 10μM cyclic GMP. The data thus indicated that cyclic GMP, IBMX and ICI 118233 act as reverible inhibitors of the 'dense–vesicle'–PDE and can be removed effectively by a simple dialysis protocol.

FIGURE 4.1:

BINDNG OF cAMP PHOSPHODIESTERASE ACTIVITY FROM A RAT LIVER HYPOTONIC EXTRACT TO DV-1-SEPHAROSE.

DV-1-Sepharose was prepared and equilibrated as in Section 2.32. Five 3.33µl aliquots of the gel were incubated with increasing amounts of a rat liver hypotonic extract in eppendorf tubes on a rotary mixer at 4°C for 30 minutes. The gel was pelleted and the unbound fraction retained. Gels were washed in 2 x 1ml aliquots of TE Buffer (10mM Tris-HCl pH 7.4, 1mM EDTA) followed by 2 x 1ml aliquots of TE Buffer/0.5M NaCl and finally in 2 x 1ml aliquots of TE Buffer. Washes were pooled and assayed for cAMP phosphodiesterase activity. The amount of activity bound to the column was estimated by substracting the unbound activities from the total applied. The activity remaining unbound was assayed for cAMP phosphodiesterase activity in the presence and absence of 10µM cGMP and the ratio +/- calculated. The data is plotted as the estimated cAMP PDE activity bound against the total activity applied.

(■) Cyclic AMP phosphodiesterase activity bound

(□) +/- ratio for unbound cyclic AMP phosphodiesterase activity.

FIGURE 4.1:

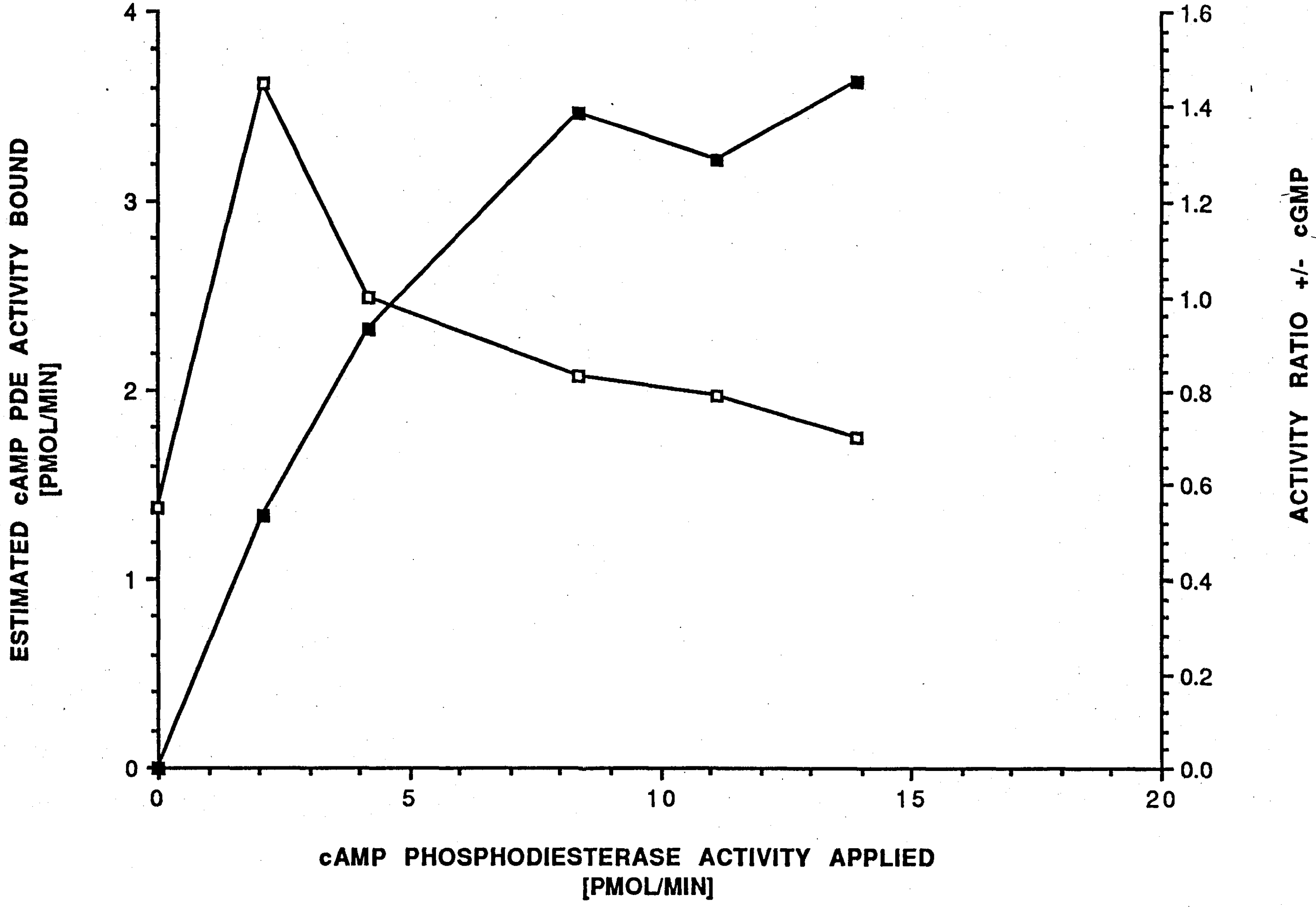


FIGURE 4.2:

ELUTION OF 'DENSE-VESICLE' PHOSPHODIESTERASE PROTEIN FROM DV-1-SEPHAROSE

600μl aliquots of Hypotonic extract containing 8.2 pmol/min of cAMP phosphodiesterase activity were bound to 3.33μl aliquots of DV1-Sepharose and gels washed as before (see legend for Figure 5.1). The average bound activity was 3.2 pmol/min ± 0.3 (39%). Elution of bound material was attempted by incubating gels for 20 minutes at 4°C in :

1. 500μl of 3.5M $MgCl_2$
2. 200μl of 1mM cAMP/1mM cGMP
3. 200μl of 1% (w/v) sodium cholate
4. 500μl of 8M urea.

Solubilised samples were removed and dialysed against 4 x 0.5 litre changes of TE Buffer (10mM Tris-HCl pH 7.4, 1mM EDTA). Samples were freeze-dried and iodinated with 200μCi of ^{125}I (see Section 2.20 for general method). Iodinated proteins were analysed by a 10% SDS-PAGE gel.

Lane 1: 3.5M $MgCl_2$
Lane 2: 1mM cAMP/1mM cGMP
Lane 3: 1%(w/v) sodium cholate
Lane 4: 8M Urea

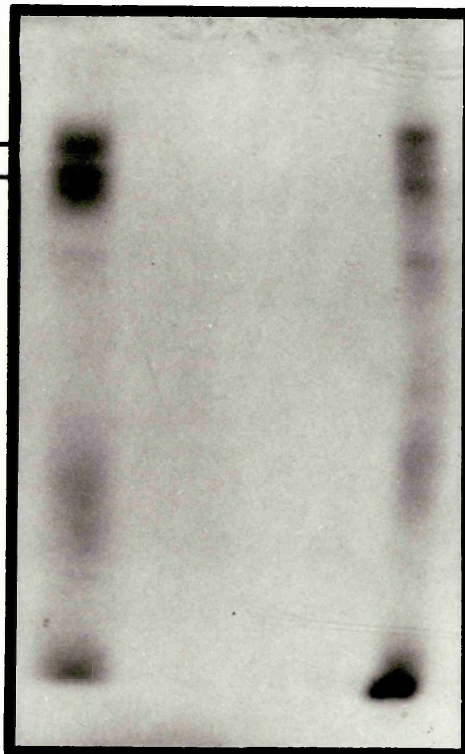

57 kDa
51 kDa
Track 1 2 3 4

FIGURE 4.3:

IMMUNOBLOTTING OF RAT TISSUES WITH ANTISERA PM-1 AND DV-1

LANE 1: LIVER

LANE 2: KIDNEY

LANE 3: HEART

LANE 4: WHITE ADIPOSE TISSUE

LANE 5: LIVER

LANE 6: KIDNEY

LANE 7: HEART

LANE 8: WHITE ADIPOSE TISSUE

Gels were loaded with equal amounts of protein (50µg) and blotting performed as in Section 2.30. Lanes 1-4 were blotted with antibody DV1 at a dilution of 1/100. Lanes 5-8 were blotted with antibody PM1 at a diluton of 1/100. Blots were developed with horseradish peroxidase stain using *o*-dianisidine as substrate

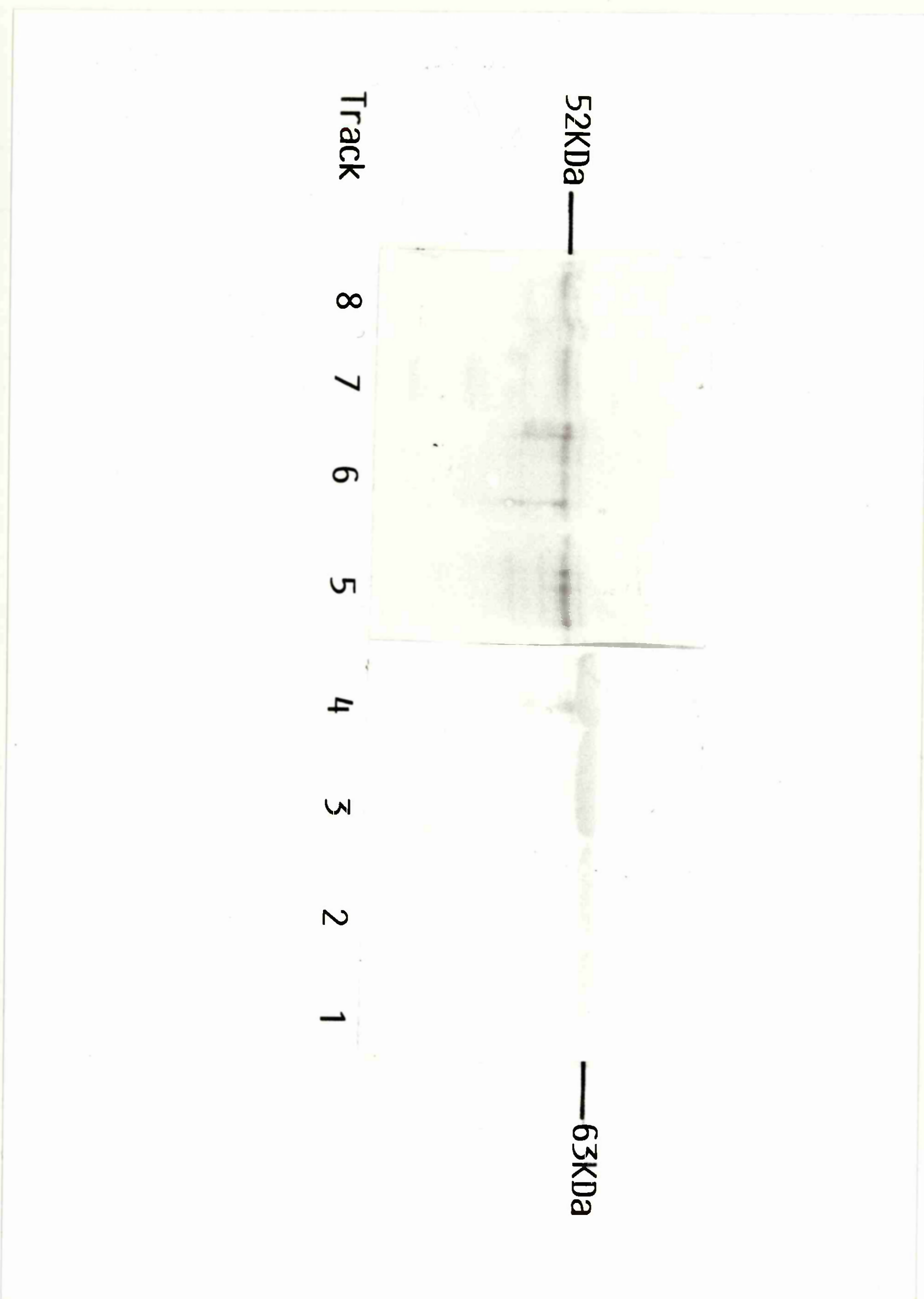

52KDa
63KDa
Track
8
7
6
5
4
3
2
1

FIGURE 4.4:

STANDARD CURVE FOR IMMUNOBLOTTING WITH ANTISERA PM-1.

Purified 'peripheral' plasma membrane phosphodiesterase (2.5ng-45ng) containing 52-kDa subunit was solubilised in Laemmli sample buffer and fractionated on a 10% SDS-PAGE gel with a 5% stacking gel (see Section 2.22). Western blotting was performed according to section 2.30 and the blot probed with 2μCi of ^{125}I-Protein-A. This was used so that the immunostained bands could be excised and counted in a γ-counter. In order to reduce the background sufficiently, it was found necessary to wash the blots in Tris-Tween buffered saline (20mM Tris-HCl pH 7.4, 0.5M NaCl, 0.05% (v/v) Tween-20. Immunostained bands were excised and counted and corrected for a background of approximately 50cpm.

FIGURE 4.4:

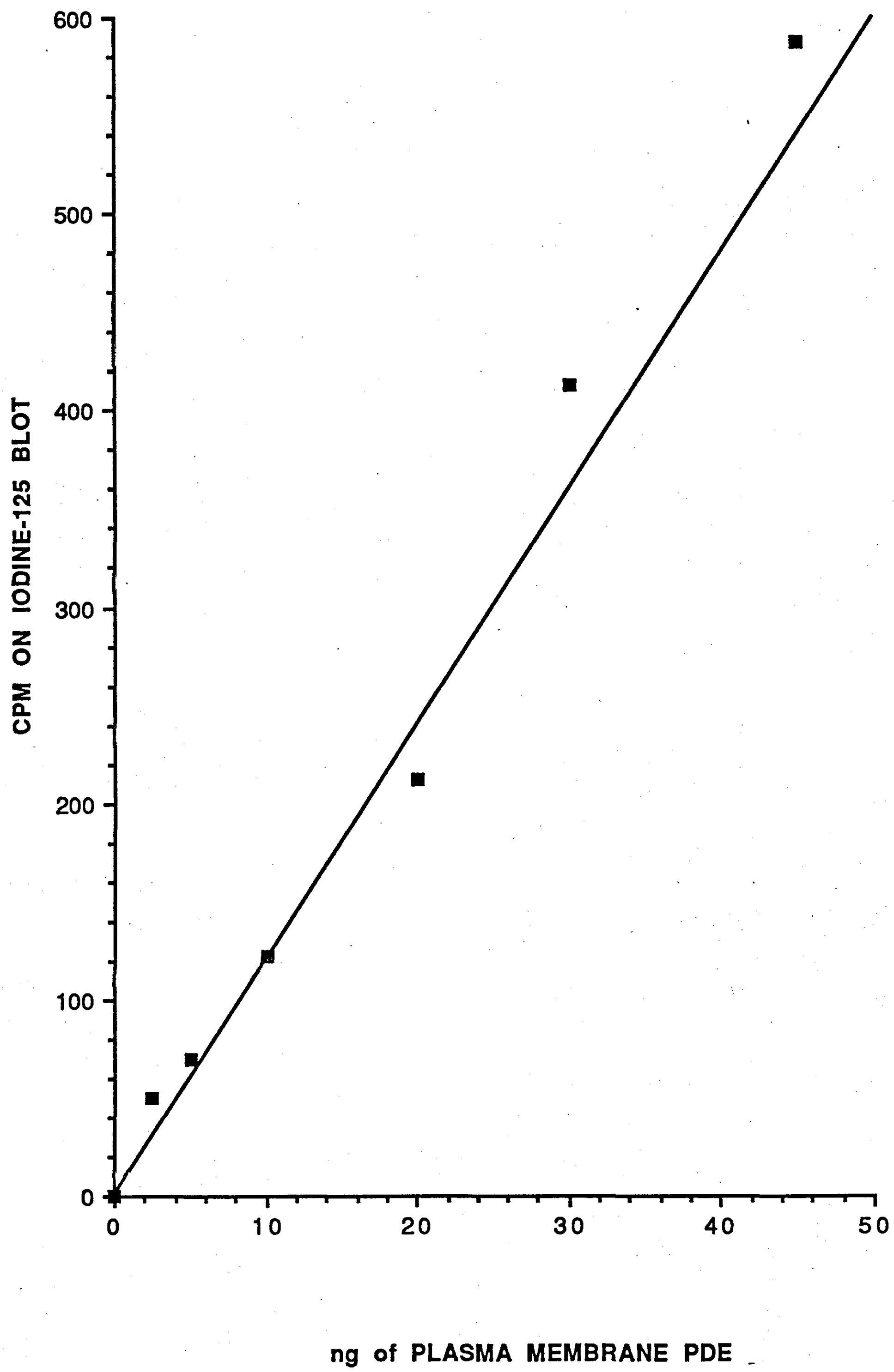


FIGURE 4.5:

STANDARD CURVE FOR IMMUNOBLOTTING WITH ANTISERA DV-1.

Purified 'dense-vesicle' phosphodiesterase (2.5ng-45ng) containing 63-kDa subunit was solubilised in Laemmli sample buffer and fractionated on a 10% SDS-PAGE gel with a 5% stacking gel (see section 2.22). Western blotting was performed according to Section 2.30 and the blot probed with 2μCi of ^{125}I-Protein-A. This was used so that the immunostained bands could be excised and counted in a γ-counter. In order to reduce the background sufficiently, it was found necessary to wash the blots in Tris-Tween buffered saline (20mM Tris-HCl pH 7.4, 0.5M NaCl, 0.05% (v/v) Tween-20. Bands were excised and counted and corrected for a background of approximately 50cpm.

FIGURE 4.5:

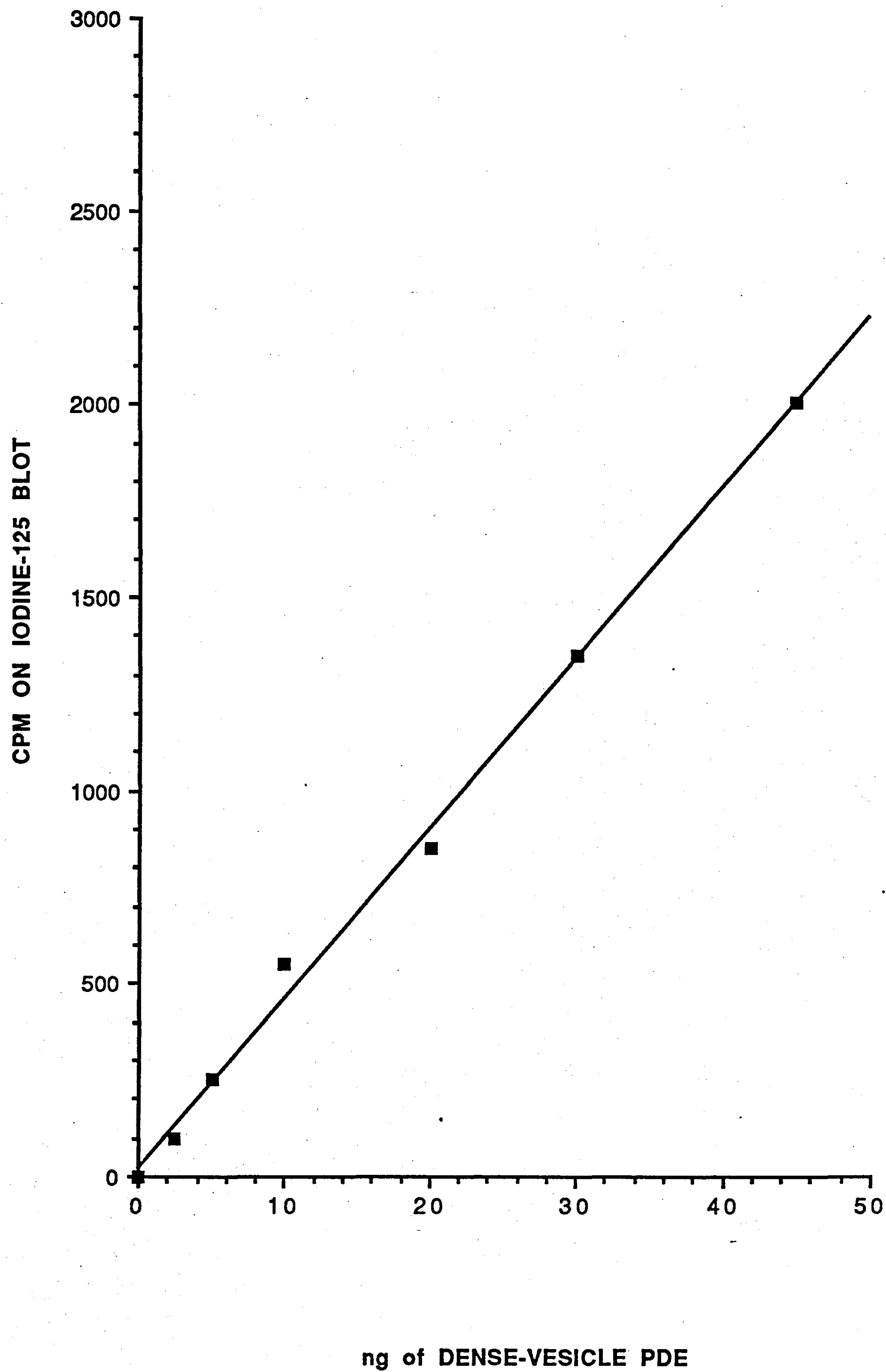


FIGURE 4.6:

INHIBITION OF THE CYCLIC AMP PHOSPHODIESTERASE ACTIVITY OF A PARTIALLY PURIFIED PREPARATION OF THE 'DENSE-VESICLE' PHOSPHODIESTERASE BY cGMP.

A partially purified preparation of 'dense-vesicle' PDE was isolated by Mono Q anion-exchange chromatography from a hypotonic extract prepared from one rat liver. Fractions were pooled and diluted to a suitable concentration in TMB buffer. Cyclic AMP phosphodiesterase activity was assessed at 0.1μM cAMP in the presence of increasing concentrations of cGMP (0-500μM). The data shown is a single representative experiment with triplicate determinations (± SD).

