The Purification and Characterisation of a Pyroglutamyl Aminopeptidase Type-1 Activity from the Cytosolic Fraction of Bovine Whole Brain

Thesis Submitted for the Degree of
Doctor of Philosophy
by

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the program of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Philip Cummins **Date:** 26/9/1995

(Candidate)

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Where do I finish?

Mum, I love you and I'll miss you

Frances, I couldn't have done this without you I owe you everything

ABBREVIATIONS

BCA Bicinchoninic Acid

BSA Bovine Serum Albumin

cDNA Complementary DNA

CNS Central Nervous System

CRF Corticotropin-Releasing Factor

cyclo(His-Pro) Histidyl-Proline Diketopiperazine

DAP Dipeptidyl Aminopeptidase

DEAE Diethylaminoethyl

DG 1,2-Diacylglycerol

DMF Dimethylformamide

DMSO Dimethylsulphoxide

DTT Dithiothreitol

E-64 N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine

EDTA Ethylenediaminetetra Acetic Acid

Fmoc-Pro-Pro-Nitrile 9-Fluorenylmethoxycarbonyl-Prolyl-Prolyl-Nitrile

GABA Gamma-Aminobutyric Acid

GH Growth Hormone

GHR-IH Growth Hormone Release-Inhibiting Hormone

Gly-Pro-MCA Glycyl-Proline-7-Amino-4-Methyl-Coumarin

GPCR G-Protein Coupled Receptor

HCL Hydrochloric Acid

His-Pro Histidyl-Proline

His-Pro-NH_2 Histidyl-Proline-Amide

HPLC High Performance Liquid Chromatography

HPT Hypothalamic-Pituitary-Thyroid

5-HT Serotonin

$InsP_3$ Inositoltriphosphate

K_I Inhibition Constant

K_m Michaelis-Menten Constant

LHRH Luteinizing Hormone Releasing Hormone/Luliberin

Lys-Ala-MCA Lysyl-Alanyl-7-Amino-4-Methyl-Coumarin

MCA 7-Amino-4-Methyl-Coumarin

ME Median Eminence

MES 2-[N-Morpholino]ethane-sulphonic Acid

mRNA Messenger RNA

ß-NA ß-Naphthylamide

NaCl Sodium Chloride

N D Not Determined

NE Norepinephrine

P_1, P_2 Pellet

PAGE Polyacrylamide Gel Electrophoresis

PAM Peptidylglycine Alpha-Amidating Monooxygenase

PAP Pyroglutamyl Aminopeptidase

PE Prolyl Endopeptidase

PEG Polyethylene Glycol

pGCK Pyroglutamyl-Chloromethyl Ketone

pGDK Pyroglutamyl-Diazomethyl Ketone/PDMK

pGlu Pyroglutamic Acid

pGlu-Ala Pyroglutamyl-Alanine

pGlu-His Pyroglutamyl-Histidine

pGlu-His-Gly Anorexigenic Peptide

pGlu-His-Gly-NH_2 Pyroglutamyl-Histidyl-Glycine-Amide

pGlu-His-Pro Acid Thyroliberin

pGlu-His-Pro-MCA Pyroglutamyl-Histidyl-Proline-7-Amino-4-Methyl-Coumarin

pGlu-His-Pro-NH_2 Thyroliberin/TRH

pGlu-MCA Pyroglutamyl-7-Amino-4-Methyl-Coumarin

pGlu-ßNA Pyroglutamyl-ß-Naphthylamide

pGlu-pNA Pyroglutamyl-p-Nitroanilide

pGlu-Pro-NH_2 Pyroglutamyl-Proline-Amide

pGlu-Val Pyroglutamyl-Valine

PHMB p-Hydroxymercuribenzoate

PMSF Phenylmethylsulphonylfluoride

PPCE Post Proline Cleaving Enzyme

PPDA Post Proline Dipeptidyl Aminopeptidase

Pro-NH_2 Proline-Amide

PVN Paraventricular Nucleus

R_f Relative Mobility

S_1, S_2 Supernatant/Crude cytosol

S D Standard Deviation

SDS Sodium Dodecyl Sulphate

Sephacryl S-200 HR Sephacryl S-200 High Resolution

SH Sulphydryl

T3 Triiodothyronine

T4 Tetraiodothyronine

TEMED N, N, N, N'-Tetramethylethylenediamine

TFA Trifluoroacetic Acid

TRH Thyrotropin Releasing Hormone/Thyroliberin/Thyrotropin Releasing Factor/TRF

TRH-R TRH-Receptor

TSH Thyroid Stimulating Hormone

$\mathbf{V_e}$ Elution Volume

VIP Vasoactive Intestinal Peptide

$\mathbf{V_o}$ Void Volume

Z-Gly-Pro-MCA *N*-Benzyloxycarbonyl-Glycyl-Proline-7-Amino-4-Methyl-Coumarin

Z-pGCK *N*-Benzyloxycarbonyl-Pyroglutamyl-Chloromethyl Ketone

Z-pGDK *N*-Benzyloxycarbonyl-Pyroglutamyl-Diazomethyl Ketone

Z-Pro-Prolinal *N*-Benzyloxycarbonyl-Prolyl-Prolinal

ABSTRACT

Since the initial isolation of pyroglutamyl aminopeptidase (PAP) from a strain of *Pseudomonas fluorescens* by Doolittle and Armentrout in 1968, similar enzyme activities have been isolated and characterised from a multitude of prokaryotic and eukaryotic sources Studies on eukaryotic PAPs have been done mainly in mammals, typically with a view to elucidating the potential role of this class of enzymes in the catabolism of various pGlu-terminating peptides, including neuropeptides, *in vivo* The central aim of this study was to undertake the complete purification and characterisation of a PAP activity observed within the soluble or cytosolic fraction of bovine whole brain Several workers have previously described the purification and characterisation of soluble PAP activities from different mammalian tissues including guinea-pig and human brain However, other than some minor details furnished by earlier studies, little was previously known of the bovine brain activity

A combination of different chromatographic methodologies subsequently generated a soluble PAP activity with a total active yield of 6 6% which had been purified to near homogeneity, as judged by SDS PAGE and silver staining techniques The unstable nature of the purified enzyme in dilute solution was very apparent, prompting the usage of 0 5%w/v BSA to stabilise PAP activity during both assay and storage Characterisation of this enzyme activity subsequently revealed a number of interesting results, many of which compared well with findings previously reported for soluble PAP activities examined in other sources In addition to a predominantly cytosolic subcellular location, this enzyme was found to exhibit a low relative molecular mass Gel-filtration chromatography revealed a native molecular mass of approximately 23,700 daltons, a value which compares well with that obtained for the enzyme under denaturing conditions via SDS PAGE (22,450 daltons), supporting the likelihood that the soluble bovine brain PAP exists as a monomer A pH optimum of 8 5-9 0, as determined with pGlu-MCA at 37°C, was also demonstrated for this enzyme, whilst the expression of PAP activity exhibited an absolute requirement for the presence of a disulphide bond-reducing agent such as DTT, suggesting the participation of active site thiol groups in enzyme activity (i e a thiol protease) Strong inhibition of purified PAP activity was observed with a number of different agents which included the transition metal ions Hg^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+}, the sulphydryl-blocking agents iodoacetate, 2-iodoacetamide, PCMB and N-ethylmaleimide and the reversible inhibitor 2-pyrrolidone Serine protease inhibitors and metal chelating agents (with the exception of 1,10-phenanthroline) as well as the compounds bacitracin puromycin and bestatin had no effect on enzyme activity
The cleavage of the N-terminal pGlu residue from a wide range of pyroglutamyl substrates including TRH, acid TRH, pGlu-His-Pro-MCA (a TRH analog), bombesin and neurotensin was clearly demonstrated for the soluble bovine brain PAP activity N-terminal pGlu cleavage of eledoisin and LHRH could not be detected however Whereas this was expected for eledoisin, a substrate which commences with the sequence pGlu-Pro, such a finding for LHRH was quite unexpected Subsequent kinetic analysis also revealed that the purified PAP activity displays K_m and K_i values within the

lower micromolar range for a number of these substrates (TRH, acid TRH, LHRH, pGlu-MCA, pGlu-βNA and pGlu-His-Pro-MCA) indicative of a strong enzyme-substrate interaction In addition, all of the pGlu-peptides for which K_I values were estimated proved to be competitive inhibitors