FIGURE 4.6:

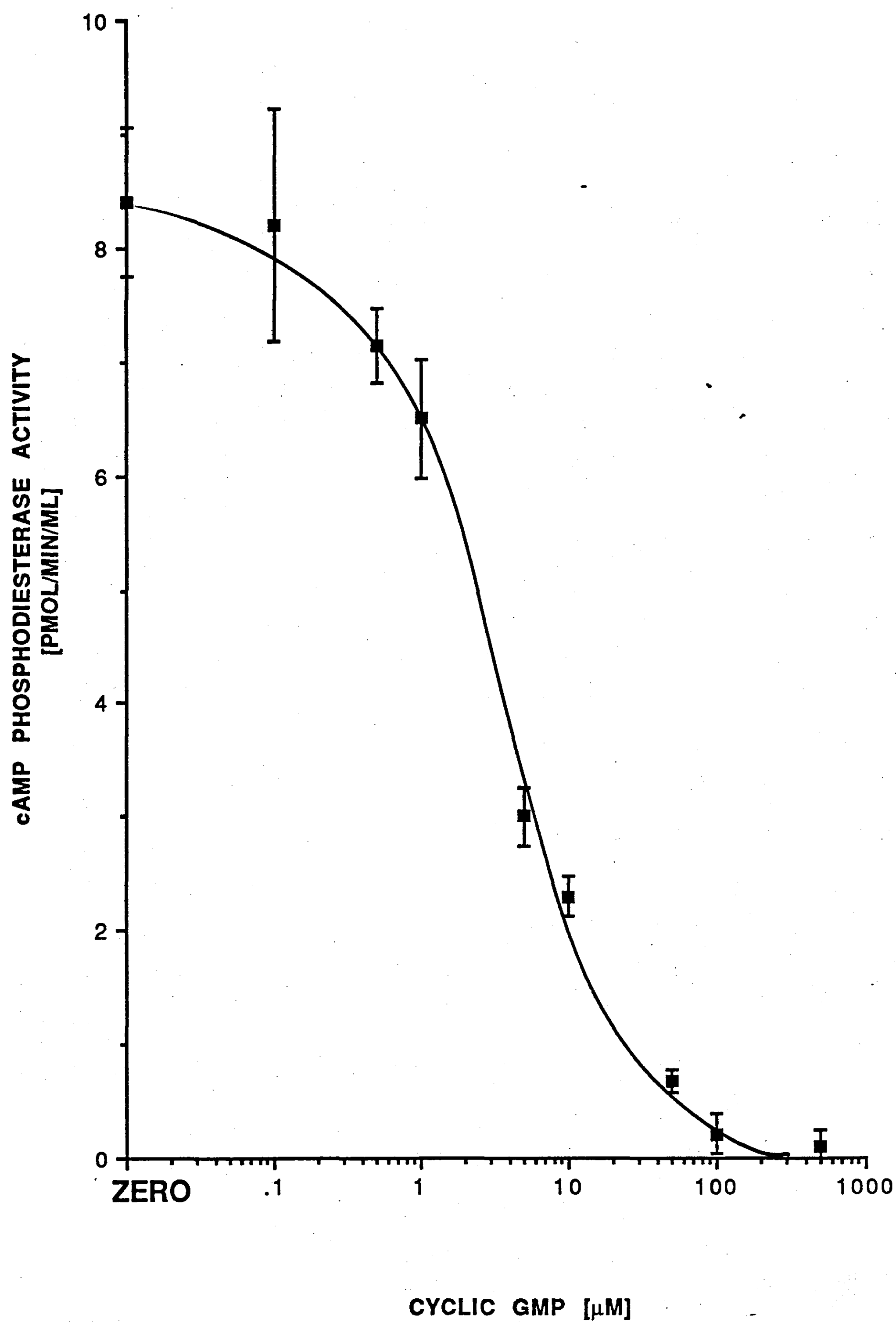


FIGURE 4.7:

INHIBITION OF THE CYCLIC AMP PHOSPHODIESTERASE ACTIVITY OF A PARTIALLY PURIFIED PREPARATION OF THE 'DENSE-VESICLE' PHOSPHODIESTERASE BY ICI 118233.

A partially purified preparation of 'dense-vesicle' PDE was isolated by Mono Q anion-exchange chromatography from a hypotonic extract prepared from one rat liver. Fractions were pooled and diluted to a suitable concentration in TMB buffer. Cyclic AMP phosphodiesterase activity was assessed at 0.1μM cAMP in the presence of increasing concentrations of ICI 118233 (0-100μM). The data is shown as the mean of triplicate determinations ± SD.

FIGURE 4.7:

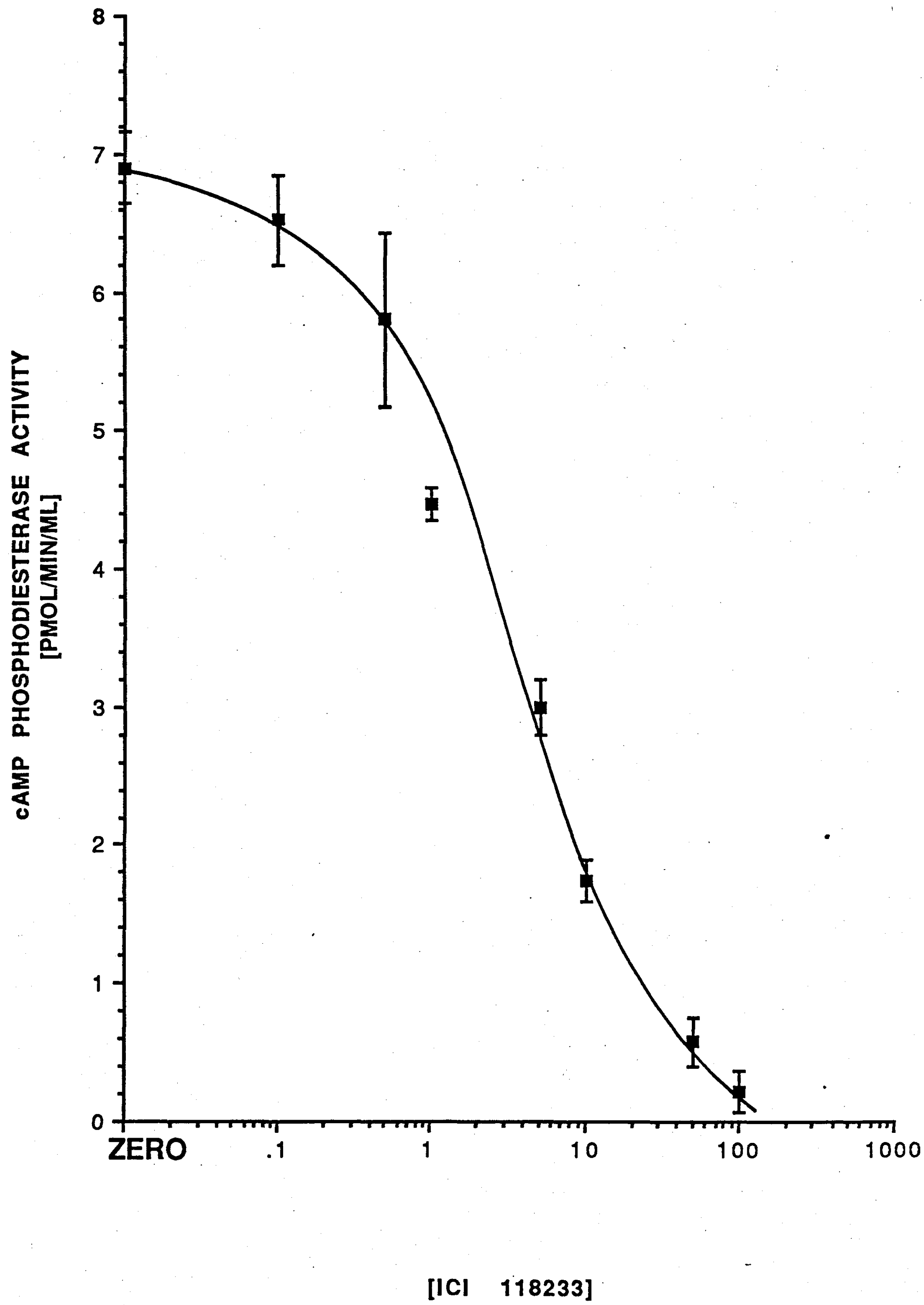


FIGURE 4.8:

KINETIC ANALYSIS OF ICI 118233 INHIBITION OF cAMP HYDROLYSIS BY THE RAT LIVER 'DENSE-VESICLE' PHOSPHODIESTERASE.

Cyclic AMP hydrolysis of a partially purified preparation of the 'dense-vesicle' phosphodiesterase (MQ-DV-PDE) was analysed over the substrate range 0.1-1.0μM in the absence and presence of increasing concentrations of the ICI 118233 inhibitor. Kinetics were analysed by Lineweaver-Burk plots of the reciprocal of the rate of cyclic AMP hydrolysis against the reciprocal of cyclic AMP substrate concentration. All points were determined in triplicate.

(▲) 0μM ICI 118233

(□) 1μM ICI 118233

(■) 10μM ICI 118233.

FIGURE 4.8:

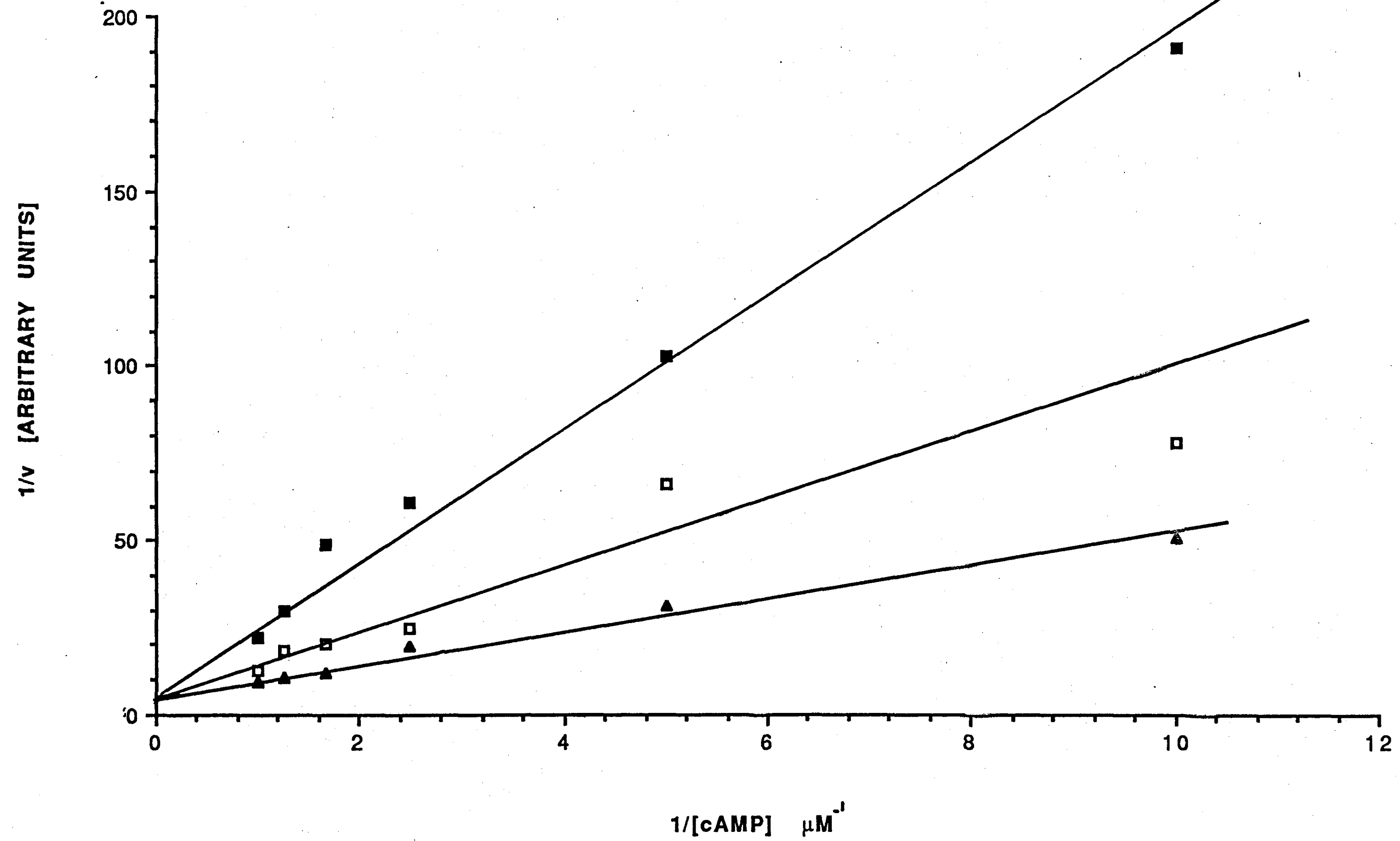


FIGURE 4.9:

DETERMINATION OF THE Ki FOR ICI 118233 INHIBITION OF CYCLIC AMP HYDROLYSIS OF THE 'DENSE-VESICLE' PHOSPHODIESTERASE.

The slope of the Lineweaver-Burk plot in Figure 5.6 was measured and plotted against the ICI 118233 concentration (μM). The straight line obtained had a correlation coefficient of 0.986. The intercept on the x-axis gave the K_i. In this typical experiment it was 4.1μM.

FIGURE 4.9:

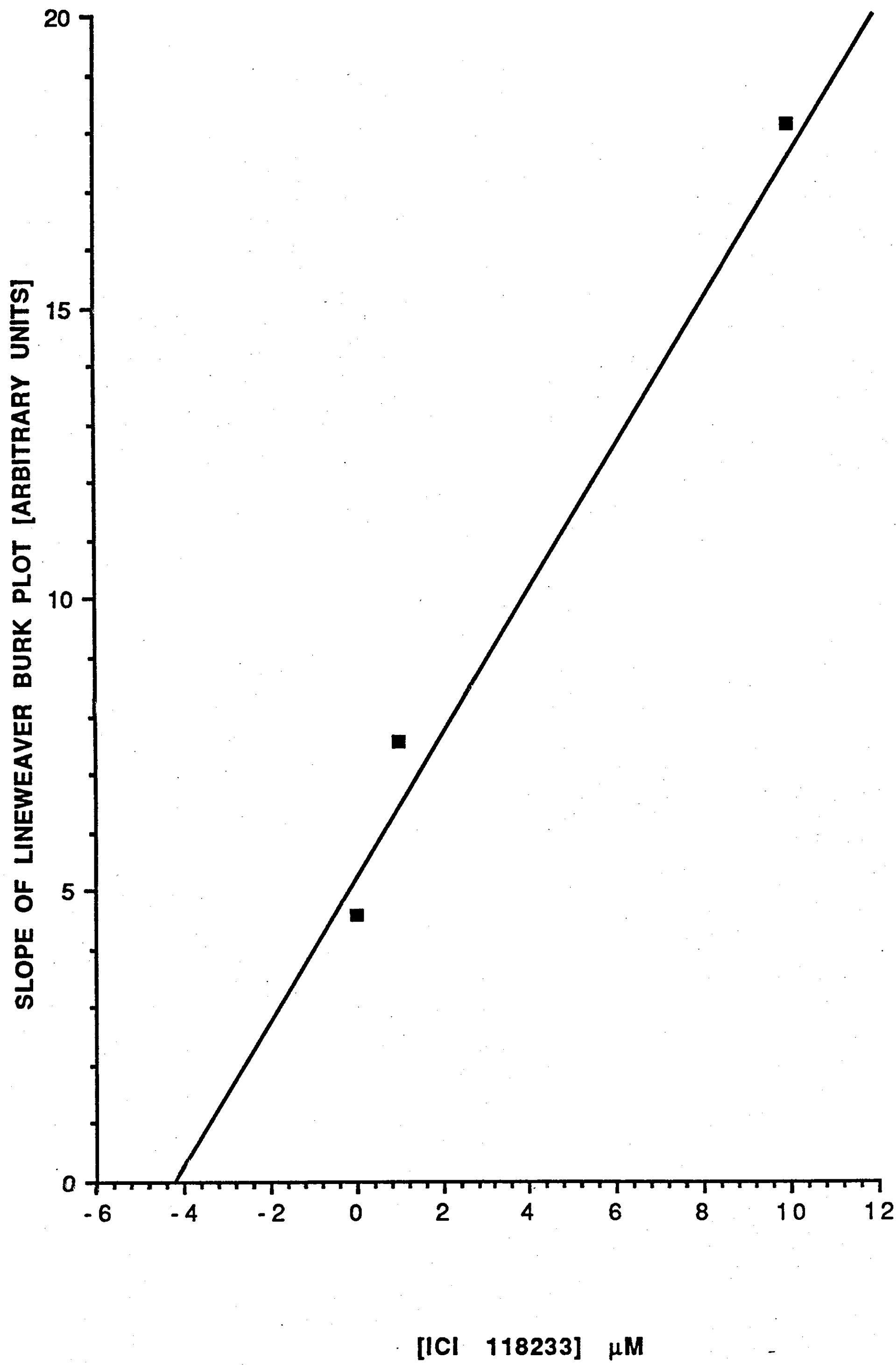


FIGURE 4.10:

CHROMATOGRAPHY OF HYPOTONIC EXTRACT ON ECTEOLA CELLULOSE.

70mls of hypotonic extract (195mg of protein) was applied to an ECTEOLA cellulose column (7.5 x 2.5 cm) at 0.5ml/min. The gel was washed with Buffer A (10mM Tris-HCl, pH 7.4, 1mM $MgCl_2$, 2mM Benzamidine, 0.1mM PMSF) at 1.5ml/min (350mls) and then with Buffer A containing 0.15M NaCl and 20% ethylene glycol (250mls). Bound phosphodiesterase activity was batch eluted at 1.5ml/min in Buffer A containing 0.4M NaCl and 36 x 2.5ml fractions were collected. cAMP phosphodiesterase activity was assessed at 0.1μM cAMP in the presence (□) and absence(■) of 10μM cGMP. Fractions containing activity were pooled and diluted 1:1 with Buffer A.

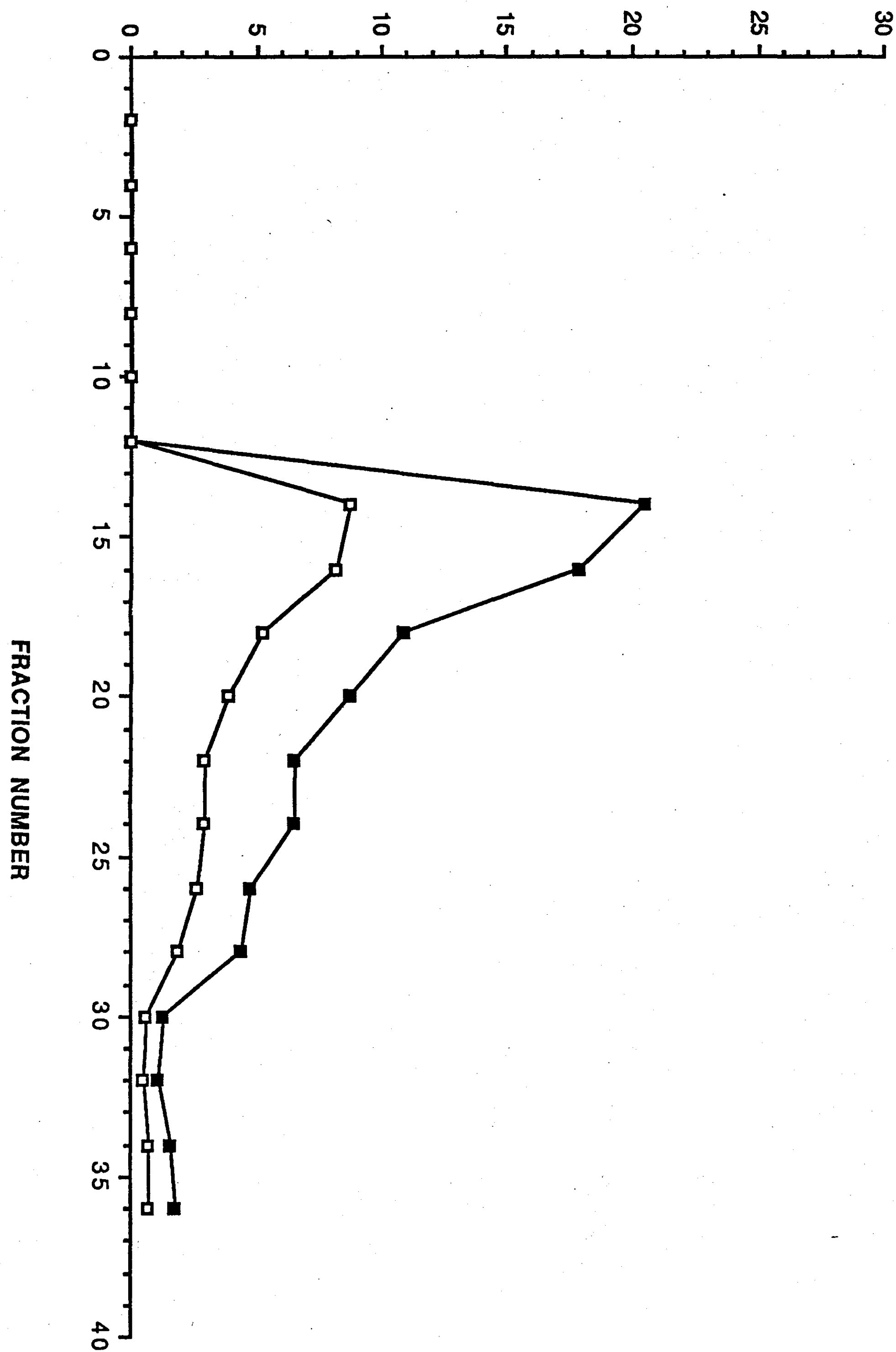


FIGURE 4.10:

FIGURE 4.11:

ELUTION OF cAMP PHOSPHODIESTERASE ACTIVITY FROM ω-AMINO-PENTYL AGAROSE.

Fractions containing activity from the ECTEOLA cellulose step (80mls, containing 6.5mg of protein) were pooled and applied to a column of ω-amino-pentyl agarose (3.1 x 1.3 cm) at 0.4ml/min. The column was washed at 1.5ml/min with 90mls of Buffer A (10mM Tris-HCl pH 7.4, 1mM $MgCl_2$, 2mM Benzamidine, 0.1mM PMSF), followed by 130mls of Buffer A containing 0.275M NaCl/20% ethylene glycol at 1.2ml/min. cAMP phosphodiesterase activity was batch eluted using Buffer A containing 0.5M NaCl/20% ethylene glycol. 15 x 3 ml fractions were collected and cAMP phosphodiesterase activity assayed in the presence (□) and absence (■) of 10μM cGMP.

FIGURE 4.11:

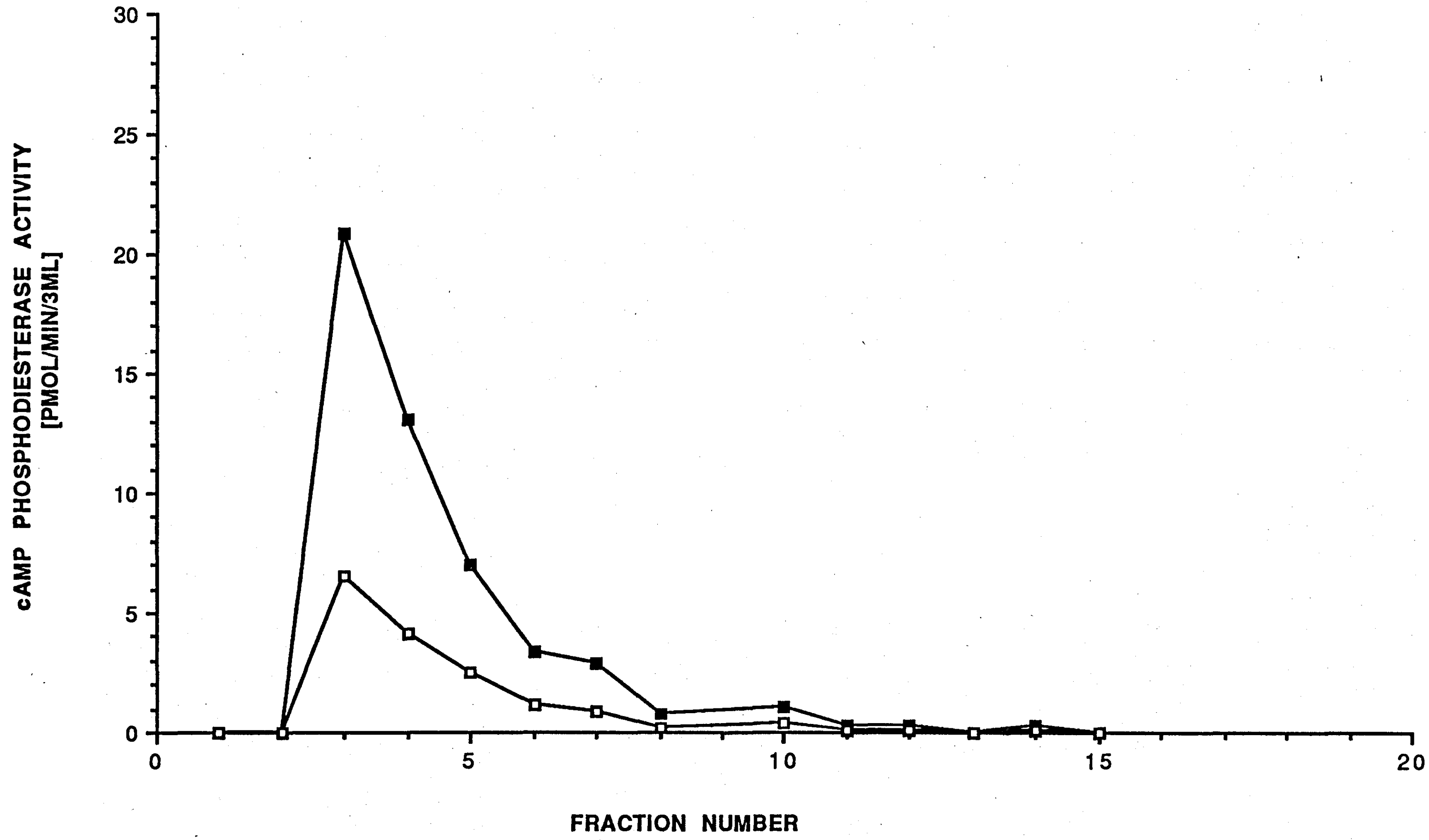


FIGURE 4.12:

ELUTION OF cAMP PHOSPHODIESTERASE ACTIVITY FROM SEPHADEX G-25.