Based on a comparison of these findings with those reported for soluble PAP activities in other mammalian tissues, the soluble PAP enzyme activity observed in bovine whole brain can tentatively be classified as a pyroglutamyl aminopeptidase type-1 or PAP-I (EC 3 4 19 3)

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1. INTRODUCTION

PREFACE

The experimental body of work outlined in this report concerns the purification and characterisation of the cytosolic neuropeptidase, pyroglutamyl aminopeptidase or PAP, from bovine brain This enzyme has previously been shown to have a relatively broad pyroglutamyl-substrate specificity *in vitro*, which includes the hypothalamic releasing factor, thyrotropin releasing hormone (TRH), among other neuropeptides Following a brief introductory segment which examines the physiological relevance of neuropeptides (section 1 1), the ensuing section (section 1 2) will subsequently endeavour to review and condense the extensive volume of literature currently available on this neuropeptidase and to report on aspects of its genetic characterisation, biochemical and enzymatic properties, potential physiological functions and its distribution both among different species and between different mammalian tissues

Since its isolation from porcine and ovine hypothalamic tissue in 1969, TRH has been the focus of intense investigation, and has been proposed as a potential substrate for a number of soluble and particulate enzyme activities, including pyroglutamyl aminopeptidase, *in vivo* In view of the physiological and pharmacological significance ascribed to this neuropeptide, a short review of TRH will also be undertaken (section 1 3) Aspects of its structure and its functional capacities as a neuroendocrine and neuroregulatory factor, as well as its inactivation by neuropeptidases will be examined In addition, some of the more recent and interesting aspects of TRH research will receive some attention These include TRH receptor signalling, the genetic characterisation and processing of the TRH prohormone and the regulation of TRH at the biosynthetic and secretory level

1.1 Neuropeptides

Neuropeptides form an ever expanding and interesting family of potent chemical messengers The previous three decades have seen the identification of well over 100 neuroactive peptides in mammalian tissues, each having either a clearly defined or proposed neurobiological function Application of the techniques of immunohistochemistry and radioimmunoassay coupled with various other techniques in biochemistry and molecular biology have greatly facilitated the initial identification and characterisation of a host of these peptides within the vertebrate brain Many of these peptides however, were previously known to exist in vertebrate tissues beyond the confines of the central nervous system, as well as in lower species (Le Roith *et al*, 1980, 1981, 1982, Schwabe *et al*, 1983, Maruo *et al*, 1979), in which they may serve as primitive elements of intercellular communication prior to the development of neuronal or endocrine systems Table 1 1 highlights the categories into which mammalian brain peptides can be grouped

Structurally, these compounds consist of polypeptide chains of up to 40 amino acid residues in length, with very specific modifications to individual residues, which govern the distinctive biological activity of each particular neuropeptide Common amino acid residue modifications include modification of the amino-terminus (e g by acetylation or by the cyclisation of glutamine to pyroglutamic acid) and the carboxy-terminus (e g by amidation) These structural modifications determine the highly specific biological activities of neuropeptides. This includes their ability to recognise and bind to receptors and their relative stability towards inactivation by peptidase enzymes (neuropeptidases) which are present in all mammalian cells, both cytosolically and at the membrane level, with the specific purpose of degrading peptides or proteins to their constituent amino acids, enabling their return ultimately to the cellular amino acid pool.

1 1 1 Role of neuropeptides

Before neuropeptides were studied thoroughly in the central nervous system, they were simply thought of as a new category of neurotransmitters With time however it became clear that a given neuropeptide could be involved in a variety of biological functions and that neurotransmitter-like actions alone were not enough to account for these functions This view was further substantiated by the observation that similar neuropeptides can occur in different tissues and organisms, suggesting therefore that the same peptide can act via different modes of intercellular communication, depending on the tissue in which it is present It is now known that neuropeptides can follow at least three different routes of action In some instances a given neuropeptide can act as (1) a local factor via autocrine or paracrine secretion, (2) a neuroendocrine substance (neurohormone or hormone) and (3) a neuroregulatory substance (neurotransmitter or neuromodulator)

Table 1 1 Categories of mammalian brain peptides

Hypothalamic-Releasing Hormones
Thyrotropin-Releasing Hormone
Luteinizing Hormone-Releasing Hormone
Somatostatin
Corticotropin-Releasing Hormone
Growth Hormone-Releasing Hormone
Neurohypophyseal Hormones
Vasopressin
Oxytocin
Neurohypophysin (s)
Pituitary Peptides
Adrenocorticotropic Hormone
ß-Endorphin
alpha-Melanocyte-Stimulating Hormone
Prolactin
Luteinizing Hormone
Growth Hormone
Thyrotropin
Invertebrate Peptides
FMRF Amide *
Hydra Head Activator
Gastrointestinal Peptides
Vasoactive Intestinal Polypeptide
Cholecystokinin
Gastrin
Substance P
Neurotensin
Methionine-Enkephalin
Leucine-Enkephalin
Insulin
Glucagon
Bombesin
Secretin
Somatostatin
Motilin
Others
Angiotensin II
Bradykinin
Carnosine
Sleep Peptides
Calcitonin
Neuropeptide Yy

(Borrowed from Krieger (1983))

* Phe-Ala-Met-Arg-Phe-Ala-NH_2

1.1 2 Neurotransmitters and neuromodulators

The latter capability of neuropeptides, that of neuroregulation, is of particular interest This refers to the processing of information in the brain and largely involves chemical communication among neurons through neuroregulatory substances (peptides, classical aminergic transmitters etc) In this respect, there has been considerable debate as to whether brain peptides should be properly classified as neurotransmitters or "neuromodulators"

A neurotransmitter has previously been described as a substance liberated at presynaptic terminals which, after diffusing across the narrow synaptic gap, acted on the post-synaptic membrane Its action is highly localised to the synaptic region, with a duration of milliseconds Termination of its action is accomplished by removal of the neurotransmitter, either by enzymatic degradation or via a reuptake mechanism into the presynaptic terminal The reader is directed to Bowman and Rand (1980) for a more in depth examination of the criteria that should be satisfied for positive identification of a substance as a neurotransmitter

The observation that many peptide actions have a slow time course (Bloom, 1979, Iverson, 1984, Lundberg and Tatemoto, 1982), that there were mismatches between locations of peptides and their receptors in the brain (Herkenham and McLean, 1986) and that many neuropeptides coexisted with other transmitter agents in individual neurons (Lundberg and Hokfelt, 1983, Hokfelt *et al*, 1986) prompted researchers to define the term "neuromodulator" to describe the nonclassical transmitter actions displayed by several neuropeptides This term has been used to define peptide actions when they (1) modify the known actions of the so-called "classical" neurotransmitters, (2) act to block the release of a given neurotransmitter via their release at presynaptic endings on the terminals releasing that transmitter and (3) alter the turnover of other neurotransmitters The net effect of neuromodulation therefore, amounts to the amplification or dampening of neuronal signalling processes
Indeed the presence in the same cell of two or more putative neurotransmitters appears to be the rule rather than the exception (Crawley, 1990) Hokfelt *et al* (1980) have, for example, demonstrated the coexistence of serotonin with substance P and TRH in neurons of the medulla oblongata, vasoactive intestinal peptide (VIP) and acetylcholine within autonomic ganglia and corticotropin-releasing factor (CRF) and vasopressin within cells of the paraventricular nucleus It is widely believed therefore that together with the small-molecule amines largely responsible for synaptic transmission, coexisting peptides, although present at concentrations several orders of magnitude less than those of the classic neurotransmitters such as acetylcholine (Krieger, 1983), may act as modulators and/or have alternative functions The coexistence of multiple transmitters and modulators within the same synapse therefore, enhances the versatility of the chemical message transmitted by neurons by providing the mechanism for more complex patterns of postsynaptic response

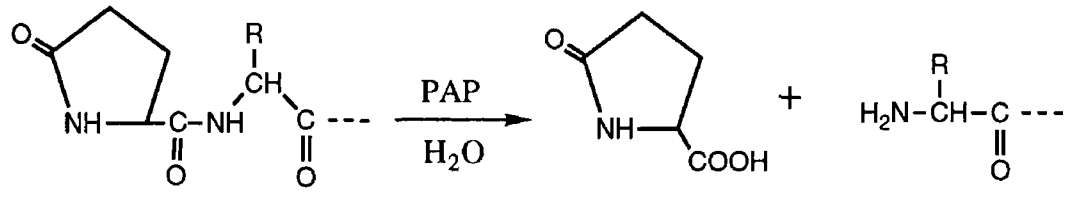
The vast scope of peptide neuromodulation within the brain, together with the fact that neuromodulation is an "indirect" process in the sense of requiring another agent, does raise questions

of specificity Therefore, most investigators dealing with peptide modulation have made at least some effort to assess the specificity of the neuromodulation in question by testing whether neuromodulation by a particular peptide can be duplicated with other, unrelated peptides With rare exception, neuropeptide-specificity has been demonstrated For example, Pan *et al* (1988) have tested preoptic neurons whose responses to norepinephrine (NE) were modulated by LHRH, with TRH as well They found that none of the neurons modulated by LHRH were also modulated by TRH This specificity was demonstrated in spite of the fact that TRH itself could modulate NE-responses in the ventromedial hypothalamus (Kow and Pfaff, 1987) For a more complete overview of neuromodulation, Kow and Pfaff (1988) is highly recommended

1.2 Pyroglutamyl aminopeptidase

Pyroglutamyl aminopeptidase or PAP (EC 3 4 19 3, formerly EC 3 4 11 8) can be classified as an exopeptidase, or more correctly, an omega peptidase (McDonald and Barrett, 1986), which hydrolytically removes the pyroglutamic acid (pGlu) residue from the amino terminus of pGlu-peptides and pGlu-proteins (Fig 1 1) This enzyme is apparently specific for L-pGlu-L-amino acid optical isomers (Uliana and Doolittle, 1969)

Fig 1 1 Hydrolysis of pGlu from the N-terminus of pGlu-peptides by pyroglutamyl aminopeptidase (PAP)



This enzyme activity was first described in a strain of *Pseudomonas fluorescens* by Doolittle and Armentrout (1968) who found that a crude extract taken from this soil microorganism could hydrolyse the pyroglutamyl dipeptide, L-pGlu-L-Ala to yield free pGlu and alanine They subsequently suggested the name pyrrolidonyl peptidase PAP has since been observed in the tissues of mammals, birds, fish, plants and bacteria and has been referred to by several other names (pyrrolidonecarboxylate peptidase, pyrrohdonecarboxylyl peptidase, pyroglutamate aminopeptidase, 5-oxoprolyl-peptidase, pyroglutamyl peptide hydrolase, PYRase and pyroglutamyl aminopeptidase)

To date, two distinct classes of PAP have been characterised The first class includes the bacterial PAPs and animal type-1 (PAP-I) PAPs These are typically sulphydryl-dependent enzymes which display a cytosolic location, broad pyroglutamyl-substrate specificity and low relative molecular mass Though similar in many respects however, bacterial PAPs are generally oligomeric whilst PAP-I

observed in different animal systems appears to be monomeric The second class is comprised of the animal type-2 (PAP-II) and serum PAPs PAP-II has been shown to be a membrane-bound metalloenzyme of high relative molecular mass with a very narrow substrate specificity centering around TRH or very closely related peptides A PAP activity observed in the serum of different animal species displays biochemical characteristics remarkably similar to those of tissue PAP-II such as a high relative molecular mass, sensitivity to metal chelating agents, insensitivity to sulphydryl agents and a narrow substrate specificity restricted to TRH or closely related peptides (Awade *et al* (1994) is recommended for review)

1 2 1 Pyroglutamic acid Occurrence in bioactive peptides

Pyroglutamic acid, also known as 5-oxo-L-proline or pyrrolidone carboxylic acid, was first described by Haitinger as a glutamic acid derivative that lacked a molecule of water (Haitinger, L , 1882) Although much evidence exists for the non-enzymatic formation of pGlu from glutamic acid (Glu), glutamine and various esters and diesters of glutamic acid (Sanger *et al* , 1955, Smyth *et al* , 1962, Winstead and Wold, 1962), the enzymatic formation of this compound is well established Meister *et al* (1963) partially purified and characterised an animal tissue enzyme capable of converting D-Glu to D-pGlu via an acylation process They subsequently termed this enzyme D-glutamic acid cyclotransferase Similarly, Akita *et al* (1959) described an L-glutamic acid cyclotransferase activity in *Pseudomonas cruciviae* Studies have also reported the ability of glutamine synthetase (Meister, 1968) and gamma-glutamylcysteine synthetase (Orlowski and Meister, 1971) to convert Glu to pGlu

The enzymatic synthesis of pGlu suggests that this residue may have important physiological functions Consistent with this is the observation that the amino terminus of many proteins and bioactive peptides terminates in pGlu (Table 1 2) and that this residue is frequently a determinant of the overall peptide activity For example, Hinkle and Tashijian (1973) have demonstrated that any structural substitution in the pGlu lactam ring of the hypothalamic releasing factor, TRH (pGlu-His-Pro-NH_2, section 1 3), significantly decreases both hormone potency and receptor-binding ability More recently, Perlman *et al* (1994) have demonstrated that TRH binds to its receptor via an interaction between the ring carbonyl of the TRH pGlu moiety with Tyr^{106} of the TRH receptor The mechanisms by which pGlu is inserted onto the amino terminus of such proteins and peptides are still largely unknown However, most current evidence would seem to indicate that, in mammalian tissues, the arisal of an N-terminal pGlu moiety results from the post-translational modification of glutamine via a cyclase-type enzyme activity (Busby *et al* , 1987, Fischer and Speiss, 1987) Pyroglutamyl peptides can also arise as an artefact of protein or peptide hydrolysis, following the liberation of glutamine-terminating peptides (Sanger and Thompson, 1953, Smyth *et al* , 1962, Sullivan and Jago, 1970)

The functions of pGlu as a free acid are less clear It has been observed in the tissues of patients with Hawkinsinuria disease (Borden *et al* , 1992), whilst elevated levels of free pGlu have been shown in

patients suffering from Huntington's disease The pharmacological properties of pGlu have also been described pGlu has been shown to prevent scopolamine-induced amnesia in the rat (Spignoli *et al*, 1987) and to improve learning and age-associated memory loss (Grioli *et al*, 1990)

Table 1 2 *Some proteins/peptides with an N-terminal pGlu residue*

Peptide/protein	Sequence
TRH	pGlu-His-ProNH$_2$
TRH-like peptide (prostate)	pGlu-Glu-ProNH$_2$
LHRH	pGlu-His-Trp-Ser-Tyr-
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-
Bombesin	pGlu-Gln-Arg-Leu-Gly-
Eledoisin	pGlu-Pro-Ser-Lys-
Fastigiatine	pGlu-Glu-GlnOH
Gastrin	
Human	pGlu-Gly-Pro-Trp-Leu-
Hog	pGlu-Gly-Pro-Trp-Met-
Fibrinopeptides B	
Human	pGlu-Gly-Val-AspNH$_2$-
Ox	pGlu-Phe-Pro-Thr-Asp-
Reindeer	pGlu-Leu-Ala-Asp-
Cow	pGlu-Phe-Pro-Thr-Asp-
Physalaemin	pGlu-Ala-AspOH-Pro-
Peptides from snake venoms	pGlu-AspNH$_2$-Trp- pGlu-GluNH$_2$-Trp-
Heavy chains of rabbit IgG	pGlu-Ser-Val-Glu-Glu- pGlu-Ser-Leu-Glu pGlu-GluNH$_2$
Eisenine	pGlu-Glu-AlaOH
Vasoactive polypeptide	pGlu-Val-Pro-Gln-Trp-
Heavy chain of human pathological IgG	pGlu-Val-Thr-
Heavy chain of human gamma G immunoglobulin	pGlu-Val-Gln-Leu-
Mouse lambda chains	pGlu-Ala-Val-Val-
alpha 2-CB1 of rat skin collagen	pGlu-Tyr-Ser-Asp-Lys-
Human apoLp-Gln-II	pGlu-Ala-Lys-Glu-Pro-
Thymic factor from pig serum	pGlu-Ala-Lys-Ser-Gln-
Hypertrehalosaemic neuropeptide	pGlu-Val-Asn-Phe-Ser-
Peptide inhibiting epidermal mitosis	pGlu-Glu-Asp-Cys-LysOH
Colon mitosis inhibitory peptide	pGlu-Glu-His-GlyOH
Caerulein	pGlu-Gln-Asp-TyrSO$_3$H-
Levitide	pGlu-Gly-Met-Ile-Gly-Thr
Anorexigenic peptide	pGlu-His-Gly
Porcine pancreatic spasmolytic polypeptide	pGlu-Lys-Pro-Ala-Ala-
Heavy chains of rabbit anti-hapten antibodies	pGlu-Ser-Leu-Glu-Glu- pGlu-Ser-Val-Glu-Glu-
Human monocyte chemoattractant	pGlu-Pro-Asp-Ala-Ile-