Fractions (18mls containing 150µg of protein) containing cAMP phosphodiesterase activity were applied to a column of Sephadex G-25 (2.5 x 19 cm) and eluted in Buffer A (10mM Tris-HCL, pH 7.4, 1mM $MgCl_2$, 2mM Benzamidine, 0.1mM PMSF) at a rate of 1.2ml/min. 3ml fractions collected from the start of loading. cAMP phosphodiesterase activity was assayed at 0.1µM cAMP in the presence (□) and absence (■) of 10µM cGMP.

FIGURE 4.12:

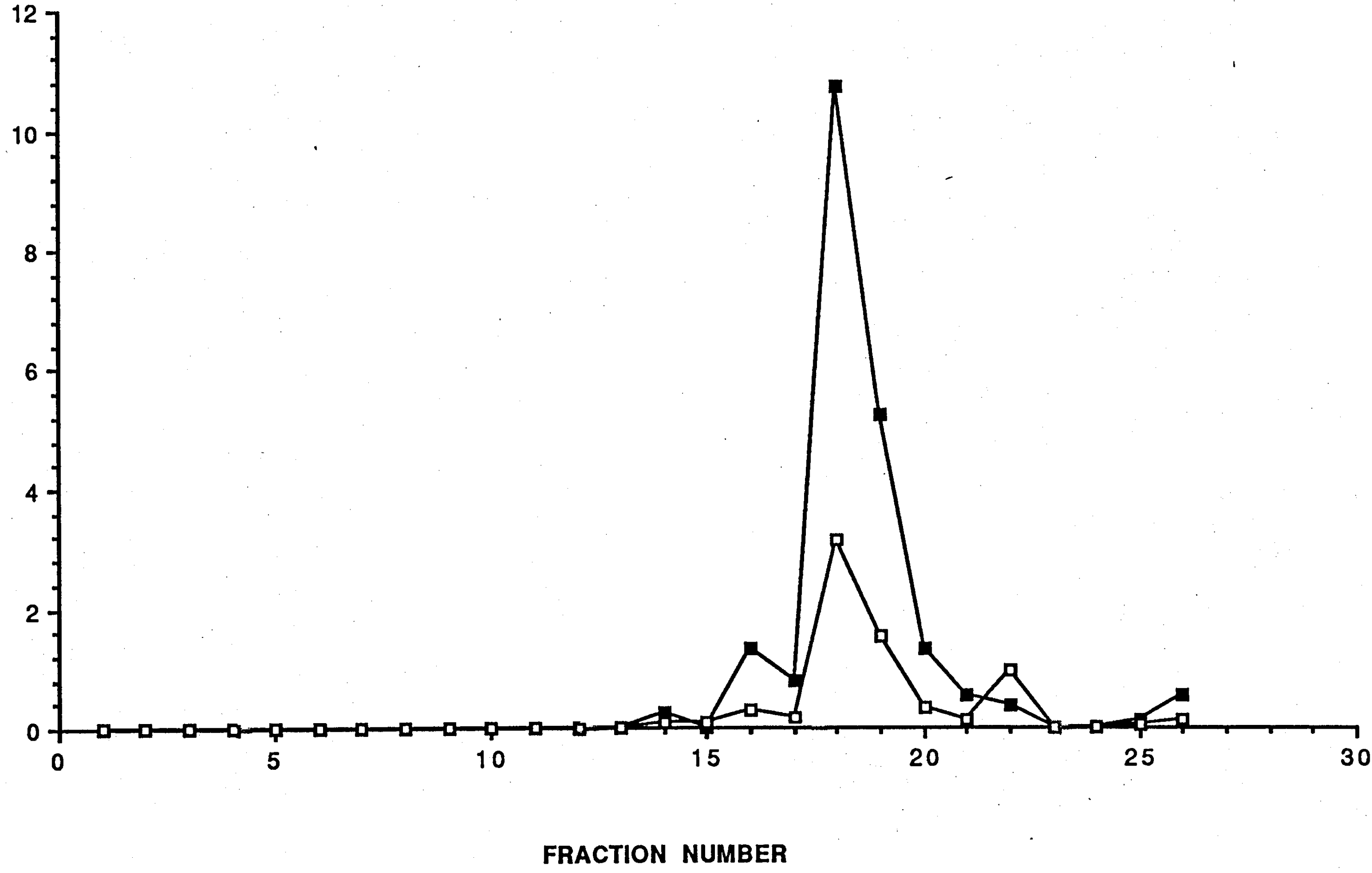


FIGURE 4.13:

ELUTION OF cAMP PHOSPHODIESTERASE ACTIVITY FROM GUANINE SEPHAROSE.

Fractions containing phosphodiesterase activity (15mls, 110μg of protein) were pooled and applied to a column of guanine-Sepharose (0.4 x 3 cm) at 0.3ml/min and washed in 48 mls of Buffer A (10mM Tris-HCL, pH 7.4, 1mM $MgCl_2$, 2mM Benzamidine, 0.1mM PMSF) followed by 15 mls of Buffer A containing 50mM NaCl. cAMP phosphodiesterase activity was eluted using Buffer A containing 0.4M NaCl/20% ethylene glycol. cAMP phosphodiesterase activity was assessed in the presence (□) and absence (■) of 10μM cGMP. 'High activity' fractions (7-9) were pooled separately from 'low-activity' fractions (10-13). The individual yields of protein were 5μg and 7μg resepectively.

FIGURE 4.13:

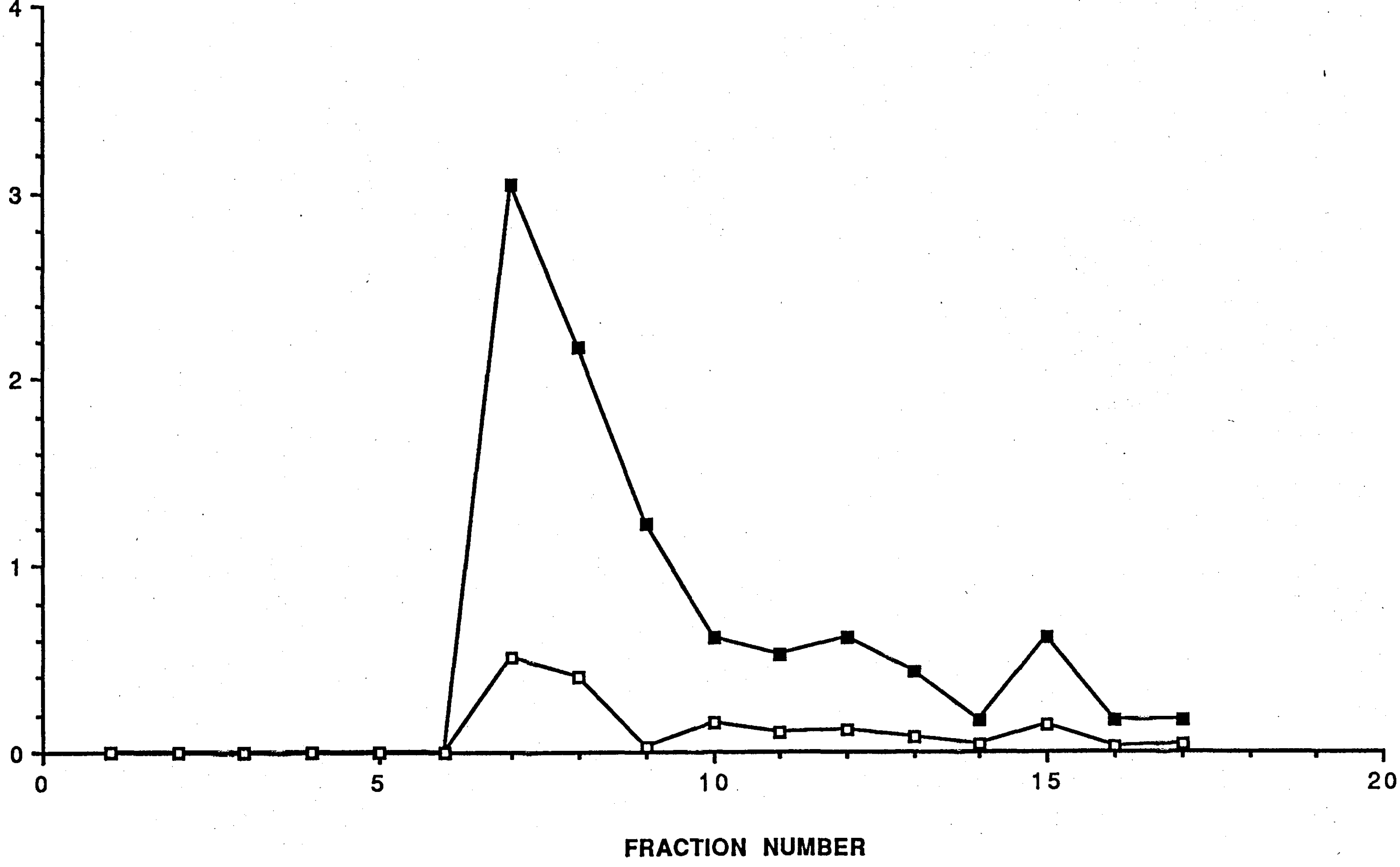


FIGURE 4.14:

SDS-PAGE ANALYSIS OF PURIFIED 'DENSE -VESICLE' PHOSPHODIESTERASE ISOLATED BY THE GUANINE-SEPHAROSE METHOD.

Samples of a purified preparation of the 'dense-vesicle' phosphodiesterase isolated by the Guanine-sepharose method were fractionated on a 10% SDS-PAGE gel with a 5% stacking gel (see Section 2.21). The Gel was stained in Coomassie Blue and destained as in Section 2.22.

LANE 1: Molecular Weight markers
LANE 2: Blank
LANE 3: 1.3µg of "low activity " shoulder from Guanine-Sepharose
LANE 4: Blank
LANE 5: Blank
LANE 6: 1.3µg of "high activity" shoulder from Guanine-Sepharose.

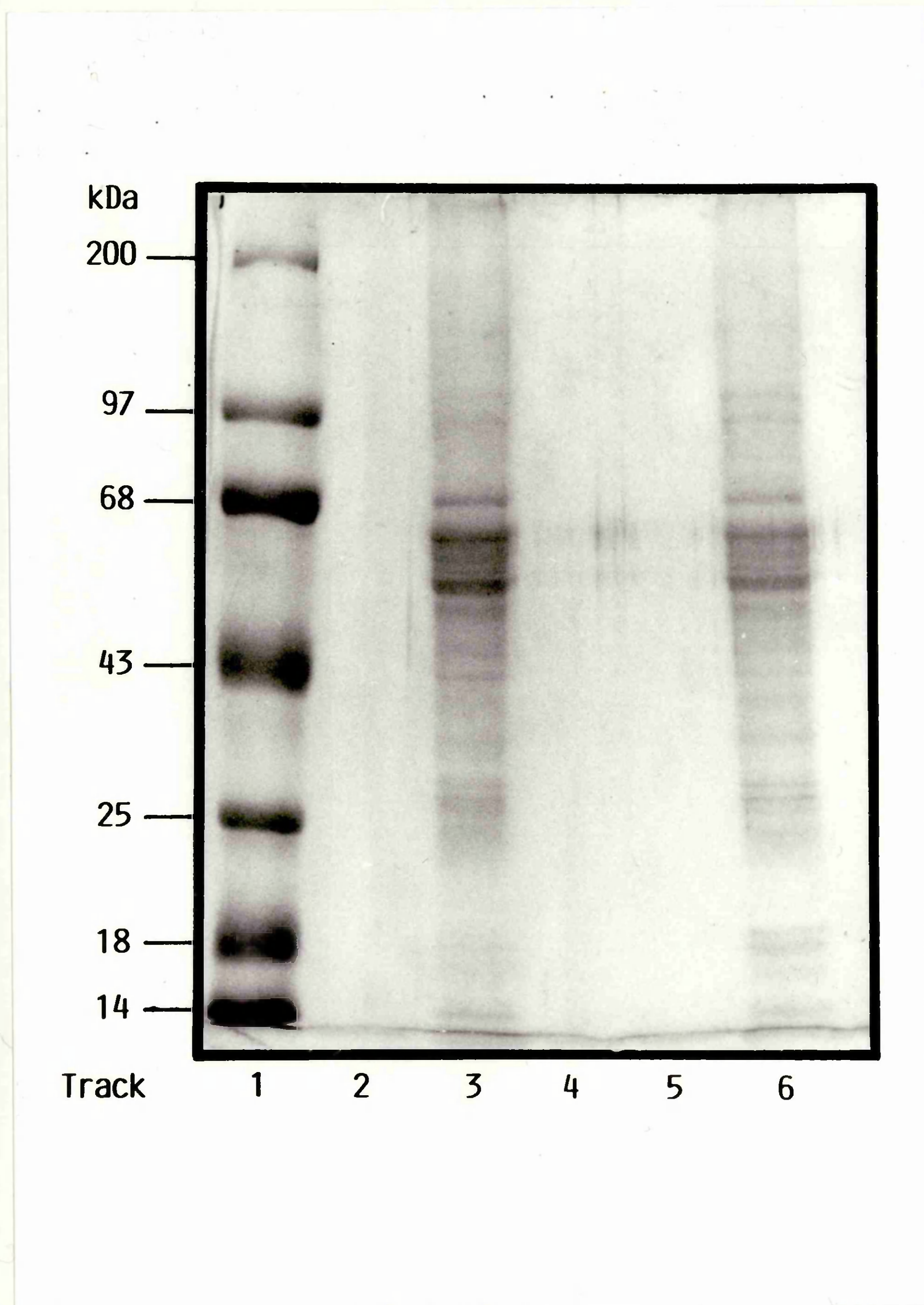

kDa
200
97
68
43
25
18
14
Track
1
2
3
4
5
6

FIGURE 4.15:

CHROMATOGRAPHY OF RAT LIVER HYPOTONIC EXTRACT ON AFFI-GEL BLUE.

A hypotonic extract was prepard from five rat livers and applied to an 8ml column of Affi-gel Blue (5 x 1.4cm) at 1ml/min and the breakthrough fraction collected as a pool. The column was washed in 48 Bed volumes of TMB Buffer (10mM Tris-HCl pH 7.4, 5mM $MgCl_2$ and the wash collected as a pool. cAMP phosphodiesterase activity was assayed in the absence (▩) or presence (▧) of 10μM cGMP. All assays were performed in triplicate and are shown as the mean ± SD.

The relative yields of protein were:

Hypotonic extract - 379mg.

Breakthrough - 214mg.

Wash - 75mg.

FIGURE 4.15:

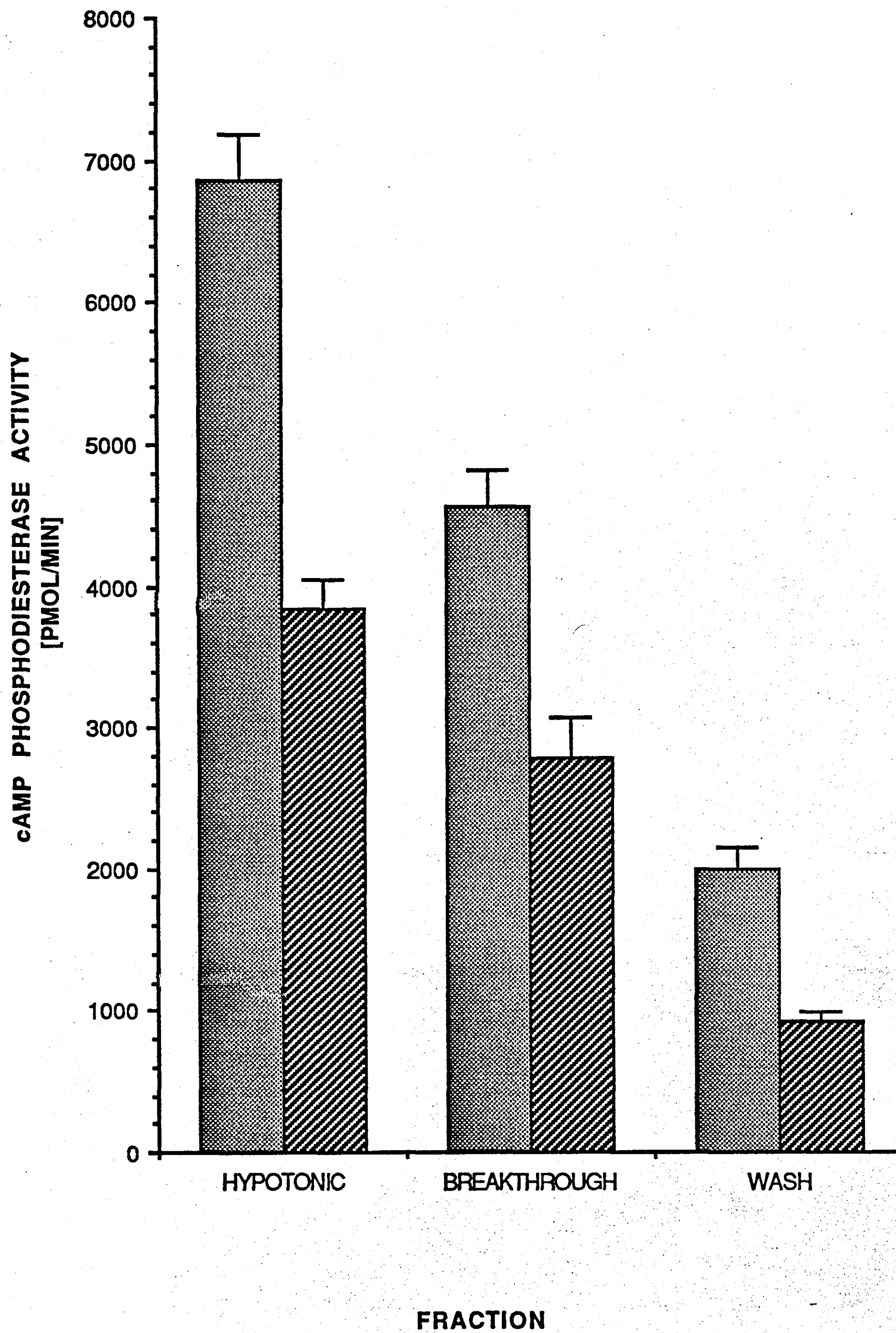


FIGURE 4.16:

ELUTION OF cAMP PHOSPHODIESTERASE ACTIVITY FROM AFFI-GEL BLUE.

Elution was initiated at 1ml/min using 200mls of 10mM Tris-HCl/1mM EDTA pH 7.4. Fractions of 4ml were collected and assayed for cAMP phosphodiesterase activity at 0.1μM cAMP in the presence (□) and absence (■) of 10μM cGMP. Activity is expressed as pmol/min/4ml.

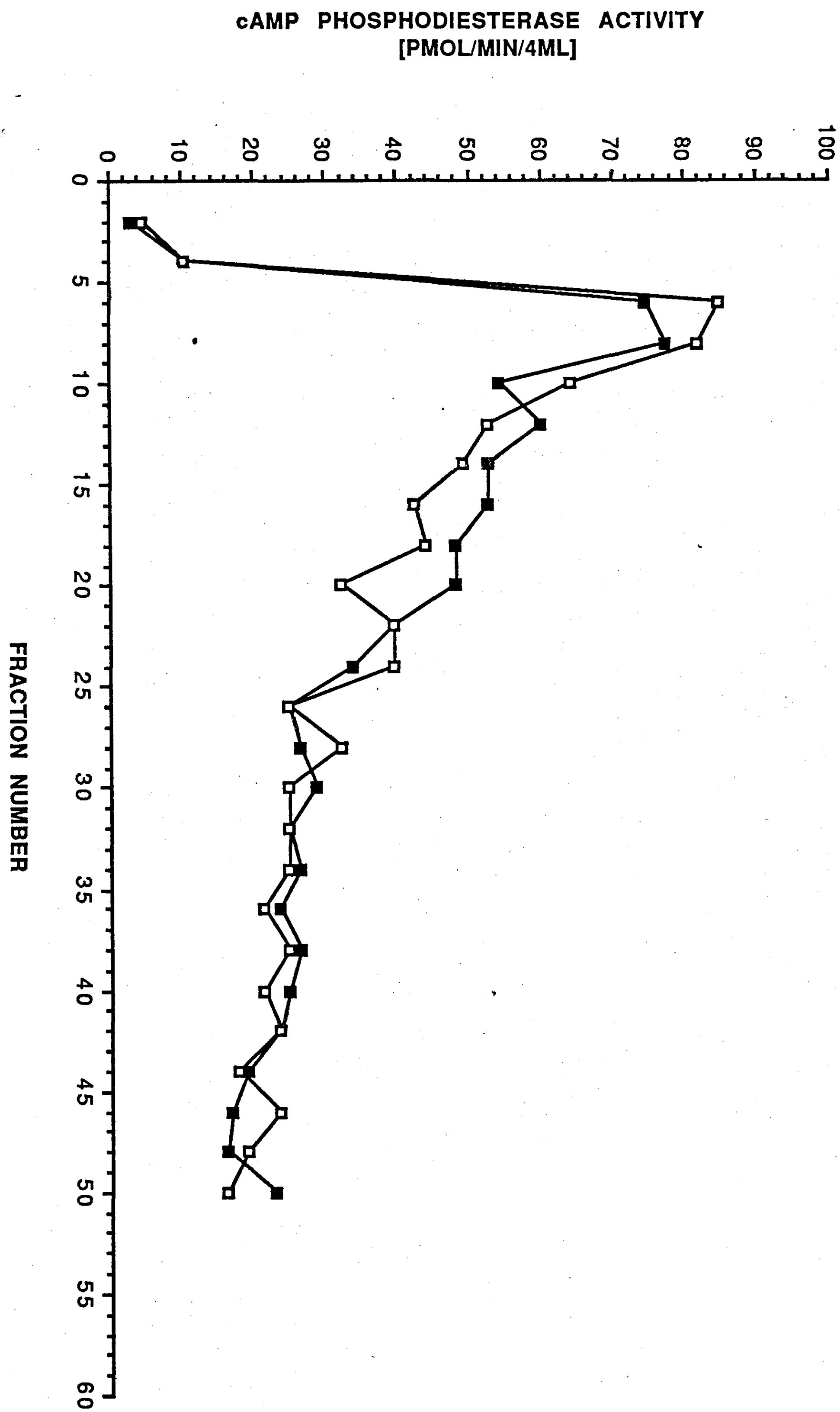


FIGURE 4.16:

FIGURE 4.17:

SCREENING OF AFFI-GEL BLUE BATCHES FOR BINDING OF 'DENSE-VESICLE' PHOSPHODIESTERASE.