(Borrowed from Awade *et al* (1994) and subsequently modified)

1.2 2 Class 1· Mammalian PAP-I and microbial PAPs

1.2.2 1 Mammalian PAP-I

Studies on eukaryotic PAP-I have been done mainly in mammals Many studies have documented the isolation and characterisation of PAP-I from different mammalian tissues with a particular view to elucidating its potential role in the catabolism of various pGlu-terminating peptides, including neuropeptides Authors have described the purification (partial or otherwise) of PAP-I from human cerebral cortex, kidney and skeletal muscle (Lauffart *et al* , 1989, Mantle *et al* , 1990, 1991), rat liver (Armentrout, 1969, Scharfmann and Aratan-Spire, 1991), bovine pituitary (Mudge and Fellows, 1973), guinea-pig brain (Browne and O'Cuinn, 1983a) and rat brain (Busby *et al* , 1982) PAP-I activity has also been reported in hamster hypothalamus (Prasad and Peterkofsky, 1976), mouse brain (Faivre-Bauman *et al* , 1981), cat brain cortex (de Gandarias *et al* , 1992), rat retina and hypothalamus (Ramirez *et al* , 1991), rat adenohypophysis (Bauer and Kleinkauf, 1980), rat pancreas (Aratan-Spire *et al* , 1986) and various rat systemic organs (Fuse *et al* , 1990) Non-mammalian animal sources such as avian, fish and amphibian tissues, have also been reported to display PAP-I activity (Albert and Szewczuk, 1972, Tsuru *et al* , 1982, Prasad *et al* , 1982a, Szewczuk and Kwiatkowska, 1970)

Studies have shown that mammalian PAP-I is a monomeric enzyme with a low relative molecular mass, a soluble or cytosolic location and an optimum pH range between pH 6 5 and 8 5 (Mudge and Fellows, 1973, Lauffart *et al* , 1989) Molecular mass estimates range from 22,000 (human kidney and skeletal muscle), 23,000 (human brain) and 24,000 (guinea-pig brain) to 60,000 (rat brain) (Mantle *et al* , 1990, 1991, Lauffart *et al* , 1989, Browne and O'Cuinn, 1983a, Busby *et al* , 1982) In addition, a strict requirement for a thiol-reducing agent such as DTT or 2-mercaptoethanol has been shown, almost-universally, for this particular enzyme activity Consequently, several reports have demonstrated the extremely inhibitory nature of sulphydryl-blocking reagents such as N-ethylmaleimide, iodoacetate, PCMB and 2-iodoacetamide towards PAP-I (Browne and O'Cuinn, 1983a, Bauer and Kleinkauf, 1980) Serine protease inhibitors such as benzamidine and PMSF usually have no effect on PAP-I (the reader is directed to section 1 2 4 for a closer examination of inhibitors of mammalian PAPs)

A distinctive biochemical feature of PAP-I is its broad pyroglutamyl-substrate specificity Thus enzyme is capable of liberating the N-terminal pGlu residue from a range of biologically active peptides which include TRH, acid TRH, LHRH, neurotensin, bombesin and anorexigenic peptide (Browne and O'Cuinn, 1983a) Synthetic substrates such as pGlu-MCA, pGlu-pNA, pGlu-ßNA and isotopic TRH are also readily hydrolysed by PAP-I, as are synthetic dipeptides such as pGlu-Ala and pGlu-Val (Browne and O'Cuinn, 1983a, Bauer and Kleinkauf, 1980, Albert and Szewczuk 1972) However, pGlu-Pro bonds are not normally hydrolysed by mammalian PAP-I (Mudge and Fellows, 1973, Browne and O'Cuinn, 1983a) although a microbial PAP isolated from *Klebsiella cloacae* has been shown to be capable of splitting pGlu-proline (Kwiatkowska *et al* 1974) As mentioned earlier, this enzyme is apparently specific for L-pGlu-L-amino acid optical isomers (Uliana and Doolittle, 1969) In

addition, the rate of hydrolysis of a given substrate generally depends on the nature of the amino acid (or other group) immediately adjacent to the pGlu residue

Despite its broad substrate specificity, PAP-I is highly specific for N-terminal pGlu residues A study by Capecchi and Loudon (1985) reports that minor alterations to the pGlu moiety of a given substrate, such as the introduction of a second ureido nitrogen into the pyroglutamyl ring or increasing the ring size from 5 to 6 members, has very deleterious effects on the ability of PAP-I to cleave this amino terminal group More recently, Bundgaard and Moss have exploited this pGlu specificity as a means of developing potentially useful "prodrugs" which are resistant to PAP-I attack This research group have clearly demonstrated that by changing the N-H group on the pGlu ring to an N-X group where X is an acyl group (N-CO-R_1), Mannich base (N-CH_2N-R_2-R_3), glyoxylic acid adduct (N-CH(OH)-COO-R_4) or one of several other derivatives also investigated, the resultant pGlu moiety is completely resistant to cleavage by PAP-I (Moss and Bundgaard, 1989, 1992, Bundgaard and Moss, 1989)

The physiological role of PAP-I currently remains unclear On the basis of its relatively ubiquitous distribution in such functionally dissimilar tissues as skeletal muscle, brain and kidney, and through comparison to other soluble aminopeptidases, it has been proposed that PAP-I may contribute to the intracellular catabolism of peptides to free amino acids, which are then released to the cellular pool (Mantle *et al* , 1990, 1991, Lauffart and Mantle, 1988, Lauffart *et al* , 1989) Thus this enzyme may, at least in part, be involved in the regulation of the cellular pool of free pGlu It is noteworthy that free pGlu is known to have pharmacological properties (see section 1 2 1 above) Thus a specific pathway for pGlu production, e g , through PAP-I activity, may exist to generate this molecule An earlier study by Albert and Szewczuk (1972) also suggests that mammalian PAP-I may participate in the absorption of peptides and proteins from the alimentary tract This view is supported by the occurrence of the enzyme in the small intestine (Pierro and Orsatti, 1970) as well as in the intestinal mucous membrane and the duodenum and the comparatively low specificity of the intestinal enzyme (Albert and Szewczuk, 1972)

The potential role of PAP-I in the metabolism of physiologically important neuropeptides such as TRH and LHRH, has been the focus of intense investigation (and speculation) for several years Although this enzyme has demonstrated an ability to hydrolyse a range of pGlu-terminating neuropeptides *in vitro*, no definitive evidence linking neuropeptide inactivation *in vivo*, with PAP-I has yet been presented A particularly interesting case study is the potential role of PAP-I in the inactivation of TRH, a hypothalamic releasing factor which regulates TSH secretion from the anterior pituitary This case study is examined more thoroughly in section 1 3 5

1 2 2 2 Microbial PAPs

Following its initial discovery by Doolittle and Armentrout (1968) PAP has since been purified and characterised from a number of bacterial sources such as *Pseudomonas fluorescens* (Armentrout and Doolittle, 1969), *Klebsiella cloacae* (Kwiatkowska *et al* , 1974), *Bacillus subtilis* (Szewczuk and Mulczyk, 1969, Fellows and Mudge, 1971a), *Bacillus amyloliquifaciens* (Tsuru *et al* , 1978, 1984,

Fujiwara *et al*, 1979) and *Enterococcus faecium* (Sullivan *et al*, 1977) Bacterial PAPs display many of the biochemical characteristics common to mammalian PAP-I With the exception of the *K cloacae* enzyme which is associated with a particulate fraction, all of the bacterial PAPs examined to date have been shown to be soluble proteins located in the cell cytosol (Awade *et al*, 1992a, 1992b, Tsuru *et al*, 1978) Bacterial PAPs also display a broad pyroglutamyl-substrate specificity as well as a strict requirement for a highly reduced environment (DTT or 2-mercaptoethanol) (Tsuru *et al*, 1984, Fujiwara *et al*, 1979, Gonzales and Robert-Baudouy, 1994)

In contrast to the monomeric nature of native mammalian PAP-I however, bacterial PAPs invariably exist as oligomeric enzymes Molecular mass determinations for the bacterial enzyme under denaturing conditions indicate an average subunit mass of 25,000 daltons, which is almost identical to the relative molecular mass of the undenatured mammalian PAP-I examined in different species Native determinations on the other hand indicate more variability in size and show an average molecular mass from 40,000 to 90,000 daltons Tsuru *et al* (1978, 1984) have proposed that the *B amyloliquifaciens* PAP, with a native molecular mass of 72,000 and a subunit molecular mass of 24,000, probably functions as a trimer More recently however, Yoshimoto *et al* (1993) have cloned the gene for the *B amyloliquifaciens* enzyme (below) and, following its over-expression in *E coli*, have shown that the recombinant enzyme appears to exist as a dimer, suggesting that the recombinant form of the enzyme differs from the natural form (possibly due to different post-translational processing patterns in the host cell) Other studies indicate that the recombinant PAPs from *B subtilis* (Gonzales *et al*, 1992, Awade *et al*, 1992b) and *S pyogenes* (Awade *et al*, 1992a) are probably tetramers, whilst the recombinant PAP from *P fluorescens* (Gonzales and Robert-Baudouy, 1994) probably functions as a dimer

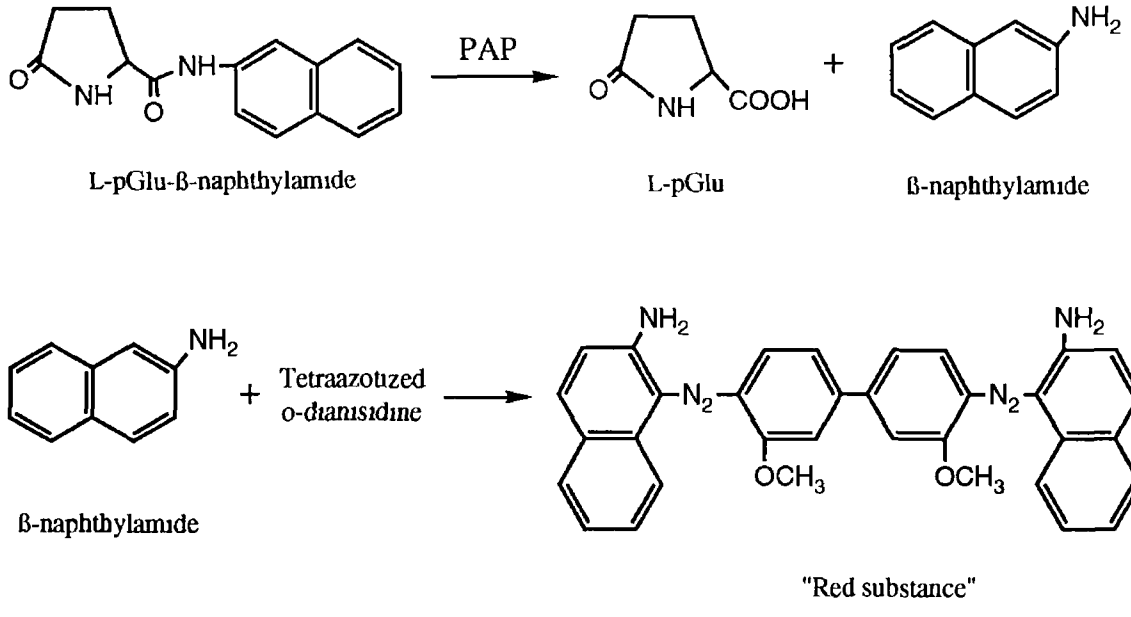
The question of the physiological role of PAP in bacteria currently remains unanswered Generally speaking, the bacterial aminopeptidases are thought to be involved in protein maturation, protein degradation and the utilisation of peptides as nutrients (Lazdunski, 1989) Therefore, one might expect that bacterial PAPs are involved in intracellular protein metabolism Awade *et al* (1994) have also suggested that PAP may be involved in detoxification, since the accumulation of peptides with a pGlu N-terminus may abnormally acidify the bacterial cell cytoplasm Such proposed roles for bacterial PAPs are weakened however, by the observation that this enzyme is noticeably absent from numerous bacterial strains Moreover, the distinctive substrate specificity of this enzyme suggests a more specific role in bacteria

1 2 2 3 Genetic characterisation of microbial PAPs

The molecular characterisation of PAP genes is a necessary step in order to develop our understanding of this particular class of enzymes Nucleotide sequence analysis and comparisons would improve prediction of conserved protein patterns involved in substrate recognition and hydrolysis and would enable researchers to examine in greater detail the factors which regulate PAP gene expression (and subsequently enzyme action) To this end, researchers have recently focused on bacterial sources to isolate and characterise the structural genes which encode for PAP activity To date, four bacterial PAP

genes have been characterised and expressed in host cells These genes have been cloned from *Streptococcus pyogenes* (Cleuziat *et al*, 1992a), *Bacillus amyloliquifaciens* (Yoshimoto *et al*, 1993), *Pseudomonas fluorescens* (Gonzales and Robert-Baudouy, 1994) and *Bacillus subtilis* (Awade *et al*, 1992b) The overexpressed *B amyloliquifaciens* enzyme has also been crystallised (Yoshimoto *et al*, 1993) and therefore a knowledge of PAP structure is imminent The strategy used for isolating these genes was to screen bacterial gene libraries for PAP activity in *E coli* This was possible because the host does not exhibit PAP activity Clones were selected using a procedure developed by Mulczyk and Szewczuk (1970) which relies on the enzymatic liberation of ßNA from pGlu-ßNA by "PAP-positive" colonies, with the subsequent conversion of ßNA into a coloured compound (Fig 1 2)

Fig 1 2 *Screening for "PAP-positive" clones*



Characterisation of the aforementioned PAP genes reveals that they have a common structure The size of their open reading frame (ORF) is similar and relatively small The PAP genes from *S pyogenes, B subtilis* and *B amyloliquifaciens* are all 645 nucleotides long, while the gene from *P fluorescens* is 639 nucleotides long These genes encode polypeptides of 215 or 213 amino acids long with deduced molecular masses of 23,135, 23,777, 23,286 and 22,441 daltons respectively The PAP gene also appears to be present as a single copy gene in the bacterial genome (this has been confirmed for *S pyogenes, B subtilis* and *P fluorescens*)

The bacterial PAP genes examined thus far do not show any significant similarity to other known nucleotide sequences, nor do the deduced amino acid sequences compare favourably with protein sequences from other prokaryotic or eukaryotic sources This lack of homology with other proteins, including other proteases, suggests that bacterial PAPs belong to a new and unique class of peptidases Conversely, a comparison of the deduced amino acid sequences of the four cloned bacterial PAPs indicates striking similarities (Gonzales and Robert-Baudouy 1994, Cleuziat *et al*, 1992a, Awade *et*

al , 1994) A multiple alignment of the four amino acid sequences reveals that the primary structure of these enzymes is highly conserved, suggesting that these genes derive from a single common ancestor The two best conserved segments, in which at least 75% of the residues are identical, lie between amino acids 81-100 and 133-145 (numbering is for *P fluorescens* PAP) The second domain contains a uniquely conserved cysteine residue (Cys-144) in the alignment, suggesting that this residue is directly involved in the catalytic site of these thiol-dependent enzymes This hypothesis has subsequently been confirmed by site-directed mutagenesis of the PAP from *B amyloliquifaciens* Changing Cys-144 to Ser resulted in total loss of enzyme activity (Yoshimoto *et al* , 1993) Further sequence analysis has also revealed that His-166 is also perfectly conserved in the four sequences, indicating that this residue may also be essential for enzyme activity or substrate binding (Gonzales and Robert-Baudouy, 1994) An examination of the hydrophobic character of the four sequences, according to the method of Kyte and Doolittle (1982), has also revealed that the charge of these four enzymes is uniformly distributed along the polypeptide chain, with a distinct absence of a long hydrophobic stretch This is consistent with the observation that these enzymes are soluble and are not therefore detected in culture medium (Awade *et al* , 1992b, Cleuziat *et al* , 1992a) This would also suggest that these enzymes lack a post-translational processed signal sequence (Kreil, 1981)