100µl portions of Affi-gel Blue were equilibrated in TMB buffer (10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 2mM β–mercaptoethanol) (see section 2.12 for general method). 1ml samples of hypotonic extract (0.43mg protein and 9.85pmol/min of cAMP PDE activity) were made up to 10mM $MgCl_2$ and incubated with 100µl portions of the equilibrated matrix and mixed on a rotary mixer at 4^0c for 30 minutes. The gel was pelletted using a refrigerated microfuge (Hettich) at full speed (14,000g*av*) for 1 minute. The supernatant containing unbound material was removed and the matrix washed in 2 x 0.5 ml aliquots of TMB buffer and elution attempted in 2 x 0.5 aliquots of TEM buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 2mM β–mercaptoethanol).

cAMP phosphodiesterase activity was assessed at 0.1µM cAMP for the breakthrough fraction (▩) wash fraction (▧) and eluted fraction (▨). All assays were perfomed in triplicate and are presented as the mean ± SD.

1: Affi-gel Blue (BioRad) 100-200 mesh (wet) 15-100µ Batch 1

2: Affi-gel Blue (BioRad) 100-200 mesh (wet) 15-100µ Batch 2

3: Affi-gel Blue (BioRad) 100-200 mesh (wet) 15-100µ Batch 3

4: Cibracon Blue 3GA-agarose (Sigma) Type 300 containing 0.4 µmoles of dye per ml of gel

5: Cibracon Blue 3GA-agarose (Sigma) Type 1000 containing 1.1 µmoles of dye per ml of gel

6: Cibracon Blue 3GA-agarose (Sigma) Type 3000 containing 5.3 µmoles of dye per ml of gel

FIGURE 4.17:

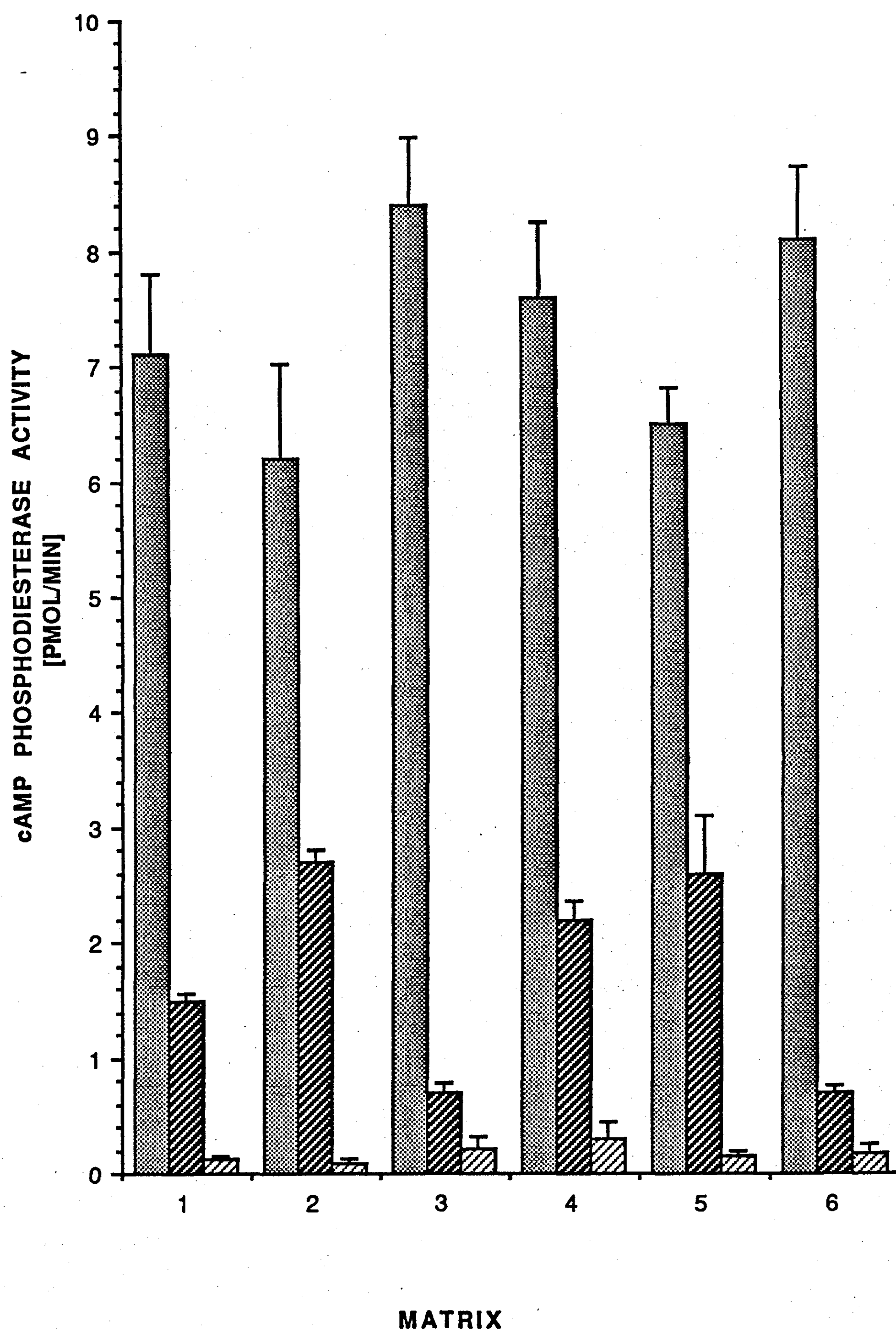


FIGURE 4.18:

ION EXCHANGE CHROMATOGRAPHY OF RAT LIVER HYPOTONIC EXTRACT ON DE-52

DE-52 resin was pepared as in Section 2.8.1 and equilibrated in TMB buffer (10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 2mM β–mercaptoethanol). A rat liver hypotonic extract in 10mM $MgCl_2$ (202mls) containing 174 mg of protein was applied at 1.35ml/min to a DE-52 column (6 x 2.4) and washed in TMB Buffer (300mls) at 1ml/min. Activity was eluted using a 400ml linear NaCl gradient between 0 and 0.55M contained in TMB buffer and 4 ml fractions collected. Cyclic AMP phosphodiesterase activity was assessed at 0.1μM cAMP in the presence (□) and absence (■) of 10μM cGMP.

The hypotonic extract contained 4820 pmol/min of cAMP PDE activity. The breakthrough fraction contained 71pmol/min and the wash fraction 131 pmol/min.

FIGURE 4.18:

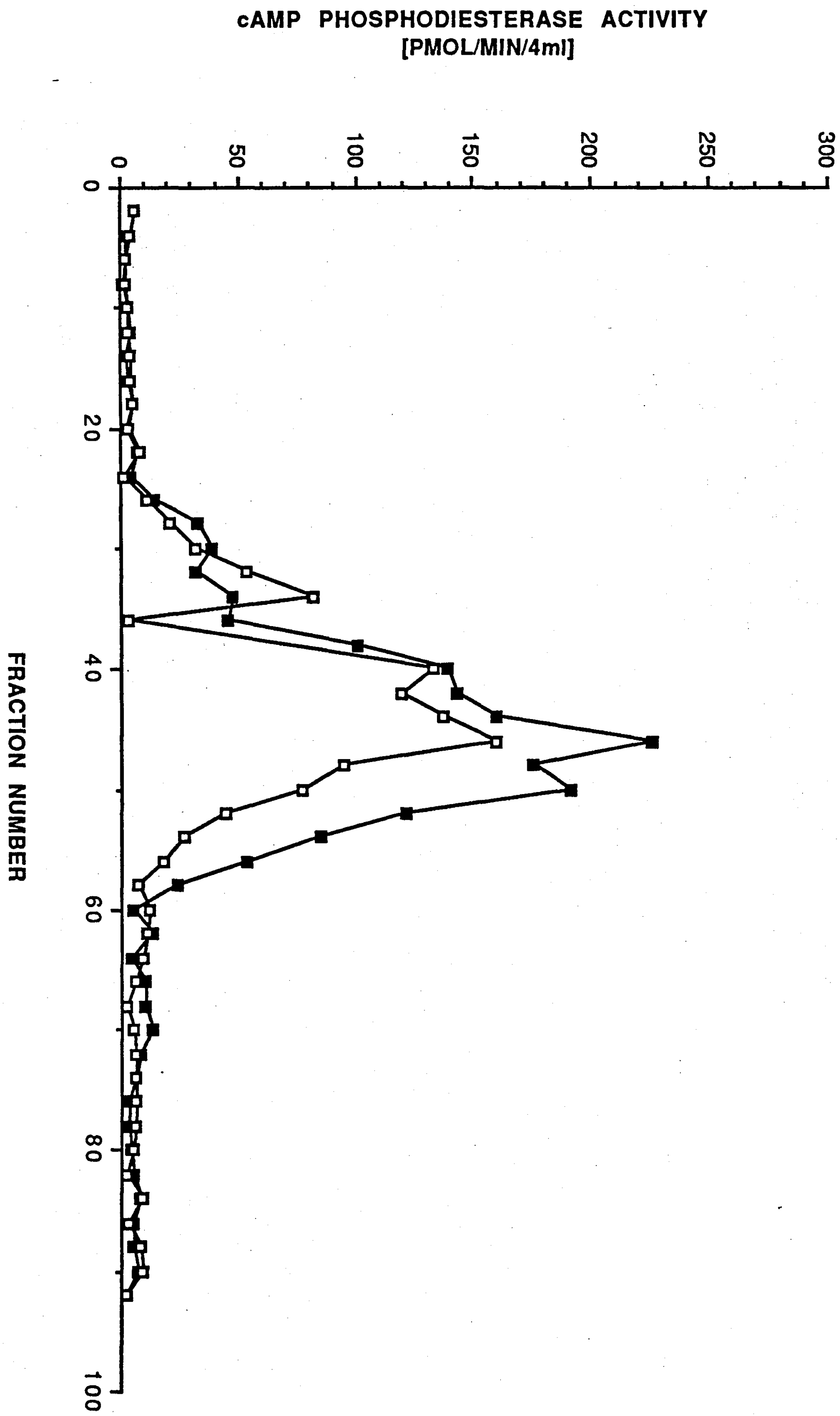


FIGURE 4.19:

ELUTION OF cAMP PHOSPHODIESTERASE ACTIVITY FROM AFFI-GEL BLUE.

Fractions from the DE-52 step (60mls, 9.2mg of protein) were dialysed at 4^{0}c against 3 x 2 litre changes of TM Buffer (10mM Tris-HCl pH 7.5) to remove sodium chloride. The dialysed sample was made up to 2mM β-mercaptoethanol and applied to a column of Affi-gel Blue (6.4 x 1.8cm) that had previously been equilibrated in TMB Buffer. The breakthrough fraction was collected and the column washed in TMB buffer (360mls). Elution of cyclic AMP phosphodiesterase activity was initiated using 200mls of TEM Buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 2mM β-mercaptoethanol) at 0.2ml/min and 50 x 4ml fractions were collected. Cyclic AMP phosphodiesterase activity was assessed at 0.1μM cAMP in the presence (□) and absence (■) of 10μM cGMP. Pooled fractions contained 200μg of protein.

FIGURE 4.19:

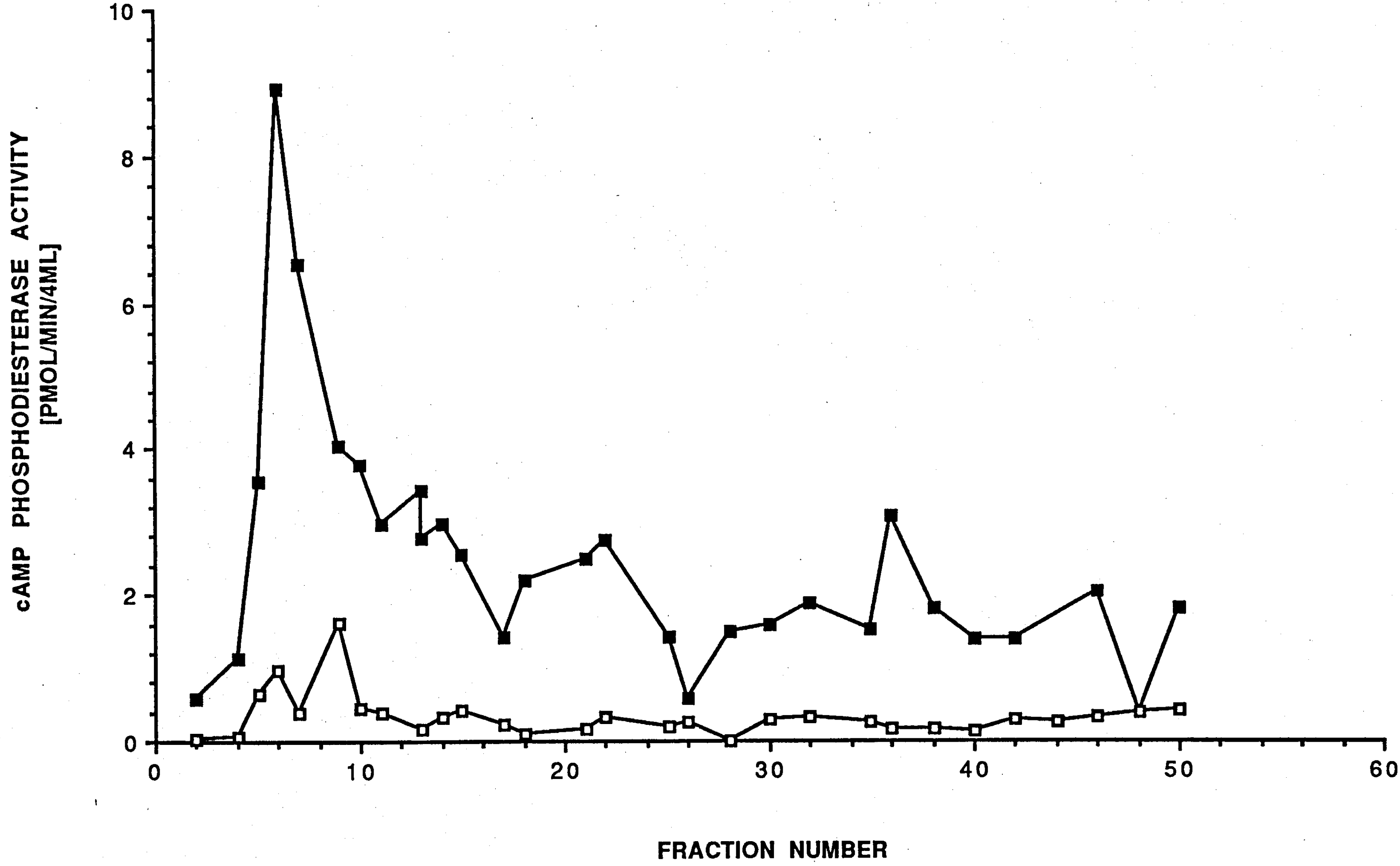


FIGURE 4.20(a):

STABILITY OF cAMP PHOSPHODIESTERASE ACTIVITY OF THE 'DENSE-VESICLE' PDE.

A partially purified preparation of the 'dense-vesicle' PDE (from the DE-52 step) was incubated at 4^0C in the presence (▧) and absence (▩) of 2mM β–mercaptoethanol for the times indicated before aliquots of enzyme were removed for assay of cAMP PDE activity.

FIGURE 5.20(b):

STABILITY OF THE CYCLIC GMP INHIBITED cAMP PDE ACTIVITY OF THE 'DENSE-VESICLE' PDE.

A partially purified preparation of the 'dense-vesicle' PDE (from the DE-52 step) was incubated at 4^0C in the presence (▩) and absence (▧) of 2mM β–mercaptoethanol for the times indicated before aliquots of enzyme were removed for assay of cAMP PDE activity in the presence of 2μM cGMP.

FIGURE 4.20(a):

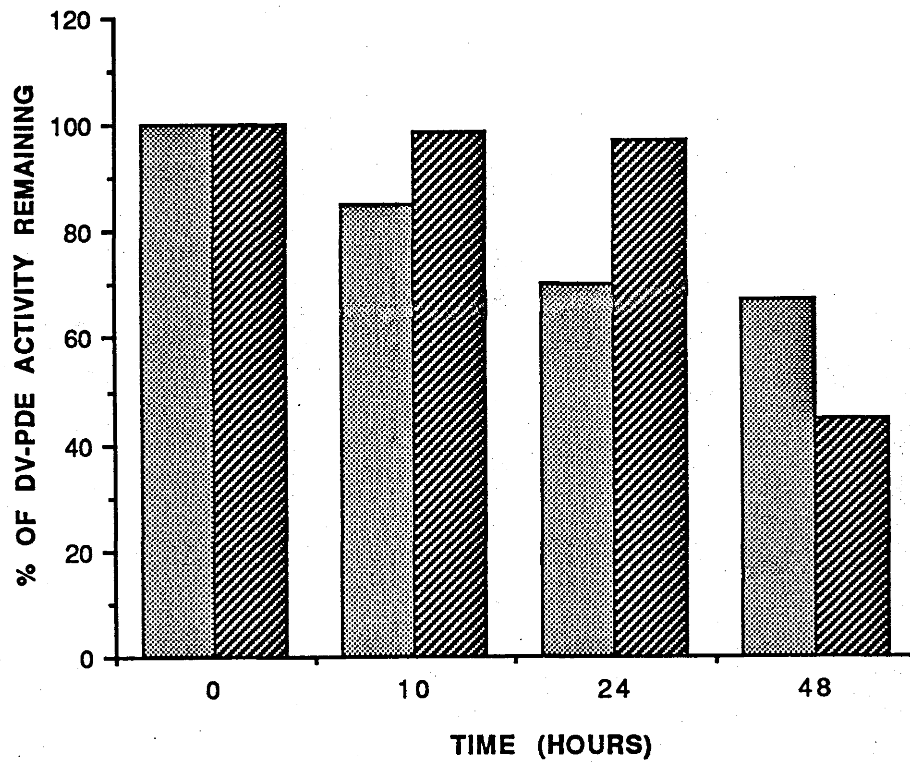


FIGURE 4.20(b):

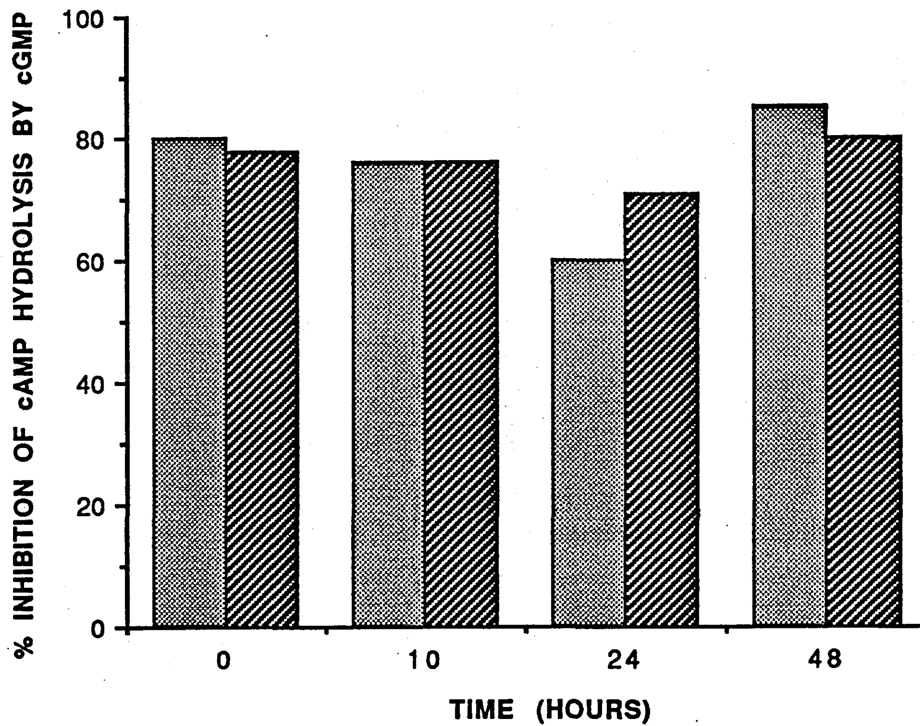


FIGURE 4.21:

SEPARATION OF RAT LIVER HYPOTONIC EXTRACT PHOSPHODIESTERASES USING MONO Q ANION-EXCHANGE CHROMATOGRAPHY.

The hypotonic extract from one rat liver (80mg of protein) was applied to a Mono Q column (HR 5/5) and PDE activity eluted using an identical 95 ml gradient to the one used for separation of soluble phosphodiesterases (see Table 3.2). 1ml fractions were collected and 25µl aliquots were assayed for cAMP PDE activity (■), cGMP PDE activity (○) and cAMP PDE activity in the presence of 2µM cGMP (□). Results are expressed as pmol/min/ml.

The hypotonic extract contained 964pmol/min of cAMP PDE activity. The yield of cAMP PDE activity was 83% and for cGMP PDE activity was 81%.

FIGURE 4.21:

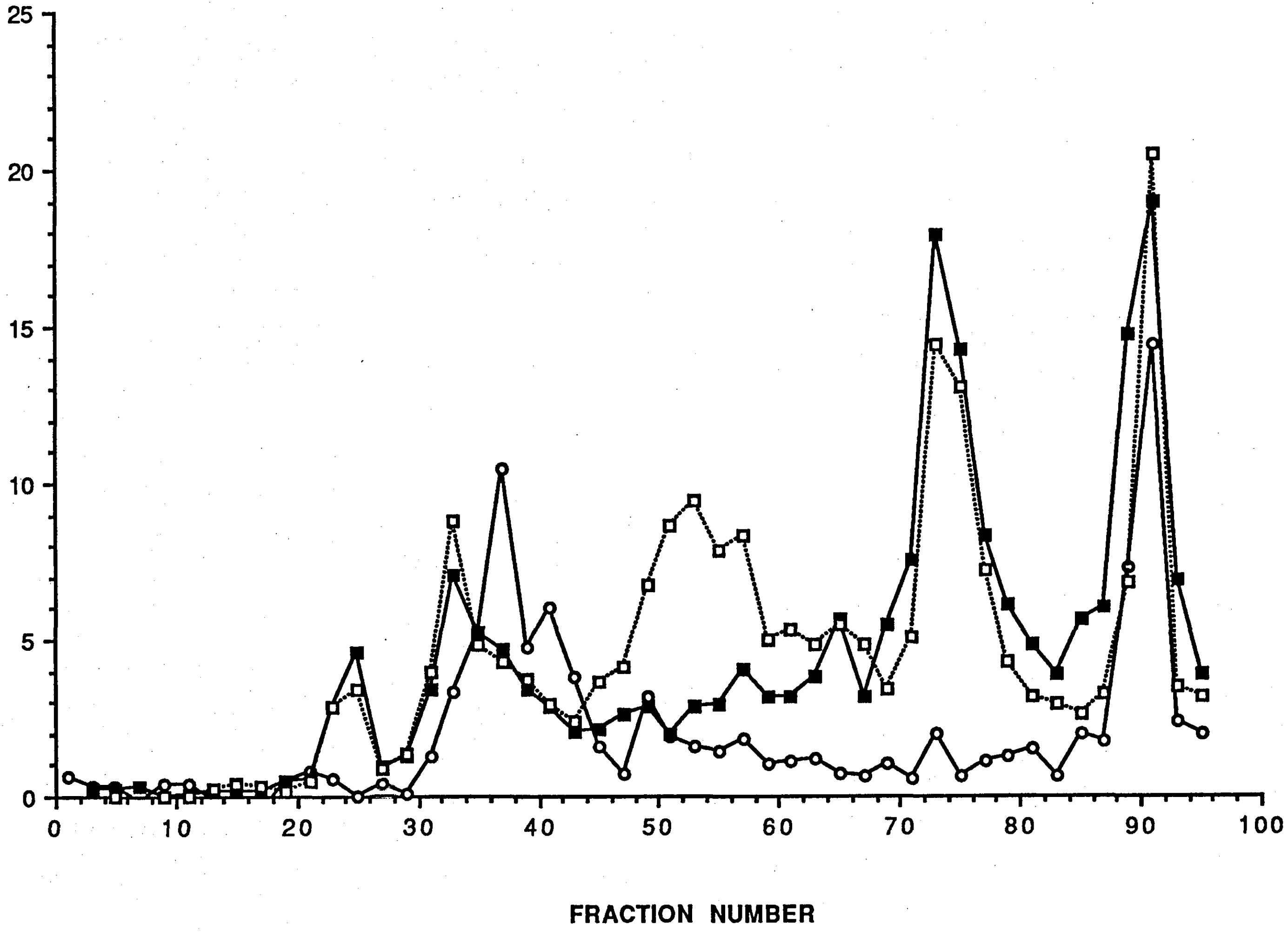


FIGURE 4.22:

CHROMATOGRAPHY OF A RAT LIVER HYPOTONIC EXTRACT ON MONO Q ION EXCHANGE USING AN EXTENDED GRADIENT

A hypotonic extract from one rat liver (120mg of protein) was applied to a Mono Q column and activity was eluted using a NaCl gradient contained in TMB Buffer (10mM Tris-HCl, pH 7.4, 5mM $MgCl_2$, 2mM β-mercaptoethanol) (see Table 5.5). 1ml fractions were collected and 25µl aliquots assayed for cAMP phosphodiesterase activity assessed at 0.1µM cAMP in the presence (□) and absence (■) of 10µM cGMP. The figure shows the cAMP phosphodiesterase activity eluted over 0.3-0.5M NaCl. Results are expressed as pmol/min/ml.

The hypotonic extract contained 768 pmo/min of cAMP PDE activity.

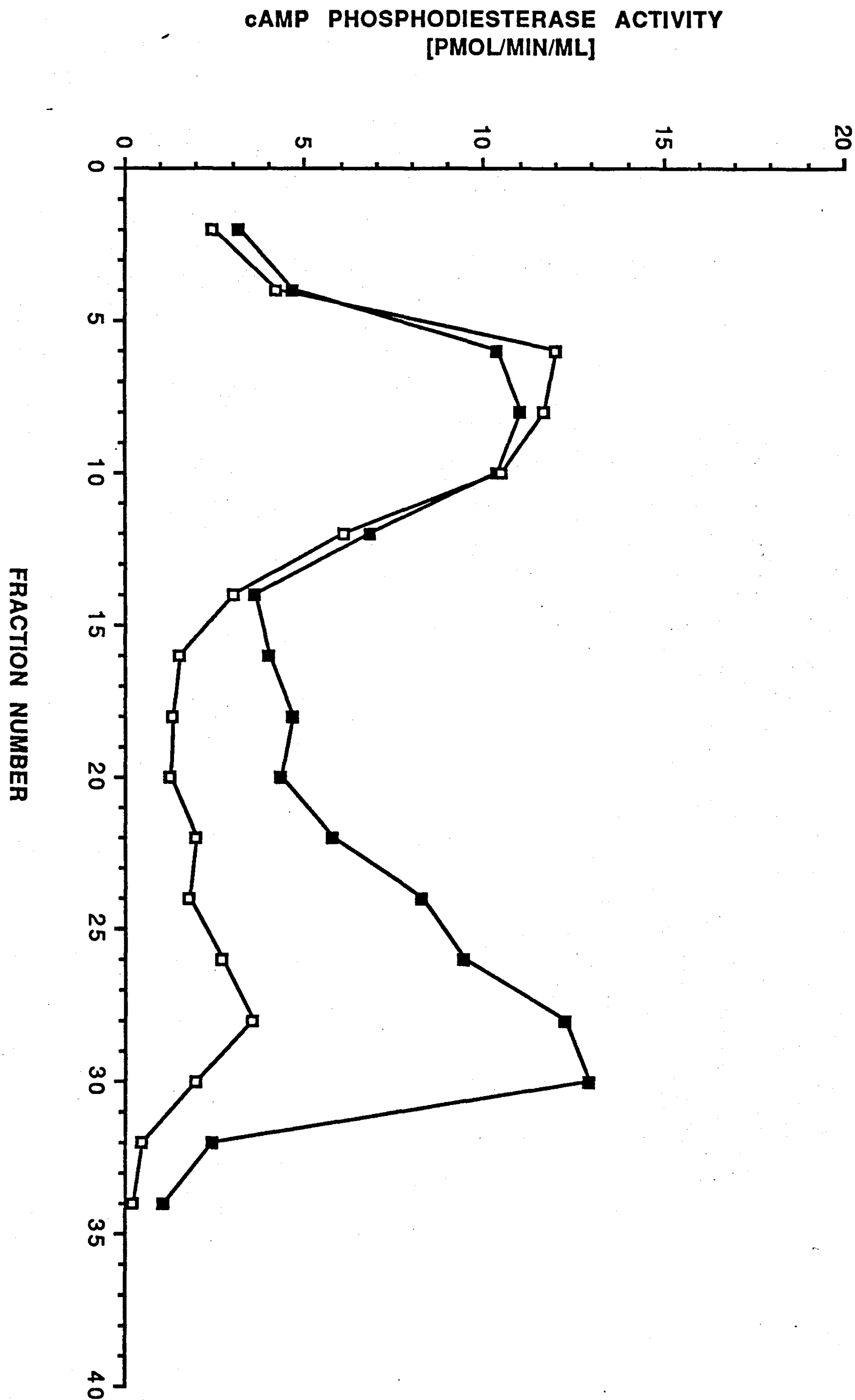


FIGURE 4.22:

FIGURE 4.23:

REVERSIBILITY OF THE INHIBITION OF THE 'DENSE-VESICLE' PHOSPHODIESTERASE ELICITED BY VARIOUS INHIBITORS.

Partially purified 'dense-vesicle' phosphodiesterase prepared using Mono Q chromatography was incubated either in TMB Buffer (10mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 2mM β-mercaptoethanol) or 100μM of each of cGMP, IBMX, or ICI 118233 in TMB buffer. Samples were assayed for phosphodiesterase activity before (▩) or after dialysis in the presence (□) and absence (▨) of 10μM cGMP. The data show a typical experiment performed three times. Results are shown as the mean ± SD.

FIGURE 4.23:

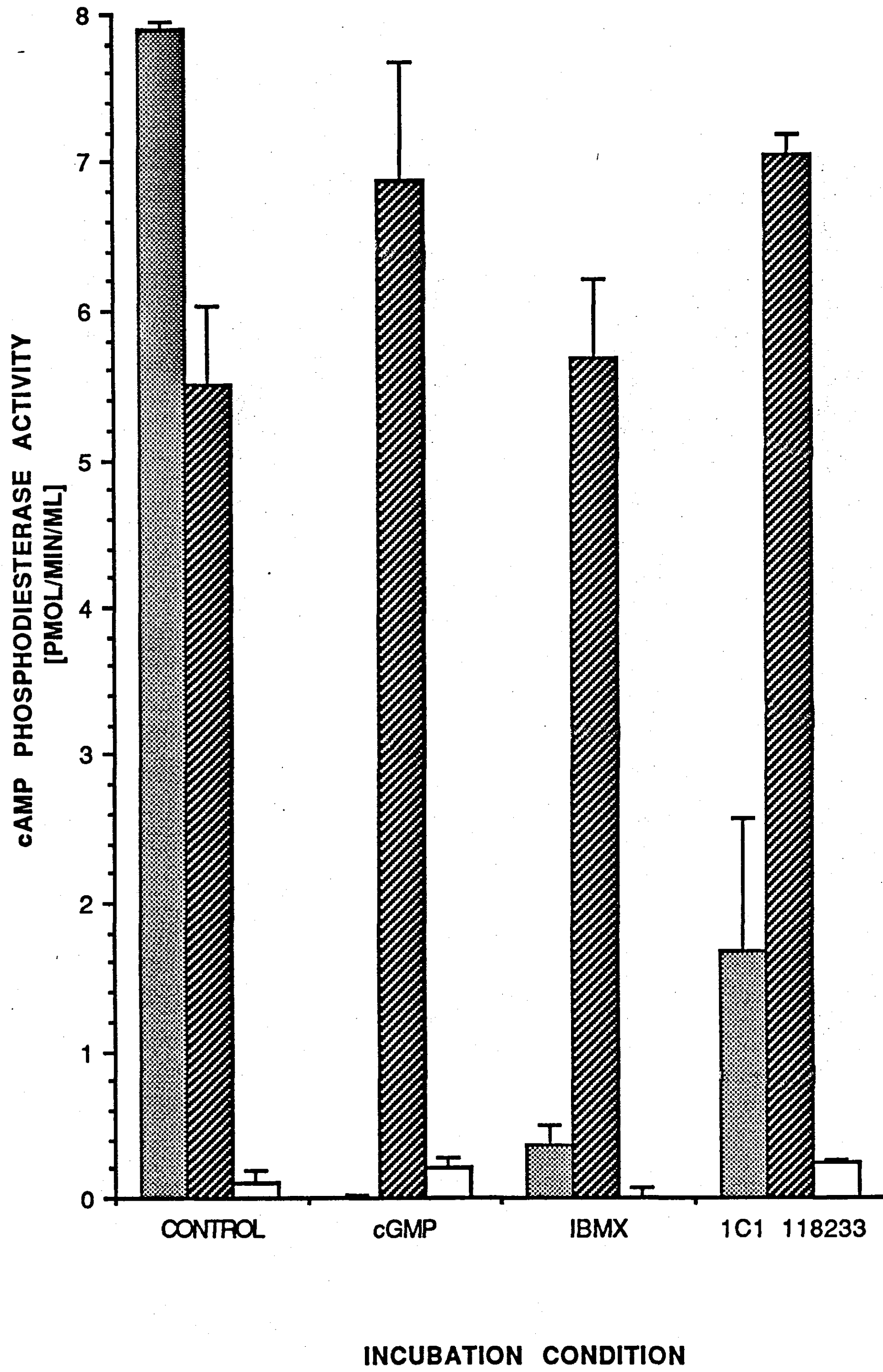


TABLE 4.1:

USE OF ANTISERA DV-I AND PM-I TO ASSESS THE DISTRIBUTION OF THE 'DENSE-VESICLE' (DV) AND 'PERIPHERAL' PLASMA MEMBRANE (PM) CYCLIC AMP PHOSPHODIESTERASES IN RAT TISSUES.

Results are expressed as the average (± SEM) of three separate experiments. Tissue distribution (TD) ratio is calculated by dividing the μg of of DV-PDE/mg of homogenate in one tissue by the μg of DV-PDE/mg of homogenate in liver.

TISSUE	DV PDE [µg/mg of homogenate]	TD RATIO	PM-PDE [µg/mg of homogenate]	RATIO [DV/PM]
LIVER	0.15±0.02	(1)	0.15±0.03	(1)
KIDNEY	0.14±0.02	0.9	0.12±0.02	1.1
HEART	0.75±0.08	5	0.11±0.03	7.1
ADIPOSE	0.48±0.06	3.2	0.11±0.02	4.6

TABLE 4.2:

USE OF THE SELECTIVE PDE INHIBITOR ICI 118233 TO ASSESS THE DISTRIBUTION OF THE 'DENSE-VESICLE' CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN RAT TISSUES.

Results are expressed as the average (± SEM) of three separate experiments. Tissue distribution (TD) ratio is calculated by dividing the DV-PDE activity/mg of tissue by the DV-PDE activity/mg in liver.

TISSUE	cAMP HYDROLYSIS [pmol/min/mg]	% INHIBITION	DV PDE ACTIVITY [pmol/min/mg]	TD RATIO
LIVER	3.33±0.21	24±10	0.8	(1)
KIDNEY	8.8±0.64	12±5	1.1	1.4
HEART	5.9±0.34	44±3	2.6	3.3
ADIPOSE	3.22±0.27	59±7	1.9	2.4

TABLE 4.3:

RECOVERY AND STABILITY OF cAMP PHOSPHODIESTERASE ACTIVITY DURING PURIFICATION BY THE GUANINE SEPHAROSE METHOD

(a) The % of cyclic AMP phosphodiesterase activity retrieved from individual columns was determined by assaying a sample of the extract before chromatography along with a sample of the pooled fraction from the particular steps indicated. Assays were performed at the same time.

(b) The % of cAMP phosphodiesterase activity remaining over approximately a 24 hour period at 4°C was assessed at individual stages of the purification by assaying the pooled samples prior to a chromatographic step and then again 24 hours later. Results are expressed as the % of activity remaining

COLUMN YIELDS ASSESSED OVER FIVE PREPARATIONS

	PREPARATION				
	1	2	3	4	5
STEP	% ACTIVITY RETRIEVED				
ECTEOLA	37%	29%	28%	38%	18%
ω-APA	19%	16%	38%	20%	22%
Sephadex	100%	20%	66%	34%	67%
Guanine	68%	94%	87%	100%	100%

DECAY OF ACTIVITY AT 4^0c FOR FIVE PREPARATIONS

	PREPARATION				
	1	2	3	4	5
STEP	% ACTIVITY REMAINING				
ECTEOLA	100%	100%	100%	100%	100%
ω-APA	91%	100%	89%	100%	100%
Sephadex	30%	100%	30%	30%	100%
Guanine	70%	50%	60%	45%	30%

TABLE 4.4:

PURIFICATION OF THE 'DENSE-VESICLE' PHOSPHODIESTERASE BY THE GUANINE SEPHAROSE METHOD

All activities were determined at 0.1μM cAMP as substrate in the absence (-) or presence of 10μM cGMP (+).

Step	Total activity (pmol/min)	cGMP ratio (+/-)	Protein (mg)	Specific activity (pmol/min/mg)	Yield (%)	Fold Purification
Hypotonic	1003	0.86	195	5.14	(100)	(1)
ECTEOLA	389	0.45	6.5	59.8	38.9	11.6
ω-APA	79.1	0.33	0.15	527	7.9	103
Sephadex	7.7	0.20	0.12	64.2	0.78	12.5
Guanine	8.1	0.25	0.012	675	0.8	131

CHAPTER 4: DISUSSION

Cyclic nucleotide phosphodiesterases are known to exist in multiple forms in mammalian tissues (Wells and Hardman, 1977; Thompson and Strada, 1978; Beavo, et al., 1982; Beavo, 1988). It appears from peptide mapping (Takemoto, et al., 1982) and immunological studies (Mumby, et al., 1982; Pyne, et al., 1987a; Harrison, et al., 1986b; Sharma, et al., 1984) that distinct classes of phosphodiesterase exist. These include the Ca^{2+}/Calmodulin-stimulated phosphodiesterases (Beavo, 1988; Wang, et al., 1990), cyclic GMP-stimulated phosphodiesterases (Beavo, 1988; Manganiello, et al., 1990b) and cyclic AMP-specific phosphodiesterases (Beavo, 1988; Beavo, 1990; Conti and Swinnen, 1990; Manganiello, et al., 1990a). The observations that the activities of specific isozymes are under different control mechanisms suggests that various forms have particular functional roles within the cell (Evans, et al., 1984; Tanner, et al., 1986; Hamet and Tremblay, 1988; Pyne and Housaly, 1988; Beavo, 1988; Corbin, et al., 1985; Heyworth, et al., 1983d; Heyworth, et al., 1984a). As such, one might expect that each tissue will exhibit a particular complement of phosphodiesterases isozymes. This is certainly true of phospho-diesterases such as the light activated cyclic GMP phosphodiesterase, which is only expressed in the retina (Baehr, et al., 1979; Gillespie and Beavo, 1989).

Further to this, it is now apparent from immunological, cloning and purification studies, that isozymes of phosphodiesterases exist within each of the major classes of phosphodiesterase. For example, it has been shown that there are distinct isozymes of the Ca^{2+}/Calmodulin-stimulated PDE (Sharma, et al., 1984; Kincaid, et al., 1984; Wang, et al., 1990), the retinal phosphodiesterases (Gillespie, et al., 1989b; Gillespie, 1990), cyclic GMP-stimulated phosphodiesterases (Pyne, et al., 1986b; Manganiello, et al., 1990a), the

cyclic AMP-specific phosphodiesterases (Weber and Appleman, 1982; Weishaar, et al., 1987a; Manganiello, et al., 1990b; Davis, 1990, Conti and Swinnen, 1990;, Degerman, et al., 1987;, Grant, et al., 1988; Davis, et al., 1989; Swinnen, et al., 1989; Collicelli, et al., 1989; Henkel-Tigges and Davis, 1990). For the case of the cyclic AMP-specific phosphodiesterases, it has been shown that the tissue distribution of mRNA species homologous to four independent cDNA clones (that have been presumed to encode cyclic AMP-specific phosphodiesterases) is different between brain, heart, kidney and testis (Swinnen, et al., 1989).

In initial studies on the isozymes of phosphodiesterase, identification of multiplicity has relied upon the ability to resolve individual activities from one another (Beavo, et al., 1982; Appleman, et al., 1973; Thompson and Appleman, 1971; Thompson and Strada, 1978). This necessarily involves the homogenisation of the tissue and in some cases can involve quite lengthy separation procedures. The conclusions based on such analyses are limited by the resolving power of the technique employed (such as DE-52 resin) as well as problems of differential extraction of various enzymes, solubilisation of membrane bound activities and susceptibility to degradation by proteolysis during extraction and purification procedures. It is well documented that species such as the Ca^{2+}/Calmodulin-stimulated phosphodiesterase (Cheung, 1971; Tucker, et al., 1981; Kincaid, et al., 1985) and the cyclic GMP-inhibited phosphodiesterase (Harrison, et al., 1986b; Grant and Colman, 1984, Grant, et al., 1988; Pyne, et al., 1987a; Loten, et al., 1978; Loten, et al., 1980) are susceptible to proteolysis. In order to address this problem it was decided to assess the expression of phosphodiesterase in tissues using immunoblotting of freshly prepared tissue homogenates. In this case, a technique was employed where tissues were disrupted rapidly in a medium containing protease inhibitors, and then boiled in SDS sample buffer and subjected to SDS/PAGE. with detection of specific isozymes by quantitative

immunoblotting with specific anti-sera. This approach also overcomes the problems of differential extraction.

This study focussed on two high-affinity cyclic AMP-specific phosphodiesterases that have previously been purified from rat liver by this laboratory. In rat hepatocytes, these two activities, the 'dense-vesicle' and 'peripheral' phosphodiesterases are stimulated by insulin, whilst the former is also stimulated by glucagon (Heyworth, et al., 1983d).

The 'peripheral' plasma membrane enzyme is a 52-kDa monomeric species which can be detected with the polyclonal antibody PM1 (Pyne, et al., 1986b, Houslay, et al., 1988). The 'dense-vesicle' enzyme possesses a native subunit of 63-kDa, which can be detected specifically with antibody DV1 in cholate extracts of membranes (Pyne, et al., 1987a). This activity can be released from it's membrane environment by proteolytic cleavage (Loten, et al., 1978; Loten, et al., 1980; Pyne, et al., 1987a). This can be achieved either by an endogenous sulphydryl protease, relesed from lysosomes by hypotonic shock procedures (Loten, et al., 1978; Loten, et al., 1980; Pyne, et al., 1987a) or by addition of exogenous protease such as chymotrypsin (Boyes and Loten, 1988). Cleavage by endogenous proteases ('hypotonic shock') releases an activity of 57-kDa presumably by cleavage of an anchoring peptide. Purified preparations containing this 57-kDa species also show evidence of a smaller form of the enzyme of molecular weight 51-kDa (Pyne, et al., 1987a). Such preparations have been used to prepare the antisera DV1.

When the antisera DV1 was covalently coupled to Sepharose, it was shown it could remove cyclic GMP-inhibited phosphodiesterase activity from a hypotonic extract. Inhibition of cyclic AMP hydrolysis is by μM concentrations of cyclic GMP is a characteristic property of this enzyme (Loten, et al., 1978; Weber and Appleman, 1982; Pyne, et al., 1987a; Degerman, et al., 1987; Grant and Colman, 1988). It could not remove all the cyclic AMP phosphodiesterase activity

however. This could have indicated that the immunoaffinity matrix had a limited capacity for this activity. However, when the phosphodiesterases present in the hypotonic extract were examined by separation on a high resolution anion-exchange matrix (Mono Q), it was apparent that there were other phosphodiesterases present in the extract. When assayed at 0.1 μM cyclic AMP and cyclic GMP it was shown that there were two cyclic AMP-specific activities in the extract which formed most of the cyclic AMP phosphodiesterase activity and were present in approximately equal amounts. However, Loten, et al., (1978) showed by chromatography on the anion-exchange resin ECTEOLA cellulose, that there was only one major peak of cyclic AMP phosphodiesterase activity present in the hypotonic extract prepared under identical conditions. From the work presented in Chapter 3 of this thesis, showing the limitations of such anion-exchange resins over that of Mono Q separations, it is probable that the one peak of cyclic AMP phosphodiesterase activity that they observed on ECTEOLA cellulose probably represents coelution of these two activities. In support of this proposal, these workers observed that this activity possessed non-linear kinetics indicative of the presence of two activities (Loten, et al., 1978).

The two Mono Q resolved activities were shown to differ in their responsiveness to cyclic GMP, with one activity (the later eluting activity) being potently inhibited by cyclic GMP and the other showing insensitivity. The former activity was presumed to be the 'dense-vesicle' phospodiesterase on the basis of it's inhibition by cyclic GMP, it's sensitivity to the inhibitor ICI 118233 and from an analysis of it's kinetic characteristics where it was shown that the K_m for cyclic AMP hydrolysis for this Mono Q resolved activity was very similar to that of the purified protein (0.5 μM compared to 0.3 μM) (Pyne, et al., 1987a). The cyclic GMP-insensitive activity was not characterised in detail.

These studies confirmed then, the ability of the anti-sera DV1 to detect native 'dense-vesicle' phosphodiesterase. Attempts to elute bound 'dense-vesicle' enzyme, indicated that only under extreme

conditions, that were likely to denature the anti-sera, could polypeptides corresponding to the presumed molecular weights of the proteolysed 'dense-vesicle' phosphodiesterase (57-kDa and 51-kDa) be observed. Further to this, these were the only two polypeptides that were attached to the anti-sera indicating that the interaction was both specific and of high affinity.

Analysis of total liver homogenates shows quite clearly that the antibodies PM1 and DV1 detected the native 52-kDa and 63-kDa subunits of the 'peripheral' and 'dense-vesicle' phosphodiesterases respectively. Indeed, that single bands were obtained indicated that these antibodies did not cross-react with other species of phosphodiesterase that are found in the liver. The single band at 63-kDa detected by immunoblotting analysis of a liver homogenate corresponds to the 63-kDa species that could be immunoprecipitated with DV1 from cholate extracts of liver membranes (Pyne, et al., 1987a). There was no evidence of the high molecular weight species that was observed upon immunoprecipitation from detergent treated membranes either upon Western blotting or from an analysis of the polypeptides retained upon the immunoaffinity matrix DV1-Sepharose. This high molecular weight species appears to be an aggregated species of the 'dense-vesicle' enzyme as indicated by tryptic peptide mapping analysis (Pyne, et al., 1987a) that may arise as an artefact of either the cholate treatment or immuoprecipitation protocol.

These two phosphodiesterases were not found exclusively in liver however. Analysis of homogenates of heart, white (epididymal) fat and kidney showed that both of these enzymes were expressed in all these tissues. Using standard curves to estimate the µgs of these two enzymes in tissue homogenates, it was possible to calculate the level of expressed protein per mg of homogenate protein. When this was done it was apparent that the levels of these two enzymes varied between the tissues examined. In order to assess that this relative distribution of these activities corresponded with activity it was necessary to determine

the contribution of these two activities to the total phosphodiesterase activity in tissue homogenates.

In the case of the 'dense-vesicle' phosphodiesterase, the inhibitor ICI 118233 was found to be a selective and potent inhibitor of this activity. Data presented in Chapter 3 show that this inhibitor did not inhibit any of the five peaks of phosphodiesterase activity prepared from the soluble fraction of hepatocytes. In this section of work, ICI 118233 was shown to be a reversible and competitive inhibitor of partially purified preparations of the cyclic GMP-inhibited phosphodiesterase prepared by Mono Q chromatography ('dense-vesicle' phosphodiesterase). Given the selectivity of this inhibitor for the 'dense-vesicle' phosphodiesterase, the amount of inhibition of cyclic AMP phosphodiesterase activity elicited by ICI 118233 in tissue homogenates might be presumed to reflect the activity of this enzyme. This approach makes the assumption that the compound acted similarly in other tissues, both in terms of it's specificity and in it's ability to inhibit the 'dense-vesicle' enzyme homologues. This analysis revealed a very similar distribution of the 'dense-vesicle' phosphodiesterase in these tissues to that found using the specific antisera DV1.