1 2 2 4 Current uses of mammalian PAP-I and microbial PAPs

The absence of an alpha-amino group in pGlu-peptides and proteins was, for many years, a major handicap in the characterisation of these materials, since amino terminal analyses, including stepwise degradation methods (Edman, 1950), could not be carried out Consequently, the initial impetus behind the discovery of PAP (Doolittle and Armentrout, 1968) was to isolate an enzyme activity that would render terminal amino groups accessible in pGlu-terminating peptides In this regard, PAP (typically commercial calf liver PAP-I or a bacterial PAP preparation) has been extremely successful (Podell and Abraham, 1978) Indeed, despite the availability of enzymatic and chemical methods to open pGlu rings (Miyatake *et al* , 1993, Van Der Werf, 1975), and physical methods such as mass spectrometry, which are available to overcome sequencing difficulties due the N-terminal pGlu block (Khandke *et al* , 1989), PAP still remains the de-blocking method of choice for many sequencers (Bieber *et al* , 1990, Lu *et al* , 1991)

The functional usefulness of PAP has been elevated by its application in bacterial diagnosis Although present in some bacterial strains, this enzyme activity is absent in others (Doolittle and Armentrout, 1968) Several bacterial diagnostic techniques have been developed based on the use of chromogenic and fluorogenic substrates which can be specifically hydrolysed by this enzyme such as pGlu-ßNA, pGlu-MCA and pGlu-pNA For example, initial applications of the "PLP" test developed by Mulczyk and Szewczuk (1970, 1972) included differentiation of the *enterobacteriacea* and *staphylococci* species whilst more recently, PAP activity has been exploited in diagnostic tests for the identification of *streptococcal* species (Wellstood, 1987, Panosian and Edberg, 1989, Dealler *et al* , 1989) The recent characterisation of bacterial PAP genes (section 1 2 2 3) may also lead to promising applications in this area Group A *streptococci* for example have already been identified using DNA probes to the

PAP gene (Cleuziat *et al*, 1992b) Using this approach, it may be possible to replace the PAP activity tests with molecular probe tests for a more definitive identification of bacterial species

1 2 3 Class 2 Serum "thyroliberinase" and Mammalian PAP-II

1 2 3 1 Serum "thyroliberinase'

In the late 1970s, an enzyme that cleaved the pGlu-His bond of TRH was partially purified from rat (Taylor and Dixon, 1978) and porcine (Bauer and Nowak, 1979) serum Unlike the previously characterised cytosolic PAP-I activity (see section 1 2 2 1), this enzyme was not inhibited by sulphydryl-blocking reagents such as 2-iodoacetamide and N-ethylmaleimide but could be inhibited by metal chelators such as EDTA and 1,10-phenanthroline This enzyme, optimally active at neutral pH, was reported to have a relative molecular mass of 260,000 daltons, one order of magnitude greater than the molecular mass of mammalian tissue PAP-I In a subsequent study probing the substrate specificity of the serum enzyme, Bauer *et al* (1981) demonstrated that the selectivity of this enzyme was directed towards TRH or closely related peptides Other pGlu substrates such as pGlu-ßNA, LHRH and neurotensin were not cleaved As a consequence of this narrow substrate specificity, the name "thyroliberinase" was subsequently proposed by these researchers

Studies have shown that the activity of this TRH-degrading serum PAP is under the influence of thyroid hormones (Bauer, 1976, White *et al*, 1976, Dupont *et al*, 1976, Emerson and Wu, 1987) and drastically alters with developmental changes (Oliver *et al*, 1974c, Neary *et al*, 1976) This enzyme might therefore be involved within regulatory mechanisms The enzymatic degradation of TRH during its transport by hypophyseal portal blood, from the hypothalamus to the anterior pituitary, might represent a functional control element within the mechanisms regulating the availability of this neuropeptide to the trophic cells of the adenohypophysis This hypothesis is supported by the high degree of substrate specificity exhibited by the enzyme For a more detailed review of the mode of action and regulation of TRH within the hypothalamic-pituitary-thyroid axis, the reader is directed to section 1 3 2

1 2 3 2 Mammalian PAP-II

The earliest indications that a proportion of total mammalian brain PAP activity might possibly be associated with the particulate fraction, stemmed from the work of several researchers (Greaney *et al*, 1980, Hayes *et al*, 1979, Joseph-Bravo *et al*, 1979, Griffiths *et al*, 1979, 1980) Browne *et al* (1981) subsequently proposed that there were two distinct PAP activities in mammalian brain This research group observed, in guinea-pig brain, the previously characterised soluble enzyme (PAP-I, EC 3 4 19 3) which required DTT and EDTA for the expression of optimal activity, as well as a membrane-bound enzyme activity (PAP-II, EC 3 4 19 -) which was inhibited by these reagents These observations were later confirmed by the findings of O Connor and O'Cuinn (1984) who localised this particulate activity to the synaptosomal membrane preparations of guinea-pig brain from which it could be solubilised by papain treatment Soluble and particulate PAP activities have also been demonstrated in rat (Garat *et*

al , 1985, Friedman and Wilk, 1986), rabbit (Wilk and Wilk, 1989) and, quite recently, bovine brain (O'Leary and O'Connor, 1995a), as well as in primary cultures of fetal mouse brain (Cruz *et al* , 1991)

In marked contrast to the thiol-dependent, cytosolic nature of tissue PAP-I, PAP-II has been found to exhibit a large relative molecular mass (approx 230,000 daltons) as well as a sensitivity to inhibition by chelating agents such as 1,10-phenanthroline, 8-hydroxyquinoline and EDTA (O'Connor and O'Cuinn, 1984, Bauer, 1994, Wilk and Wilk, 1989) No sensitivity to sulphydryl-blocking reagents (O'Connor and O'Cuinn, 1984) or to the specific PAP-I inhibitor, pGlu-diazomethyl ketone (Friedman and Wilk, 1986), could be observed Bauer (1994) has also recently demonstrated using SDS PAGE analysis, that PAP-II purified to homogeneity from rat and porcine brain is comprised of two identical subunits of 116,000 daltons each, this dimeric feature being a general property of many cell-surface peptidases The tissue distribution of PAP-II strongly suggests that it is located primarily in the central nervous system with significantly smaller levels of membrane-bound PAP activity observed in other mammalian tissues (Friedman and Wilk, 1986, Vargas *et al* , 1992a) Within the central nervous system, PAP-II appears to have a neuronal, as opposed to a glial, location (Cruz, 1991, Bauer *et al* , 1990) in addition to a relatively unhomogeneous distribution (Vargas *et al* , 1987)

More significant was the finding that PAP-II, like the previously observed serum "thyroliberinase" enzyme activity, has a substrate specificity restricted to TRH or very closely related peptides (O'Connor and O'Cuinn, 1985, Elmore *et al* , 1990, Wilk and Wilk, 1989) O'Connor and O'Cuinn (1985) have demonstrated a K_m of 40μM for PAP-II isolated from the synaptosomal membranes of guinea-pig brain when TRH was used as the substrate LHRH, although not hydrolysed by this enzyme, was found to inhibit the hydrolysis of TRH competitively with a K_i value of 20μM Indeed, PAP-II isolated from this source has been shown to have a substrate specificity restricted to tripeptides, tripeptide amides and tetrapeptides commencing with the sequence pGlu-His (Elmore *et al* , 1990) (it is worth noting however, that Bauer (1994) has recently demonstrated the ability of PAP-II, purified from rat and porcine brain membrane preparations, to hydrolyse pGlu-ßNA, a substrate typically used to assay for PAP-I)

This unprecedented degree of specificity for a particular peptide configuration, combined with the knowledge that (1) the active site of PAP-II appears to be exposed extracellularly (Charli *et al* , 1988), (2) it is primarily located within neuronal elements of the central nervous system, (3) it has a differential distribution within the central nervous system and (4) the inhibition of PAP-II specifically increases recovery of TRH released from rat brain tissue (Charli *et al* , 1989), would serve to indicate that the particulate PAP activity is responsible for "specifically" inactivating neuronally released TRH within the extracellular vicinity of target cells Several studies reporting on the influence of thyroid hormones on PAP-II activity support this observation Suen and Wilk (1989) and Bauer (1987a, 1988) have clearly demonstrated the significantly increased TRH-degrading action of PAP-II in the anterior pituitary following acute treatment with triiodothyronine (T3) whilst Emerson and Wu (1987) and Bauer (1987a) were unable to detect any effect of thyroid hormones on whole brain PAP-II activity Such tissue-specific regulation of adenohypophyseal PAP-II by thyroid hormones suggests that it may

serve an integrative function in modulating the response of adenohypophyseal target cells to TRH by terminating the biological activity of the tripeptide at this location, the increased TRH-degrading activity subsequently contributing to the negative feedback effect of thyroid hormones on the hypothalamic production of TRH (see section 1 3 2) Wilk (1986) has subsequently proposed that the membrane-bound PAP-II be considered the first characterised neuropeptide-specific peptidase