Using these values of total protein/mg of homogenate and total activity/mg of homogenate it was possible to calculate a specific activity for the 'dense-vesicle' phosphodiesterase in the different tissues. In liver this value was estimated to be 5333 pmol/min/mg at 0.1 μM cyclic AMP. The specific activity of the purified protease 'clipped' form of the enzyme has been estimated to be 17,553 pmol/min/mg (Pyne, et al., 1987a). In kidney, the estimated specific activity of the enzyme was estimated to be 7857 pmol/min/mg, whilst in heart it was 3,446 pmol/min/mg. All of these values were somewhat lower than the estimated values for the purified 'dense-vesicle' phosphodiesterase from rat liver determined by Pyne et al., (1987a). The reasons for these differences is not apparent. It is possible that they reflect the fact that in measurements of total homogenate activity, the activity determined in the presence of ICI 118233 is that of the holoenzyme

attached by it's membrane anchor to it's native membrane. Upon proteolytic release this anchor is removed and this may result in a change in the catalytic activity of the solubilised (purified) species. This situation is somewhat analagous to the situation in the bovine brain form of the Ca^{2+}/Calmodulin-stimulated phosphodiesterase where limited proteolysis has been proposed to remove an inhibitory domain of the enzyme that either is or is closely associated with the Calmodulin binding domain (Kincaid, et al., 1985) resulting in an activity that is both activated and insensitive to Ca^{2+}/Calmodulin (Tucker, et al., 1981; Kincaid, et al., 1985).

If such a domain exists in the 'dense-vesicle' phosphodiesterase, this data suggests that it would act to maintain the activity in a lower catalytic form when attached to the membrane and that protease action either removes or partially destroys it. In suppport of this proposal, Loten et al., (1980) have shown that solubilisation of the activity from liver membranes caused a modest activation of the activity and indeed have also suggested the presence of an inhibitory fragment of the enzyme when attached to the membrane. The range of estimated specific activities observed in the tissues might also suggest that these enzymes, although immunologically related, might possess intrinsically different specific activities suggesting the presence of tissue specific isozymes of the 'dense-vesicle' phosphodiesterase. Alternatively, some of the enzyme in the tissues may be sequestered in some way so that they would not be detected in a functional assay, but would be upon Western blotting. This would also explain the lower specific activities that are encountered in these analyses.

The 'dense vesicle' enzyme thus appears to account for a major fraction of the total cyclic AMP phosphodiesterase activity in heart and adipose tissue. Part of insulin's major anti-lipolytic action on white adipose tissue is believed to be exerted through an effect on cyclic AMP metabolism (Loten and Sneyd, 1970; Fain, 1980; Londos, et al., 1985; Wong and Loten, 1980; Lonroth and Smith, 1986; Kather and Scheurer, 1987; Wong and Loten, 1981; Belfrage, et al., 1984). At

least part of this effect on cyclic AMP metabolism is believed to be due to the activation, by insulin, of cyclic AMP phosphodiesterase activity (Lonroth and Smith, 1986; Kather and Scheurer, 1987; Manganiello and Vaughan, 1973; Zinman and Hollenberg, 1974). Therefore the 'dense-vesicle' phosphodiesterase may provide a physiological target for insulin's action in this tissue. The observation that this activity provides a very substantial proportion of the total cyclic AMP phosphodiesterase activity in fat tissue may account for reports by various investigators that insulin elicits a very marked stimulation of cyclic AMP phosphodiesterase in this tissue

Rat adipose tissue has been shown to possess an activity that resembles the rat liver 'dense-vesicle' phosphodiesterase. Like the rat liver enzyme this activity is activated by insulin (Kono, et al., 1975), has a high affinity for cyclic AMP, is associated with the P2 fraction (Manganiello and Vaughan, 1973; Sakai, et al., 1974; Zinman and Hollenberg, 1974). Cyclic AMP hydrolysis can also be inhibited by cyclic GMP (Weber and Appleman).

This activity has been purified from rat adipose tissue by a number of workers (Saltiel and Steigerwelt, 1986c; Degerman, et al., 1987). The most convincing purification of this activity was obtained by Degerman, et al., (1987). The enzyme isolated by these workers was shown to be sensitive to inhibition by cyclic GMP, Cilostamide and Cilostamide derivatives and possesses high affinities for both cyclic AMP and cyclic GMP but shows selectivity for cyclic AMP as substrate. In agreement with this study, the subunit molecular weight of the enzyme was 64-kDa. One point of difference that occured between this purified preparation and that of the rat liver 'dense-vesicle' PDE lies in the kinetics of hydrolysis of cyclic nucleotides. Whilst the rat liver 'dense-vesicle' phosphodiesterase exhibited anomalous kinetics, the rat adipocyte cyclic GMP-inhibited phosphodiesterase demonstrated linear kinetics and also exhibited a much larger specific activity (Pyne, et al., 1987a; Degerman, et al., 1987). Whether this difference reflects the occurence of tissue specific isozymes of this activity is uncertain but

the data presented here would suggest that this is not the case. Alternatively, the difference in properties may reflect the fact that purified adipocyte phosphodiesterase is probably the holoenzyme, whilst the rat liver purified enzyme is a proteolytically 'clipped' version of the holoenzyme. This is a consequence of the fact that the adipocyte phosphodiesterase is not released from it's membrane environment by a hypotonic shock (Loten, et al. , 1980) method as was the liver enzyme (Loten, et al., 1978; Loten, et al., 1980; Pyne, et al., 1987a).

It has been shown by numerous workers that heart tissue contains a cyclic GMP – inhibited phosphodiesterase. For example this activity has been identified in human (Reeves, et al.,, 1987), bovine (Donnely, 1978; Harrison, et al., 1986a; Harrison, et al., 1986b), canine (Weishaar, et al., 1987a; Weishaar, et al., 1987b; Weishaar, et al., 1987c), guinea pig (Weishaar, et al., 1987a; Weishaar, et al., 1987b; Weishaar, et al., 1987c) and rat (Weishaar, et al., 1987b) hearts.

This study suggests that the rat heart cyclic GMP – inhibited phosphodiesterase is related to the rat liver 'dense – vesicle' phosphodiesterase both immunologically and in terms of it's sensitivity to ICI 118233 and it's molecular weight. It appears to contribute a large proportion of the cyclic AMP phosphodiesterase activity in the rat heart. This has also been shown to be true of this activity in the hearts of other species (Weishaar, et al., 1987a; Weishaar, et al., 1987b; Weishaar, et al., 1987c). These activities are different however in terms of their subcellular distribution. Whilst the bovine and canine activities are membrane bound, the activities in rat and guinea pig hearts are both soluble (Weishaar, et al., 1987b). This might imply that there are isozymes of this activity which differ in terms of their subcellular location. The molecular weight of the cyclic GMP – inhibited phosphodiesterase in rat heart appears to be identical to that observed in other tissues, namely about 63 – kDa. In contrast the molecular weight of the bovine cardiac cyclic GMP – inhibited phosphodiesterase has been shown to be 110 – kDa by immunoblotting with monoclonal

antibodies raised to highly purified (but proteolysed) preparations of the enzyme (Harrison, et al., 1986b).

Similarly, the subunit molecular weight of the human platelet enzyme has been shown to be 110-kDa by two groups (MacPhee, et al., 1986; Grant, et al., 1988). It is of interest to note that the human platelet 110-kDa subunit containing enzyme is also soluble as the guinea pig and rat cardiac activities are (MacPhee, et al., 1986; Grant and Colman, 1984; Weishaar, et al., 1987a; Weishaar, et al., 1987b; Weishaar, et al., 1987c). It seems unlikely then that a soluble location is correlated with the smaller subunit molecular weight. The observations that the molecular weight of this activity in rat tissues appears to be uniform between all tissues examined (63-kDa) whilst the bovine and human tissues demonstrate an activity that has a much larger molecular weight (110-kDa) does however suggest that species-specific isozymes of this activity exist.

The observation that about 12% of the total cyclic AMP hydrolysis in the homogenates of kidney appears to be due to the 'dense-vesicle' like activity agrees with the observation that about 90% of the total cyclic AMP phosphodiesterase activity in kidney homogenates is soluble (Hoey and Houslay, 1990). In this tissue the cyclic GMP-inhibited phosphodiesterase is membrane bound (Hoey and Houslay, 1990) and this study would suggest that it forms nearly all of the membrane bound cyclic AMP phosphodiesterase activity in kidney homogenates.

The 'peripheral' plasma membrane phosphodiesterase can be distinguished from the 'dense-vesicle' phosphodiesterase by a number of crteria including it's insensitivity to cyclic GMP as an inhibitor of cyclic AMP hydrolysis (Pyne, et al., 1986b; Pyne, et al., 1987a). Such cyclic GMP-insensitive phosphodiesterases have also been observed in a number of tissues in addition to liver and hepatocytes namely heart (Weishaar, et al., 1986; Weishaar, et al., 1987a; Weishaar, et al., 1987b), kidney (Thompson, et al., 1979; Thompson, et al., 1988) and

adipose tissue (Weber and Appleman, 1982). For example, heart tissues from a number of species have been shown to possess such an activity. It has, however, been suggested that, unlike the cyclic cardiac GMP–inhibited 'dense–vesicle' like phosphodiesterase, these are not observed in all species. Guinea pig cardiac tissue for example has been suggested not to contain a cyclic GMP–insensitive phosphodiesterase (Weishaar, et al., 1986; Weishaar, et al., 1987b). In these studies the cyclic GMP–inhibited phosphodiesterase was the major cyclic AMP–specific activity observed after chromatography of the soluble fraction on DE–52. This view has been questioned, however, since work from Reeves, et al., (1987) has shown that using the higher resolving power of DEAE–Sepharose, it is possible to identify an activity that has properties which are similar to the cyclic GMP–insensitive subclass of phosphodiesterase in soluble extracts of guinea pig cardiac tissue. Interestingly, this activity appeared to have a molecular weight of approximately 45,000 upon gel filtration which is very similar to the 52–kDa subunit molecular weight of the rat liver 'peripheral' phosphodiesterase (Marchmont, et al., 1981b; Pyne, et al., 1986b; Houslay, 1988) which is also cyclic GMP–insensitive. Similarly, the rat heart has been found to possess a soluble cyclic GMP–insensitive phosphodiesterase (Weishaar, et al., 1987b). Whilst the relationship between this activity and the 52–kDa species identified upon Western blotting of rat heart homogenates is not clear, it is tempting to speculate that these activities are the same.

A soluble cyclic AMP–specific phosphodiesterase has been purified from canine kidney by Thompson and co–workers which has properties that are similar to the rat liver 'peripheral' plasma membrane phosphodiesterase (Thompson, et al., 1979; Thompson, et al., 1988). However this activity has a subunit molecular weight of 82–kDa on SDS–PAGE (Thompson, et al., 1988), which is clearly different to that of the 52–kDa suggested for this activity in rat kidney. Whether this is due to the existence of species–specific isozymes of this activity is uncertain. It should be noted, however, that there appear to be

multiple forms of this activity in rat kidney. Hoey and Houslay (1990) have identified two soluble cyclic AMP-specific phosphodiesterase activities in rat kidney that are insensitive to inhibition by cyclic GMP. Either one or both of these activities may be related to the rat liver 'peripheral' phosphodiesterase and equally as well to the canine 82-kDa subunit containing form.

A number of distint cDNA clones have been isolated from brain and testis cDNA libraries (Davis, et al., 1989; Swinnen, et al., 1989; Colicelli, et al., 1989) that have been shown in some instances to encode cyclic-AMP specific phosphodiesterases that are insensitive to inhibition by cyclic GMP (Henkel-Tigges and Davis, 1990). The predicted molecular weights of these activities range from 60-kDa to 68-kDa (Davis, et al., 1989; Swinnen, et al., 1989; Colicelli, et al., 1989). As yet no clone has been isolated capable of encoding a 52-kDa protein. This study implies then that the number of cyclic AMP-specific phosphodiesterases of this type may include even further members than have been identified by cloning analyses.

The difficulties that I observed in reproducing the previously published procedures for the purification of the rat liver 'dense-vesicle' phosphodiesterase (Pyne, et al., 1987a) are difficult to explain. Whilst I could clearly identify this activity in hypotonic extracts by Mono Q chromatography and it was possible to follow it's purification by at least one of the methods (the Guanine-Sepharose method), the yields from this procedure were very low. This could in part be explained by the instability of the 'dense-vesicle' phosphodiesterase particularly at the later stages of the preparation. This variation in properties from the published procedure may be related to the proteolytic release of the activity from it's membrane location. The rat liver 'dense-vesicle' phosphodiesterase can be solubilised either by the endogenous proteases (Loten, et al., 1978; Loten, et al., 1980; Pyne, et al., 1987a) or by the exogenously added proteases such as trypsin, chymotrypsin and papain (Loten, et al., 1980). It was observed by Loten, et al., (1980), that the particulate activity was much less labile than the 'hypotonically'-

released activity, whilst the chymotrypsin–released activity was even more labile still. The trypsin–released activity was intermediate in labililty between these latter two species. These results suggest that the stability of the enzyme depends on the precise proteases used to cleave the membrane bound activity. The hypotonic shock method will release a number of proteases and it may be that more than one protease can act on the enzyme to produce a slightly different clipped activity with increased lability. This may also alter the chromatographic properties so that the activity does not bind to Affi–gel Blue. Altered chromatographic properties on Affi–gel Blue have been reported for the bovine brain Ca^{2+}/Calmodulin–stimulated phosphodiesterase (Tucker, et al., 1981) although in this instance proteolysed and non–proteolysed phosphodiesterase could bind to this matrix. Indeed Affi–gel Blue is presumed to bind to the nucleotide binding site of this enzyme since it can act as a competitive inhibitor of cyclic nucleotide hydrolysis (Morril, et al., 1979) so proteolysis would not be expected to directly affect the ability of this enzyme to bind to this matrix. Whilst the mechanism by which the 'dense–vesicle' phosphodiesterase binds to Affi–gel Blue is not known it may be that it does not bind through the catalytic site so that proteolysis may remove or partially destroy the Affi–gel Blue binding site.

CHAPTER 5.

DISCUSSION.

CHAPTER 5: FINAL DISCUSSION.

This work presented in this thesis has involved the identification and characterisation of the soluble phosphodiesterase species contained in the soluble fractions derived from both rat liver and hepatocytes as well as an assessment of the tissue distribution of activities immunologically related to the insulin-sensitive 'peripheral' and 'dense-vesicle' phosphodiesterases. It is becoming apparent that the multiplicity of phosphodiesterases previously observed in several tissues is even more complex than originally envisaged. This multiplicity is observed both within a single tissue and between different species. From both purification and cloning analyses the number of distinct forms of phosphodiesterase that have been identified has increased from the original three types that were observed by workers such as Russell et al., (1973) and Thompson and Appleman (1971). What is also becoming apparent is that different cell/tissue types exhibit different multiplicity of forms from each other. The work presented here demonstrates the existence of at least five soluble phosphodiesterases in the soluble fractions of rat liver and hepatocytes. These were identified by the use of high resolution anion-exchange chromatography. This system, in addition to providing a much greater resolution of forms, is also more rapid than conventional chromatography on matrices such as DE-52 or DEAE-Sepharose, which have been used by numerous workers in this type of study (Russel, et al., 1973; Thompson and Appleman, 1971; Reeves, et al., 1987; Turnbull and Hickie, 1984; Weishaar, et al., 1987b; Hidaka and Asano, 1976; Grady and Thomas, 1986; Lugnier, et al., 1986; Erneux, et al., 1980).

The five soluble activities that have been described are, PDE MQ-I, a cyclic AMP and cyclic GMP hydrolysing activity, PDE MQ-II, a cyclic GMP-specific activity that contains at least the Ca^{2+}/calmodulin-stimulated phosphodiesterase, PDE MQ-III a cyclic

GMP and cyclic AMP hydrolysing activity that is the soluble cyclic GMP–stimulated phosphodiesterase and PDE MQ–IV and PDE MQ–V which are both specific for cyclic AMP as substrate. One of the aims of phosphodiesterase research is to identify and characterise the individual isozymes contained in a particular cell or tissue and this is a necessary prelude to the eventual purification of all of the individual species.

A largely unanswered question concerning this occurence of multiple forms of cyclic nucleotide phosphodiesterase in single cell types such as hepatocytes is, what role do the individual isozyme forms play in controlling the levels of cyclic AMP and cyclic GMP in unstimulated and stimulated cells ? Furthermore, if all of the phosphodiesterase activities do hydrolyse cyclic AMP and cyclic GMP *in vivo* why is such a diversity of species needed ?

It is well documented that the individual phosphodiesterae isozymes are compartmentalised in the cell. For example, in hepatocytes, phosphodiesterase activity is observed in both the particulate and soluble fractions (Heyworth, et al., 1983d). Furthermore, within the particulate fraction, activity is observed in subcellular fractions containing the plasma membrane (Marchmont, et al., 1980a; Marchmont, et al., 1980b), the endoplasmic reticulum (Cercek and Houslay, 1983a; Wilson and Houslay, 1983), the mitochondria (Cercek and Houslay, 1983b), and another unidentified location that has been called the 'dense–vesicle' (Heyworth, et al., 1983d). No activity is observed in the nucleus, lysosomes or peroxisomes however (Heyworth, et al., 1983d). The soluble fraction of cells is usually considered to be a single compartment, within which molecules are freely diffusable. However, it is quite conceivable that some or all of the soluble phosphodiesterases identified here may not be free to move but may be associated, albeit very weakly, with membrane bound organelles so that they are in effect constrained. This compartmentalisation of phosphodiesterase activity within the cell raises the possibility that cyclic AMP/cyclic GMP may also be

compartmentalised within the cell as well. If this is the case this would suggest that a number of different activities are required to control these functionally discrete pools of cyclic nucleotide.

There is some evidence to suggest that discrete functionally distinct pools of cyclic AMP do exist in cells. Studies on the ability of the inotropic agent imazodan to produce an inotropic response in rat, guinea pig and canine hearts have indicated that subclasses of phosphodiesterase exist and that these may be 'coupled' to functionally distinct pools of cyclic AMP. In species such as the dog, where the imazodan-sensitive cyclic AMP-specific PDE (cyclic GMP-inhibited PDE) is membrane bound (Weishaar, et al., 1987a; Weishaar, et al., 1987d), then agents that inhibit this activity such as imazodan (CI-914), CI-930 and amrinone exert positively inotropic effects, whilst agents such as Ro-20-1724 and rolipram which are weak inhibitors of this activity but potent inhibitors of the soluble imazodan-insensitive (cyclic GMP-insensitive) cyclic AMP-specific PDE, do not. In rat and guinea pig hearts, where the imazodan-sensitive PDE is soluble rather than membrane bound (Weishaar, et al., 1987a; Weishaar, et al., 1987d), then these agents were found to exert either a small or no positive inotropic effect (Weishaar, et al., 1987b; Weishaar, et al., 1987d)). This localisation of the imazodan-sensitive phosphodiesterase in cellular compartments other than the sarcoplasmic reticulum (Kaufman, et al., 1986) has been suggested (Weishaar, et al., 1987b) to have the effect of partially 'uncoupling' the enzyme in terms of it's ability to regulate contractility. Such 'uncoupling' would mean that the increases in cyclic AMP that result from inhibition of the cyclic GMP-inhibited phosphodiesterase would produce lesser increases in contractility than would otherwise occur since such increases would not be in the most appropriate compartments within the cardiac cell. Implicit in this model is the hypothesis that cyclic AMP is somehow compartmentalised in the cell and that a distinct pool is intimately involved in regulating contraction.

Similarly, studies on 3T3-L1 adipocytes have suggested the presence of at least two functionally distinct pools of cyclic AMP. Elks and Manganiello (1984a) showed that the cilostamide-sensitive subclass of cyclic AMP specific phosphodiesterase (cyclic GMP-inhibited PDE) may be more important in regulating lipolysis in 3T3-L1 adipocytes than the Ro-20-1724-sensitive form (cyclic GMP-insensitive). These workers have also reported that both the non-selective phosphodiesterase inhibitor IBMX and Ro-20-1724 (selective for the cyclic GMP-insensitive subclass of cyclic AMP-specific phosphodiesterase) enhance cellular differentiation, whereas cilostamide (selective for the cyclic GMP-inhibited subclass of cyclic AMP-specific phosphodiesterase) had no effect on differentiation (Elks and Manganiello, 1985). These results once again imply that distinct pools of cyclic AMP exist within the cell that are regulated by distinct phosphodiesterase isozymes and that they are involved in mediating discrete effects on cellular processes.

Whilst there is not the same evidence that compartmentalisation of cyclic nucleotides may occur in hepatocytes there is some evidence to suggest that there are two types of hormone-responsive adenylate cyclase in the rat liver (Yamatani, et al., 1987). These workers showed that one of these, the less abundant form, is linked to glycogenolysis and the other is not. Glucagon stimulates mainly the glycogenolysis-linked fraction, whilst secretin stimulates the other adenylate cyclase that is not linked to glycogenolysis. On this basis these workers have suggested that there is a functional compartmentalisation of cyclic AMP either within the hepatoctye or among distinct hepatocytes.

Whilst compartmentalisation of cyclic nucleotides and cyclic nucleotide-mediated processes may be considered to be one possible role of the multiple forms of phosphodiesterase it is also possible to hypothesise, in addition, that such multiplicity of phosphodiesterases allows the cell to control cyclic nucleotide levels in response to a number of factors. A number of different phosphodiesterases that are individually regulated by different mechanisms would suit this purpose

very well. This study has suggested that, in both hepatocytes and liver, cyclic AMP disposal can be rapidly increased by the presence of low (μM) concentrations of cyclic GMP. The abundance of the cyclic GMP–stimulated phosphodiesterase in both the soluble and particulate fractions provides a means whereby cyclic AMP metabolism can be controlled by the levels of cyclic GMP. In addition, raised cyclic GMP will inhibit the activity of the membrane bound 'dense–vesicle' phosphodiesterase allowing for an increase in the cyclic AMP levels surrounding the membrane location of this activity. This picture is further enhanced by the observation that the cyclic GMP specificity of the soluble Ca^{2+}/calmodulin–stimulated phosphodiesterase allows for a potential control of cyclic GMP levels themselves by an alteration in Ca^{2+} levels. Thus the presence of the Ca^{2+}/calmodulin–stimulated phosphodiesterase and the cyclic GMP stimulated phosphodiesterase may allow for a direct control of cyclic AMP metabolism by cyclic GMP (through the cyclic GMP–stimulated phosphodiesterase) and an indirect modifying control by levels of intracellular Ca^{2+} (through the Ca^{2+}/calmodulin–stimulated phosphodiesterase).

Further factors that control cellular phosphodiesterase activity have been identified both in hepatocytes and other cell types. For example, it is well documented that the 'dense–vesicle' phosphodiesterase and activities that are related to it (cyclic GMP–inhibited phosphodiesterases) can be controlled by hormones such as insulin, glucagon, isoproterenol and PGE_1 (Loten, et al., 1978; Heyworth, et al., 1983d; MacPhee, et al., 1988; Makino and Kono, et al., 1980; Alvarez, et al., 1981). The latter hormones have been suggested to activate this enzyme by means of a cyclic AMP–dependent phosphorylation (MacPhee, et al., 1988; Reifsnyder, et al., 1987; Kilgour, et al., 1989; Gettys, et al., 1988). In hepatocytes this has been suggested to be a means by which raised cyclic AMP levels can negatively feed–forward to promote a return to basal levels of cyclic AMP (Corbin, et al., 1985). The observation that this activity is also expressed in tissues such as heart, fat and kidney may also imply

that one such role of this enzyme is to provide these tissues with this control mechanism. Other phosphodiesterases have also been shown to be under the control of hormones, in particular in hepatocytes, the insulin-stimulated 'peripheral' plasma membrane phosphodiesterase. This enzyme, although unable to decrease cyclic AMP levels in intact hepatocytes following glucagon challenge, can do so if hepatocytes are first challenged with PIA (an adenosine analogue). This effect of PIA seems to involve a reversal of the glucagon-induced block on insulin's ability to activate this enzyme (Heyworth, et al., 1984a). All these observations suggest that depending on the 'hormonal status' of the hepatoctye, different phosphodiesterases can be activated by insulin to promote decreases in the intracellular levels of cyclic AMP in the activated hepatocyte.