Schauder *et al* (1994) have recently cloned a cDNA encoding for the TRH-degrading PAP-II activity Fragments of this enzyme isolated from rat or pig brain were generated by enzymatic digestion or cyanogen bromide cleavage, purified by reverse-phase HPLC and sequenced Subsequent PCR amplification and screening of cDNA libraries from rat brain and pituitary led to the identification and isolation of a cDNA that encodes a protein of 1025 amino acids Analysis of the deduced amino acid sequence was consistent with the identification of the enzyme as a glycosylated, membrane-anchored Zn metallopeptidase Furthermore, using Northern blot analysis, these researchers demonstrated that mRNA transcript levels in pituitary tissue increased rapidly when the animals were treated with triiodothyronine, confirming the earlier findings of Bauer (1987a) Transient transfection of COS-7 cells with this cDNA led to the expression of an active ectopeptidase that displayed the characteristics of the TRH-degrading ectoenzyme

1 2 4 PAP inhibitors

The rational design of potent and specific peptidase inhibitors (including PAP inhibitors) generally proceeds from a knowledge of the enzymes mechanism of action and from detailed mapping of its substrate specificity This enables structural features to be incorporated into the inhibitor, facilitating its interaction with substrate-binding subsites on the enzyme Such inhibitors may be of potential value in a number of studies For example, (1) they may increase the half-life of-endogenous neuropeptides such as TRH and therefore be of value in exploring the physiological effects of these neuropeptides, (2) they may potentiate the effects of exogenous neuropeptides, (3) they can be used to prevent the degradation of neuropeptides such as TRH in radioligand binding assays and radioimmunoassays and finally (4) they are excellent tools for probing the physiological significance of the targeted enzyme, in this case, PAP

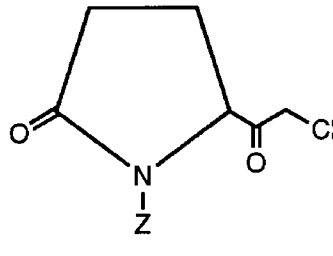
Active site-directed PAP inhibitors were first synthesised by Fujiwara *et al* (1981a, 1981b, 1982) for the *B amyloliquifaciens* PAP These were pGlu-chloromethyl ketone (pGCK), Z-pGlu-chloromethyl ketone (Z-pGCK) and Z-pGlu-diazomethyl ketone (Z-pGDK) The chloromethyl ketone derivatives were found to be highly specific, potent and irreversible inhibitors of this PAP In addition, the rate of PAP inactivation by pGCK was found to be over 10-fold greater than that of Z-pGCK (the lower potency of Z-pGCK can be accounted for by the presence of the Z group on the pGlu ring) Fujiwara *et al* (1981a) have also achieved complete and rapid inactivation of PAP-I from rat liver and kidney *in vitro* with pGCK (more recently, Svoboda and Currie (1992) have reported the irreversible inhibition of calf liver PAP-I by chloromethyl ketone analogues of TRH) Unfortunately, thiol-reducing agents

such as DTT and 2-mercaptoethanol had to be excluded from the assay mixture to avoid their reactivity with these inhibitors, subsequently making accurate kinetic analysis of the inactivation reaction very difficult The diazomethyl ketone derivative was also found to be a specific and irreversible PAP inhibitor, although far less potent than pGCK or Z-pGCK (Fujiwara *et al*, 1982) However, Z-pGDK was found to be relatively inert toward -SH reagents The *in vivo* effects of these inhibitors have yet to be ascertained

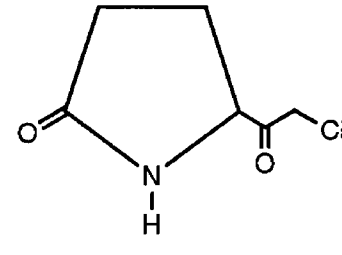
Wilk *et al* (1985) have synthesised the active site-directed inhibitor pGlu-diazomethyl ketone (pGDK or PDMK) PDMK was found to be significantly more inhibitory than its Z-derivative described above Preincubation of partially purified bovine brain or calf liver PAP-I with nanomolar quantities of this compound led to rapid and irreversible inactivation of the enzyme in a time and concentration-dependent manner In addition, inhibitor concentrations five orders of magnitude higher did not inactivate other endo- or exopeptidases tested, including PAP-II (Wilk, 1989), indicating that this inhibitor is highly specific for PAP-I *in vitro* This inhibitor was also found to be extremely effective and long-lasting *in vivo* When administered intraperitoneally to mice, it totally inactivated PAP-I in all tissues studied (brain, heart, muscle, lung, spleen, liver and kidney) at doses as low as 0 1mg/kg with as much as 50% inhibition still observed in most tissues 24 hours after administration Significant inhibition was also observed when the inhibitor dose was decreased to 10µg/kg The chemical structures of the aforementioned chloromethyl ketone and diazomethyl ketone derivatives as well as their Z-counterparts are illustrated in Fig 1 3

Fig 1 3 *Chemical structures of active site-directed PAP inhibitors*

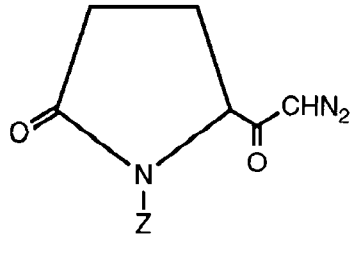
(1) Z-pGlu-chloromethyl-ketone



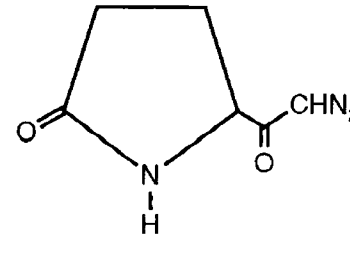
(2) pGlu-chloromethyl-ketone



(3) Z-pGlu-diazomethyl-ketone

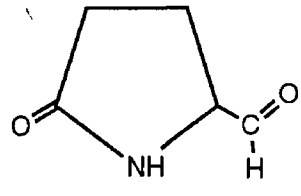


(4) pGlu-diazomethyl-ketone



Peptide aldehyde analogues of the acyl portion of protease substrates have been reported to be potent competitive inhibitors of thiol and serine proteases (Westerik and Wolfenden, 1972, Thompson, 1973) To this end, Friedman *et al* (1985) have synthesised 5-oxoprolinal (Fig 1 4), the aldehyde analogue of pGlu, as an active site-directed transition-state inhibitor of PAP-I This inhibitor was shown to be a potent and specific competitive inhibitor of calf liver PAP-I *in vitro* (K_I = 26nM) 5-oxoprolinal however, is far less effective *in vivo* Intraperitoneal injection into mice at a dose of 50mg/kg resulted in greater than 60% inhibition of enzymatic activity in all organs tested 10 minutes after inhibitor injection After 30 minutes however, the degree of inhibition had significantly decreased This relatively weak and transient action *in vivo* contrasts with PDMK examined above, and may possibly be accounted for by the reversible nature of enzyme-inhibitor binding and/or metabolic inactivation of the inhibitor