Other classes of phosphodiesterases may also be regulated, for example, the activity of the Ca^{2+}/calmodulin-stimulated phosphodiesterase can be regulated by phosphorylation, as has been shown for the isozymes in brain. It has been demonstrated, *in vitro*, that the 60-kDa isozyme is a substrate for the cyclic AMP-dependent protein kinase (Sharma and Wang, 1985; Sharma and Wang, 1986c), whilst the 63-kDa isozyme is a substrate for the auto-phosphorylated form of Ca^{2+}/calmodulin-dependent protein kinase II (Sharma and Wang, 1986a; Sharma and Wang, 1986c; Hashimoto, et al., 1989). Both of these events lead to a decrease in the affinity of the enzyme towards calmodulin and have been suggested to occur *in vivo*. It is not known if the isozyme of the Ca^{2+}/calmodulin-stimulated phosphodiesterase in hepatocytes undergoes a similar phosphorylation however.

All these different mechanisms of regulating phosphodiesterase activity provide a potential explanation for the occurence of multiple forms of phosphodiesterase within a single cell type. In order to be able to examine the mechanisms by which regulation of distinct species is achieved it is necessary to identify, purify, characterise and determine

the amino acid sequence of, each of the individual species present in a single cell type.

This work described in this thesis has provided the first detailed identification and characterisation of the soluble phosphodiesterases present in hepatocytes. The PDE MQ−I species appears to be an unusual activity that is very similar to an activity that has been purified from pig liver by Helfman and his colleagues (Helfman, et al., 1981). In order to be able to assess this for certain it is necessary to purify this activity from rat liver. It should be possible to do this, based on the properties determined in this study, namely by chromatography on DE−52, the use of Affi−gel Blue as a negative step to remove other nucleotide binding proteins, gel filtration and Mono Q chromatography. If an affinity step is required it should be possible to use 8−$H_2N(CH_2)_2NH$−cyclic AMP Sepharose since the pig liver enzyme has been shown to bind to this matrix (Helfman, et al., 1981). Other potential affinity steps such as other dye ligand columns and cyclic AMP derivatives may also be tried and, given the ability to rapidly isolate this activity from other phosphodiesterase, matrices can be readily screened for their ability to bind this activity. It would be hoped that a specific anti−sera could then be prepared in order to allow an immunological comparison of this activity both with the pig liver enzyme and with the other soluble and particulate phosphodiesterases in hepatocytes. A purified preparation of this enzyme could then be used to obtain partial amino acid sequence it should be possible to obtain partial amino acid sequence so that cloning of the enzyme from an hepatocyte cDNA library could be achieved. If one assumes that the specific activity of the purified PDE MQ−I phosphodiesterase is similar to the pig liver enzyme (6,565 pmol/min/mg), one can estimate using the figures of the total activity of PDE MQ−I in one rat liver (at 1 μM this was 1570 pmol/min) that one rat liver should contain approximately 240 μg of this enzyme. This would be more than sufficient to carry out the suggested studies.

An alternative method for the production of antibodies that would be applicable to all of the hepatocyte-derived PDE MQ species would be the production of monoclonal antibodies against the individual activities. This could be achieved either by using the Mono Q fractions as they were or further purifying them further by various affinity steps. In the case of the Ca^{2+}/calmodulin-stimulated phosphodiesterase this could be achieved by chromatography on calmodulin-Sepharose which has been used by other workers to purify the enzyme from brain or heart (Ho, et al., 1977; Sharma, et al., 1980) or calmodulin derivatives such as 3-(2-pyridylithio) propionyl-substituted calmodulin linked to thiol-Sepharose (Kincaid, et al., 1984). The cyclic GMP-stimulated phosphodiesterase could be similarly chromatographed on a column of cyclic GMP-Sepharose as has been shown for both the soluble and particulate forms of this enzyme from rat liver (Pyne, et al., 1986a). Whilst little is known about the chromatographic properties of the two soluble cyclic AMP-specific activities, if these activities are related to the membrane-bound 'peripheral' phosphodiesterase, then an affinity step such as Affi-gel Blue or Theophylline-Sepharose could be used. These two steps have been used with some success for the purification of this activity either from rat liver plasma membranes (Marchmont, et al., 1981b) or a total particulate fraction derived from rat liver (Pyne, et al., 1986b).

Since it is unlikely that these activities would be pure after such steps it would not be possible to screen for monoclonal antibodies using purified protein. One possible method of screening would be to assess the ability of antisera to affect the phosphodiesterase activity of the various species. This could result in antisera that were inhibitory, activatory or affected the ability of Ca^{2+}/calmodulin or cyclic GMP to stimulate their target enzymes. Alternatively, the ability of antisera to precipitate phosphodiesterase activity from the PDE MQ fractions could also be used as a potential screening method.

Recent purification and cloning analyses have indicated that there are multiple isozymes of each of the individual phosphodiesterase

classes. For example, it has been demonstrated that the Ca^{2+}/calmodulin-stimulated phosphodiesterase in bovine brain can exist as either a dimer of 61-kDa subunits or 63-kDa subunits (Sharma, et al., 1984; Sharma, et al., 1986c; Wang and Sharma, 1990) and that the bovine heart 59-kDa isozyme is a distinct species from these two activities (LaPorte, et al., 1979). Similarly, cloning analyses have indicated the existence of a number of similar, but distinct, clones encoding high affinity cyclic AMP-specific phosphodiesterases in both rat brain and rat testis (Davis, et al., 1989; Swinnen, et al., 1989; Colicelli, et al., 1989; Henkel-Tigges and Davis, et al., 1990; Davis, 1990; Conti and Swinnen, 1990).

This study has suggested that there are also multiple isozyme forms of the soluble phosphodiesterases in rat hepatocytes. In particular, the existence of two soluble high affinity cyclic AMP-specific phosphodiesterases, a Ca^{2+}/calmodulin-stimulated activity that is highly selective for cyclic GMP and a form of the cyclic GMP-stimulated phosphodiesterase which appears to have a different substrate selectivity than the soluble rat liver derived form, have all been noted. It is important that the relationship between these identified activities and cloned phosphodiesterases be established in order for comparisons to be drawn between them. This can be achieved initially by comparing their immunoreactivities but ultimately will involve cloning of each of the individual species.

The production of specific anti-sera to these activities should also answer some unresolved issues concerning the precise molecular weight of rat liver derived activities compared to the bovine activities. For example, the cyclic GMP-stimulated enzyme from bovine tissues (liver, adrenals and heart) appears to have a molecular weight of between 102-kDa and 107-kDa (Beavo, et al., 1982; Martins, et al., 1982; Yamamoto, et al., 1983a), whilst those of the rat liver derived activities have smaller molecular weights (66-kDa to 67-kDa) (Pyne, et al., 1986a). Whether this is related to the observation that the rat hepatocyte-derived activity is different to the liver-derived activity is

uncertain but is worthy of attention. Whilst the bovine heart and human platelet homologues of the 'dense–vesicle' phosphodiesterase appear to have much larger molecular weights (110–kDa) than the rat liver activity (Harrison, et al., 1988; Grant, et al., 1988), the study on the tissue distribution of the 'dense–vesicle' and 'peripheral' phosphodiesterases has shown that the molecular weight of these two activities (63–kDa and 52–kDa respectively) is invariant between the rat tissues examined (heart, liver, fat and kidney) suggesting that the difference in molecular weight is due to a species difference rather than a tissue difference. This issue will need to be clarified however by cloning of the 'dense–vesicle' phosphodiesterase from each of these rat tissues.

Previous work from this laboratory has suggested the presence of an insulin–stimulated phosphodiesterase that is not the 'dense–vesicle' or 'peripheral' plasma membrane phosphodiesterases. The validity of this suggestion needs to be determined. One potential method to identify the putative third insulin–stimulated phosphodiesterase and confirm it's existence in hepatocytes may be to assess the ability of a range of different phosphodiesterase inhibitors to block the insulin–stimulated decrease in intracellular cyclic AMP levels. This work has characterised the sensitivities of the soluble phosphodiesterases in hepatocytes to a large number of inhibitors and, coupled with the known sensitivities of the membrane–bound activities (Pyne, et al., 1987a), this proposal should be a viable prospect. At the presesnt time it is known that this activity is sensitive to the non–selective phosphodiesterase inhibitor IBMX (Heyworth, et al., 1983d). If the ability of selective phosphodiesterase inhibitors to block the insulin effect on phosphodiesterase could be determined then a correlation may exist between this and the inhibitor sensitivity of the individual phosphodiesterase species in hepatocytes. This would thus allow an identification of this insulin–stimulated activity. This approach has been used by other workers in adipocytes (Kather and Scheurer, 1987), although these workers only assessed the ability of a limited number of

inhibitors to abolish insulin-stimulated phosphodiesterase activity. They did suggest that the insulin effect was sensitive to inhibition by theophylline but not by Ro-20-1724, consistent with the activted species being the proposed cyclic GMP-inhibited phosphodiesterase (Manganiello and Vaughan, 1973; Zinman and Hollenberg, 1974; Weber and Appleman, 1982; Degerman, et al., 1987). However, it is not known if, in addition to the known insulin-stimulated cyclic GMP-inhibited phosphodiesterase, there are other insulin stimulated activities in adipocytes as there are in hepatocytes. This study has suggested that fat tissue contains a phosphodiesterase activity that is immmunologically related to the rat liver 'peripheral' cyclic AMP phosphodiesterase. It is not known if this activity is also located at the plasma membrane or if it is stimulated by insulin as the liver activity is (Tria, et al., 1976; Marchmont and Housaly, 1980; Heyworth, et al., 1983d; Pyne, et al., 1987a).

The observation that a protein that is immunologically related to the 'peripheral' plasma membrane phosphodiesterase is found in tissues other than liver such as kidney, fat and heart suggests that this activity is indeed a cyclic AMP-specific phosphodiesterase. Whilst for the cyclic GMP-inhibited 'dense-vesicle' phosphodiesterase there is a selective inhibitor available, namely ICI 118233, no such selective inhibitor of the 'peripheral'cyclic GMP-insensitive activities exists. Inhibitors of this activity such as ICI 63197 and Ro-20-1724 (Pyne, et al., 1986b; Pyne, et al., 1987a) also inhibit the soluble PDE MQ-IV and PDE MQ-V activities. Whether these activities are distinct from or derived from the peripheral plasma membrane activity has yet to be determined. Therefore it was not possible to assess if this activity was also present in these tissues.

The development of suitable affinity matrices should aid in the purification of specific phosphodiesterases and will involve a thorough characterisation of the inhibitor sensitivities of individual phosphodiesterase types, perhaps even the identification of isozyme specific inhibitors is a possibility. Affinity matrices based on inhibitors

have been used by a number of workers to purify the cyclic GMP-inhibited phosphodiesterase from adipose tissue (Degerman, et al., 1987) and human platelets (Umekawa, et al., 1984). In this respect, the observation that interactions of the rat liver 'dense-vesicle' phosphodiesterase with selective and non-selective inhibitors, such as IBMX and ICI 118233, are reversible may imply that if these could be coupled successfully to a matrix such as Sepharose they may be used as an affinity step to replace the Affi-gel Blue with which problems were encountered. Other workers have shown that IBMX can be coupled to Sepharose, so this may be a useful approach. The lack of a primary amino group in ICI 118233 (see Figure 1.6(e)) precludes the use of Cyanogen Bromide-activated Sepharose to couple this activity to Sepharose, so an alternative protocol may have to be devised. An alternative strategy to the use of these two inhibitors would be to use cilostamide or it's derivatives to prepare an affinity column. This approach has been used successfully to prepare a 64-kDa protein that resembles the rat liver 'dense-vesicle' phosphodiesterase in most of it's properties (Pyne, et al., 1987a; Degerman, et al., 1987). Whilst the 'dense-vesicle' enzyme has not been shown to be inhibited by cilostamide, it seems certain that it is highly related to the adipose tissue cyclic GMP inhibited phosphodiesterase. Indeed, this study has shown that adipose tissue expresses an activity that it sensitive to ICI 118233 and is immunologically related to the rat liver activity, and on this basis will probably found to be sensitive to cilostamide. If this is the case, this step would be a promising affinity step and coupled with the high resolution anion-exchange system would provide an excellent means of purification.

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APPENDIX.

DMSO CORRECTION CURVES

Since all inhibitor studies were carried out using inhibitors diluted in the solvent dimethylsulphoxide (DMSO) it was decided to assess the effect of DMSO alone on the activity of the individual PDE MQ peaks resolved from both rat liver and rat hepatocytes. All inhibitors tested were diluted from a stock solution of 25mM in DMSO and were added at a concentration of 4x to the assay mixture. The standard curves can thus be used to correct for the effects of solvents provided that an identical dilution protocol is followed. The standard curves are presented as the mean % effect on the basal cyclic AMP phosphodiesterase activity observed over a number of different experiments. The SEM values are also shown on the individual standard curves.

The standard curves for the PDE MQ peaks from rat liver are shown in Figures I-V and those from rat hepatocyte in Figures VI-X.

FIGURE I

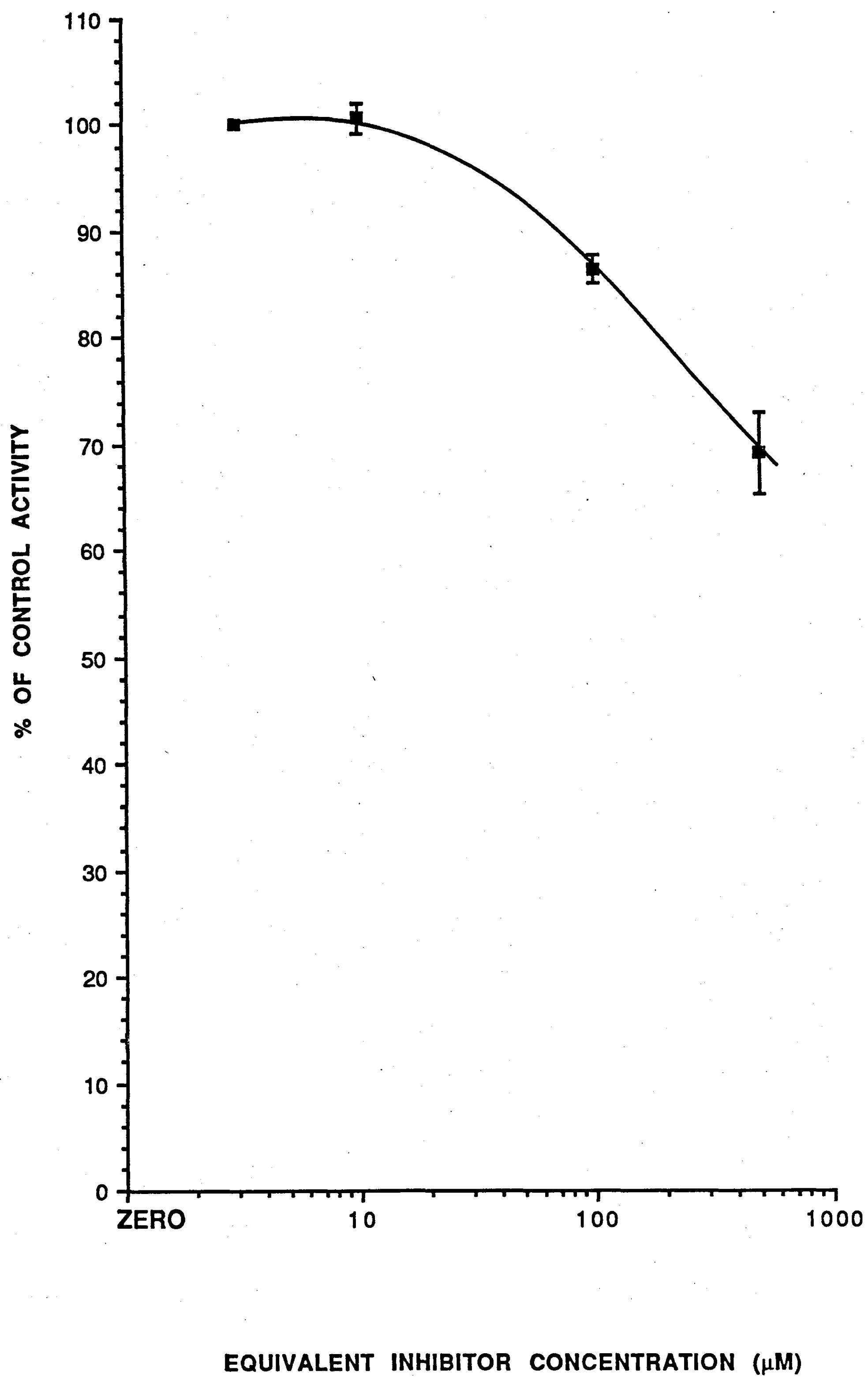
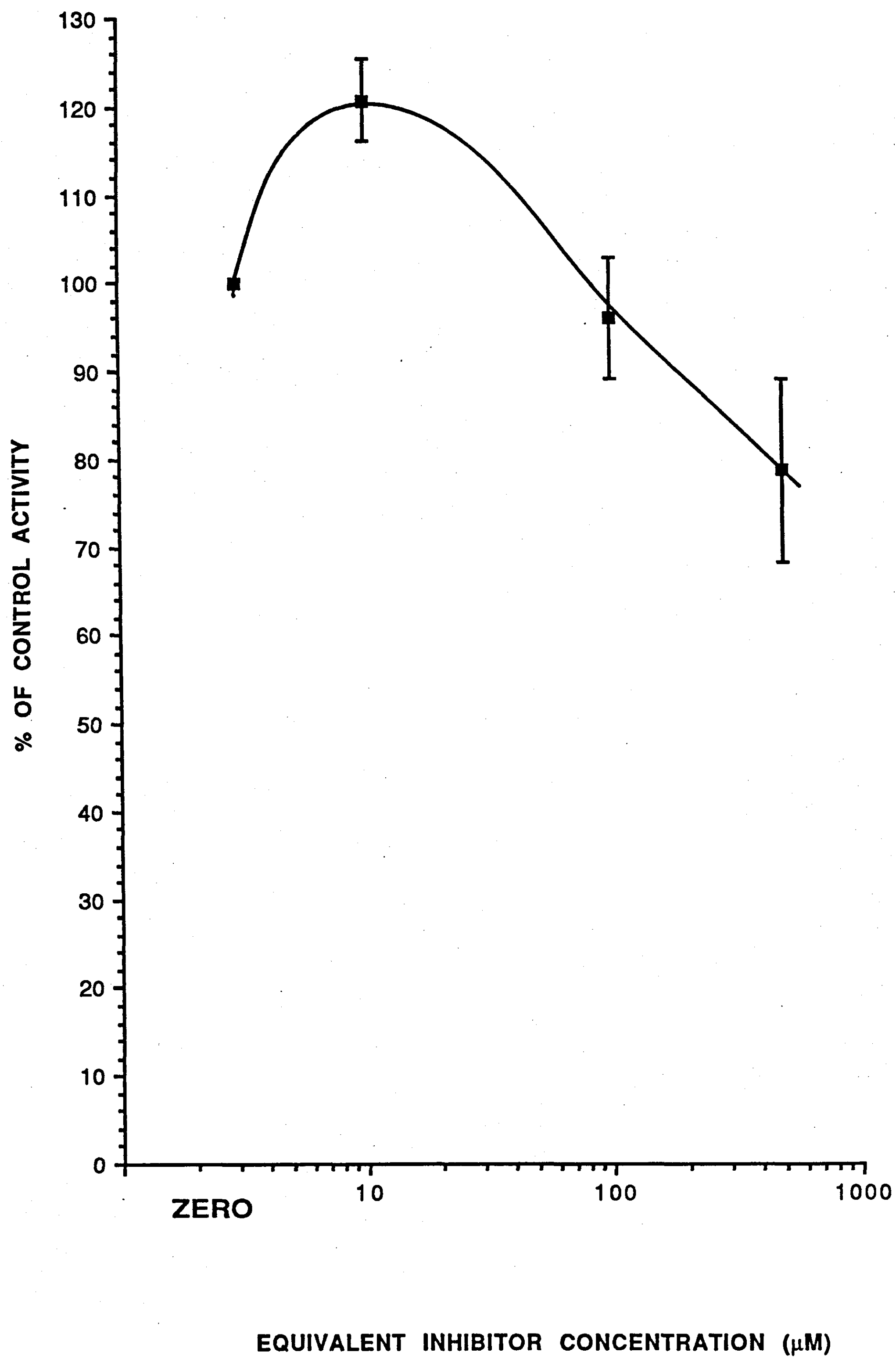


FIGURE II



<u>FIGURE III</u>

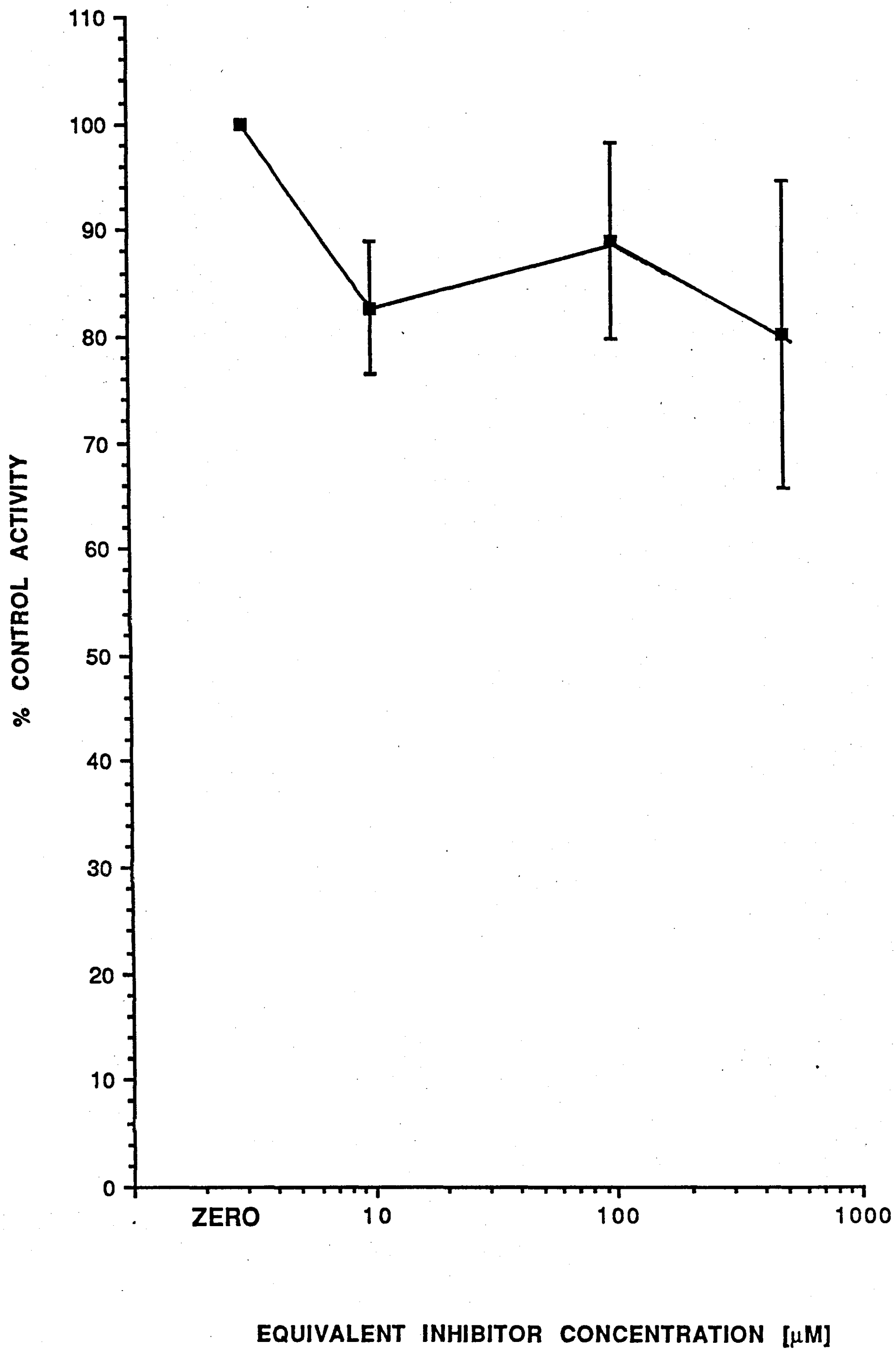


FIGURE IV

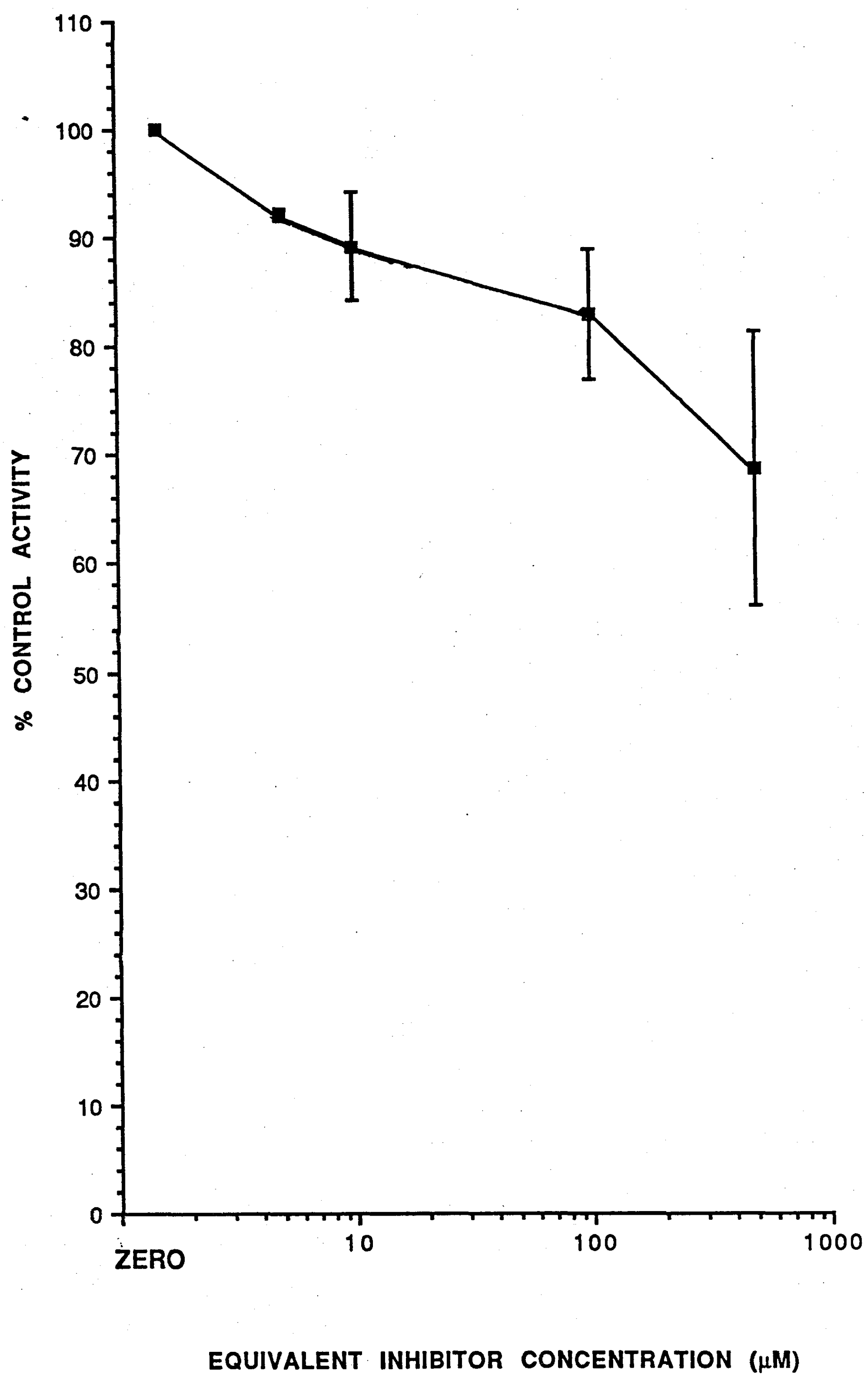


FIGURE V

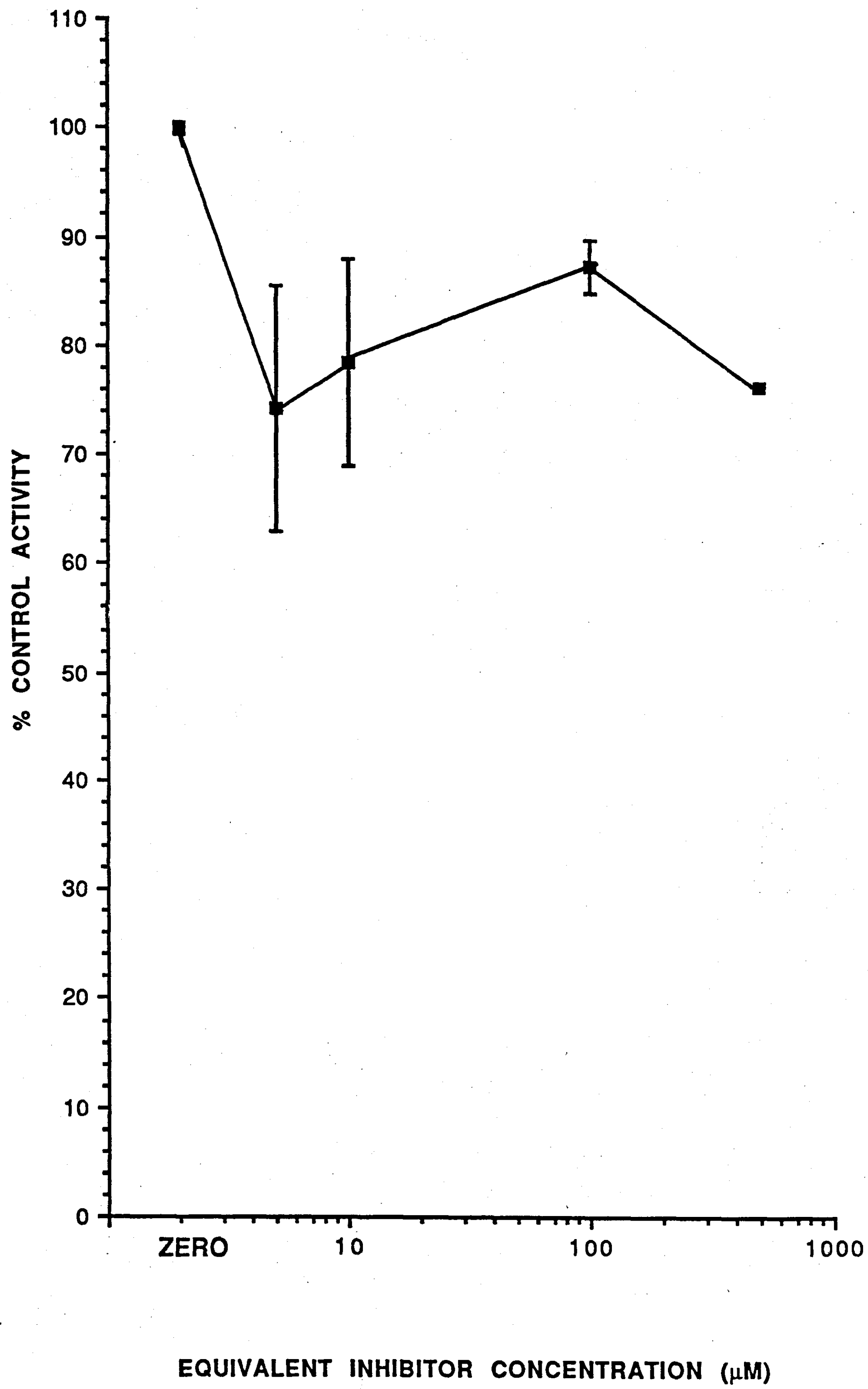


FIGURE VI

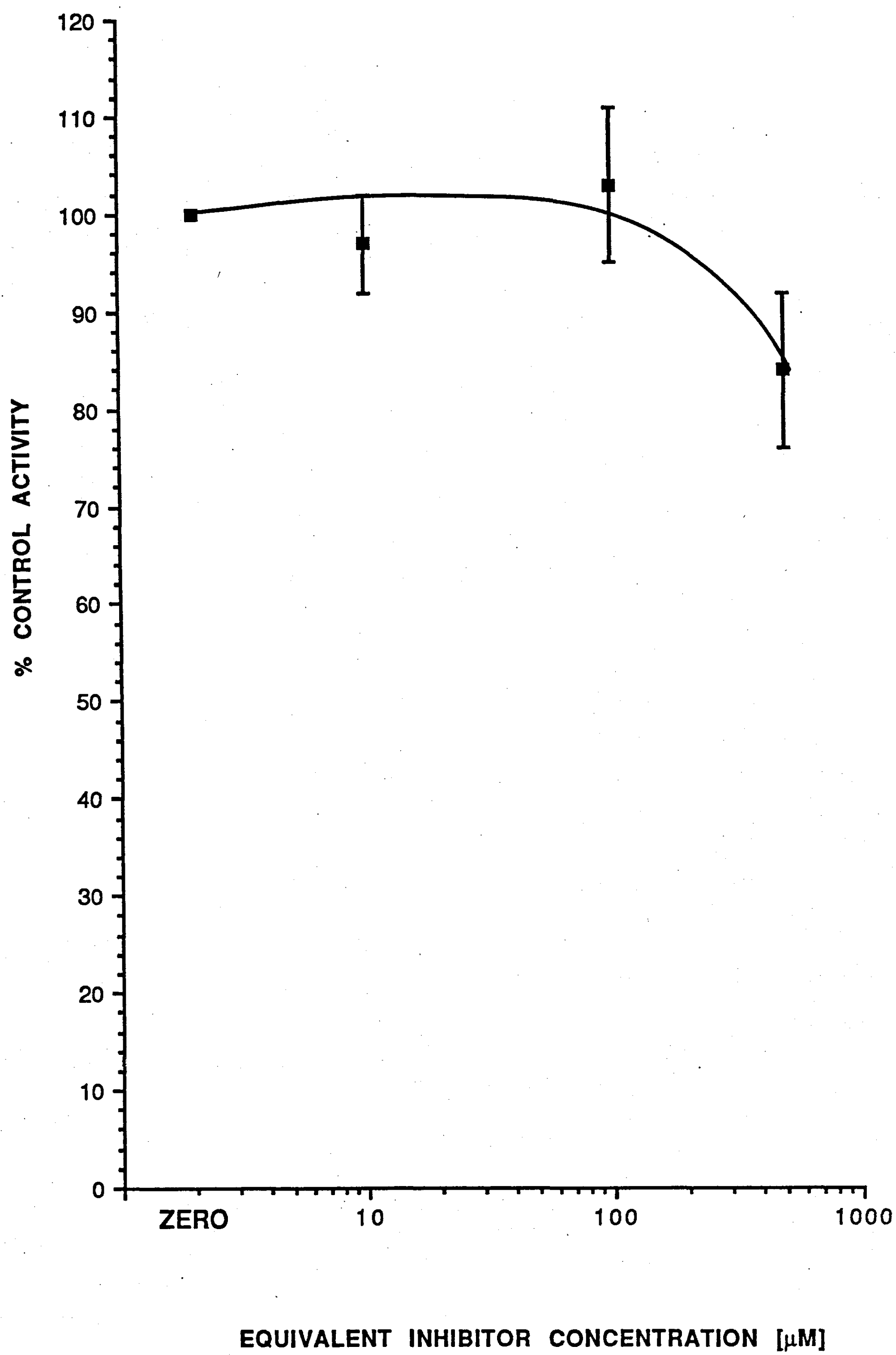


FIGURE VII

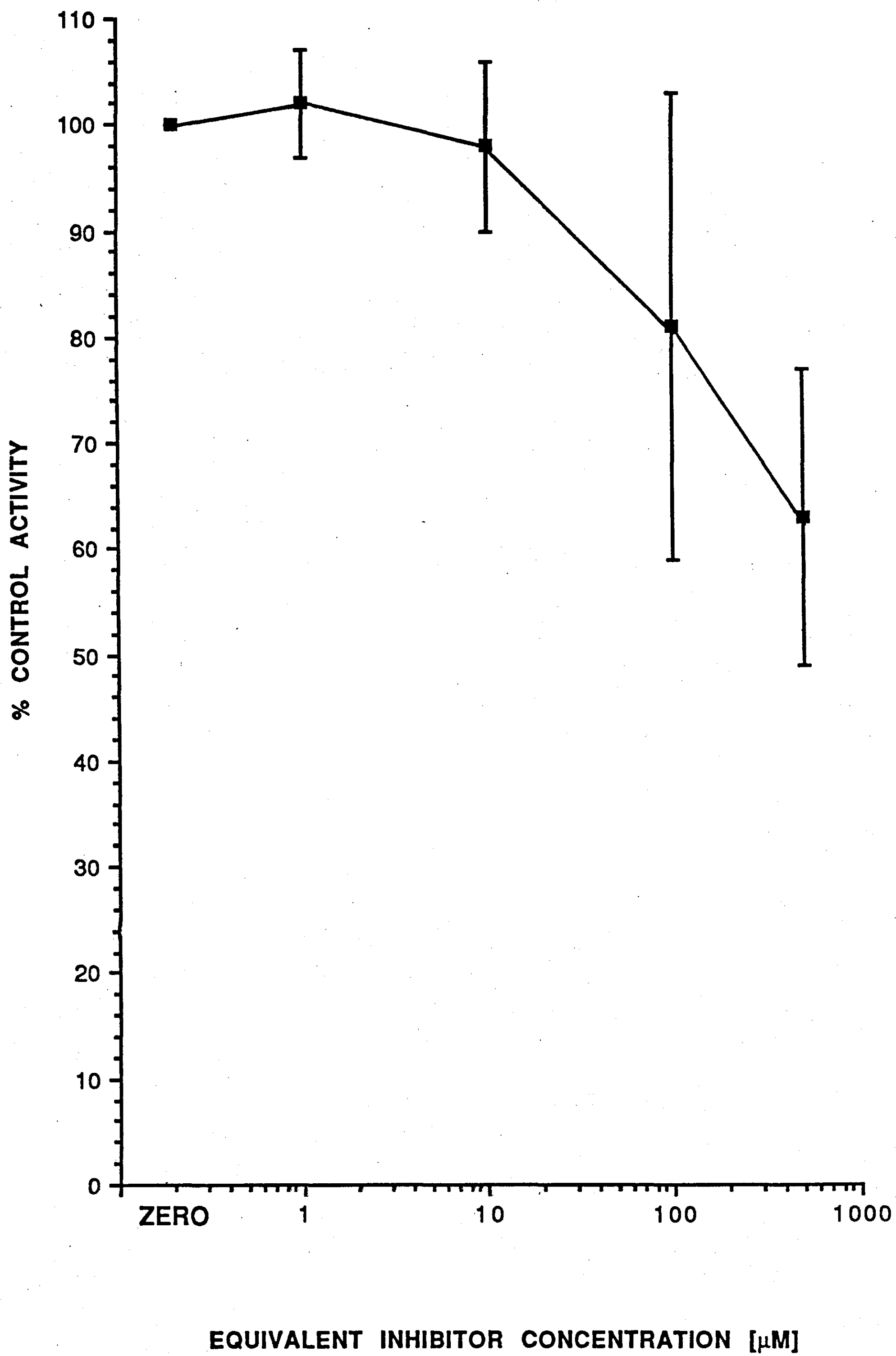


FIGURE VIII

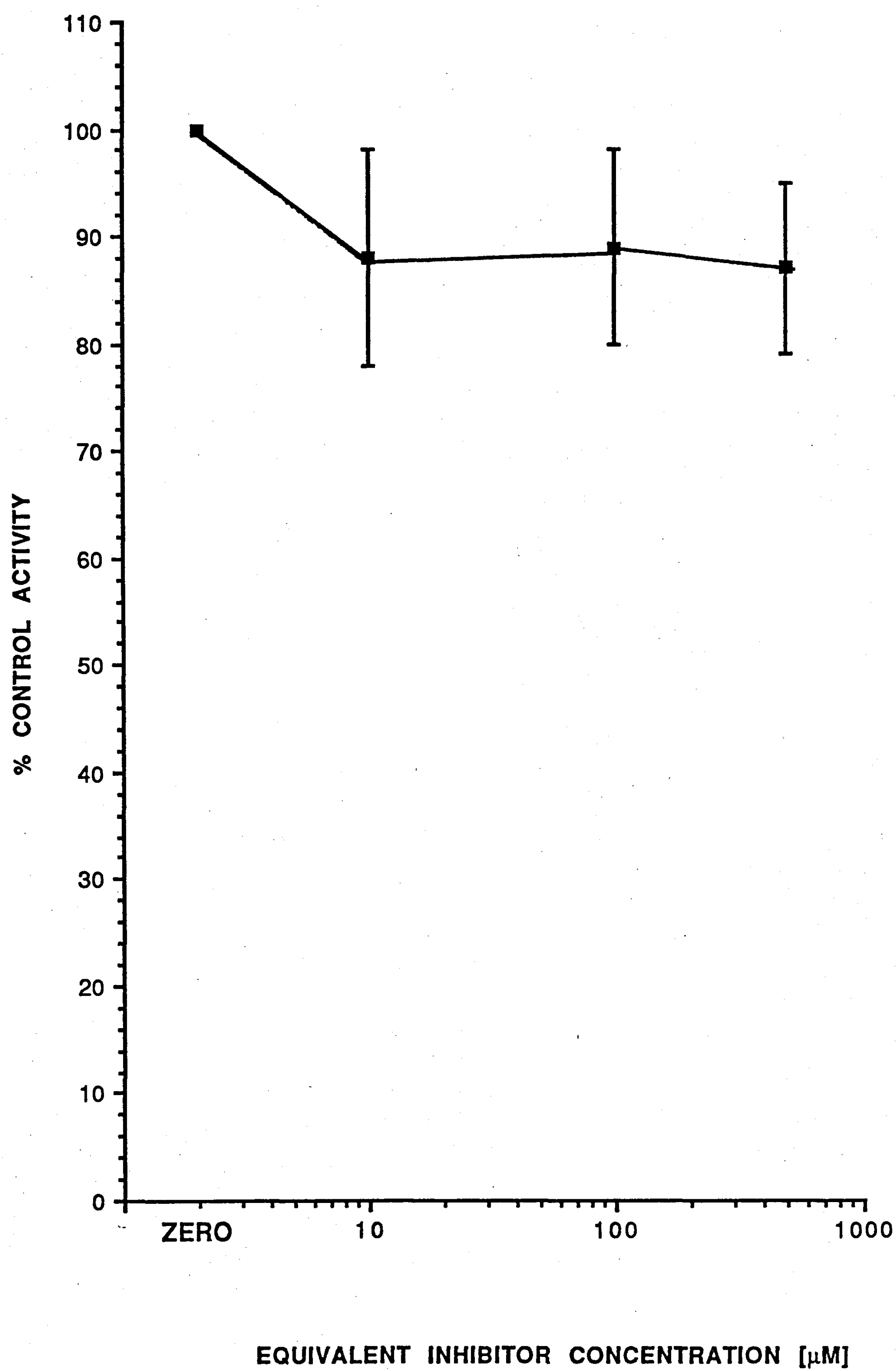


FIGURE IX

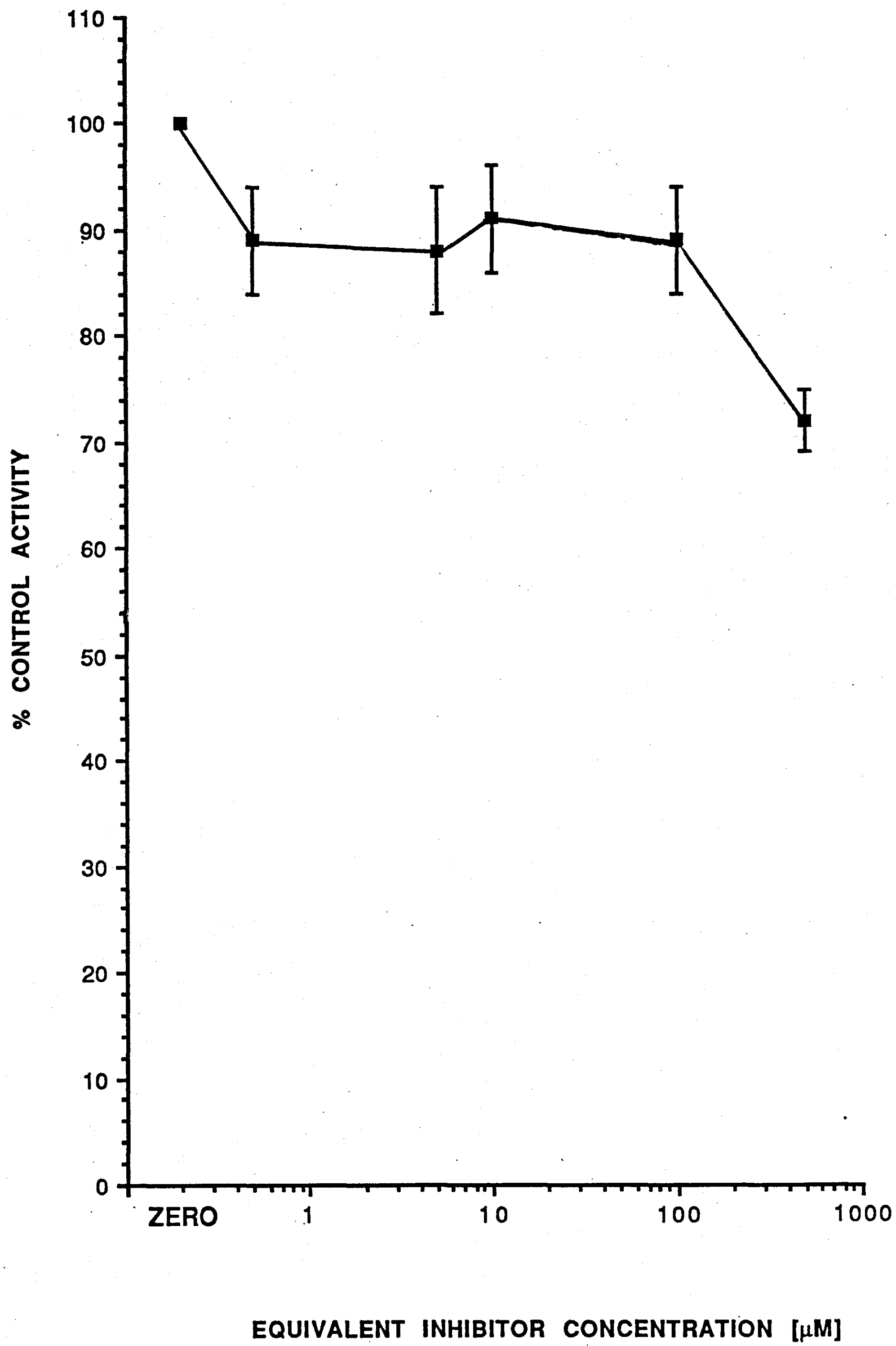


FIGURE X

110
100
90
80
70
60
50
40
30
20
10
0

% CONTROL ACTIVITY

ZERO 1 10 100 1000

EQUIVALENT INHIBITOR CONCENTRATION [μM]