Fig 1 4 Chemical structure of 5-oxoprolinal

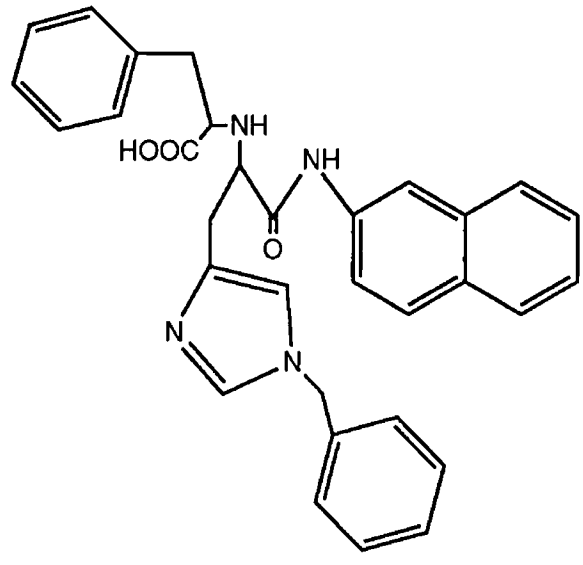


PAP-II has been classified as a TRH-specific metalloenzyme (see section 1 2 3) This suggests that an inhibitor should incorporate the structural features of TRH together with a group capable of chelating the active site metal ion Reductive amination of peptides with an alpha-keto acid has proved to be a fruitful approach to the synthesis of active site-directed inhibitors of metalloenzymes The most prominent of such compounds is enalpril, the inhibitor of angiotensin-converting enzyme (Patchett *et al*, 1980) Charli *et al* (1989) have recently synthesised such an inhibitor via the reductive amination of His(N^{im}benzyl)-NA with phenylpyruvate to yield N-[1(R,S)-carboxy-2-phenylethyl]-N-imidazole benzyl-histidyl ß-naphthylamide or CPHNA (Fig 1 5) A pyroglutamyl-containing keto acid would obviously have presented more optimal binding features Unfortunately, the aforementioned research group were unable to synthesise this intermediate Irrespective of this, CPHNA has proved to be a potent, reversible inhibitor of purified PAP-II with a K_I of 8µM

Other specific PAP inhibitors worthy of mention include benarthin, pyrizinostatin and 2-pyrrolidone (Fig 1 6) The former two inhibitors, benarthin and pyrizinostatin, have only recently been isolated from culture filtrates of the genus *Streptomyces* and represent a new structural class of PAP inhibitors (Aoyagi *et al*, 1992a, 1992b, Hatsu *et al*, 1992a, 1992b) More recently, pyrizinostatin has been synthesised from the antibiotic, 2-methylfervenulone (Tatsuta and Kitagawa, 1994) The latter compound, 2-pyrrolidone, is a pyroglutamyl substrate analogue which acts as a reversible, non-competitive inhibitor of mammalian PAP-I and microbial PAPs Despite being a specific and potent inhibitor of PAP activity however, 100% inhibition of PAP activity has never been obtained with this

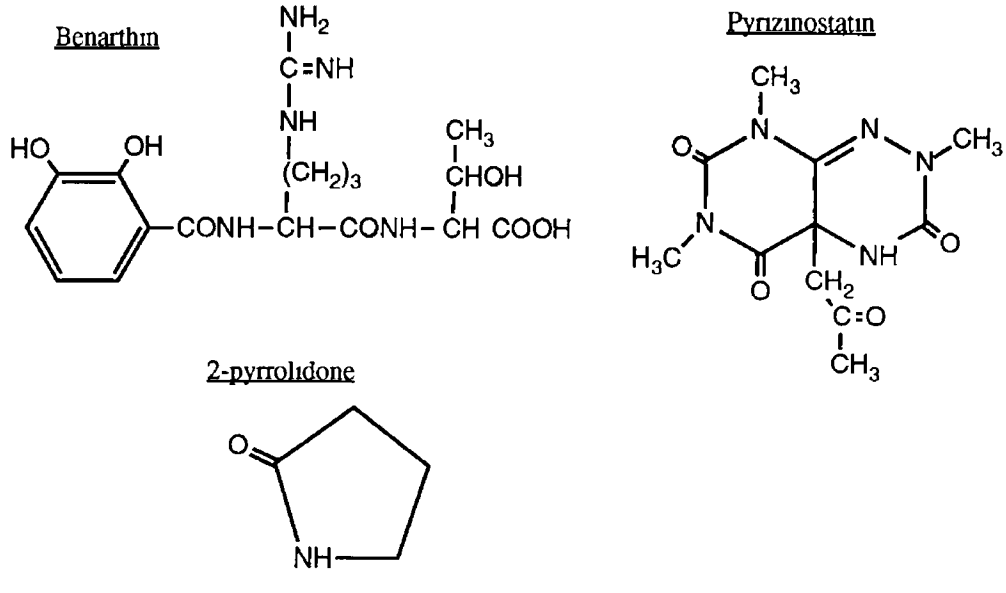
inhibitor, even at concentrations as high as 0 1M In addition, several studies have reported on the use of 2-pyrrolidone to stabilise PAP activity in solution during purification and storage (Mudge and Fellows, 1973, Armentrout and Doolittle, 1969, Armentrout, 1969)

Fig 1 5 *Chemical structure of CPHNA*



N-[1(R,S)-carboxy-2-phenylethyl]-N-imidazole benzyl-histidyl ß-naphthylamide

Fig 1 6 *The chemical structures of benarthin, pyrizmostatin and 2-pyrrolidone*



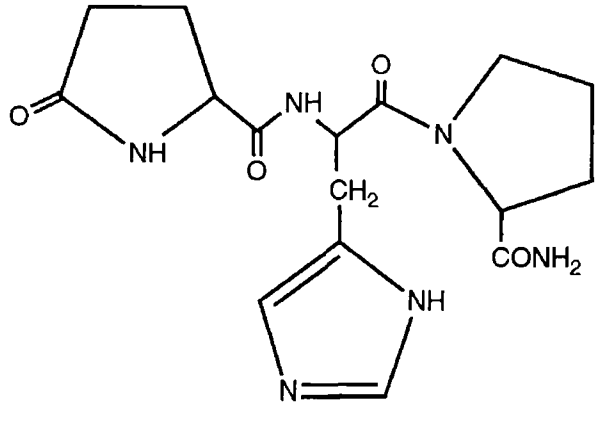
1.3 Thyrotropin Releasing Hormone

The modern era of neuroendocrinology was ushered in just over 25 years ago with the isolation and characterisation from more than 100,000 porcine (Boler *et al*, 1969) and ovine (Burgus *et al*, 1969) hypothalamic tissue fragments, of a tripeptide amide (pyroglutamyl-histidyl-proline-amide) which was designated thyrotropin-releasing hormone (TRH, TRF, thyroliberin) by virtue of its capacity to stimulate the release of thyroid-stimulating hormone (TSH, thyrotropin) from the mammalian anterior pituitary Indeed the existence of this neuropeptide was first demonstrated several years prior to this (Guillemin *et al*, 1962) and since this time, the importance of this hypothalamic releasing factor in the regulation of the hypothalamic-pituitary-thyroid axis in human beings and other mammals has been well established (Reichlin *et al*, 1978)

1 3 1 Structure of TRH

Following the exhaustive purification of TRH from porcine hypothalami by Schally *et al* (1966, 1968, 1969), degradation of the hormone by acid hydrolysis yielded three amino acids, histidine, glutamic acid and proline, present in essentially equimolar amounts, suggesting a tripeptide structure Studies with synthetic Glu-His-Pro, as well as several alternative sequences of the three amino acids showed no hormonal activity of TRH (Schally *et al*, 1968) Since it was known that TRH did not have a free amino or carboxyl group, synthetic experiments were carried out on Glu-His-Pro, the most probable moiety of TRH (Schally *et al*, 1969), to modify both the amino and carboxyl groups It was discovered by Folkers *et al* (1969) that a synthetic preparation, presumably pGlu-His-Pro-NH_2, resulting from the methylation and ammonation of the Glu-His-Pro tripeptide, exhibited hormonal activities, at nanogram-dose levels *in vivo* and at picogram levels *in vitro*, which were qualitatively indistinguishable from those of TRH This structure was subsequently verified by nuclear magnetic resonance and a range of chromatographic techniques (Boler *et al*, 1969) and can be seen in Fig 1 7

Fig 1 7 The chemical structure of TRH



(pGlu - His - ProNH$_2$)

A separate research group, reported the purification and characterisation of hypothalamic TRH of ovine origin (Burgus *et al*, 1969) and also subsequently reported its molecular structure as being established as pGlu-His-Pro-NH_2 (Burgus *et al* 1970) Indeed this structural interpretation of TRH has since been found to be applicable to all mammalian species studied to date This research group have also reported on the strict conformational requirements of TRH for biological activity and that almost any departure from the native structure of TRH results in substantial if not complete loss of biological activity (Guillemin and Burgus, 1972)

1 3 2 Neuroendocrine role of TRH

The nervous and endocrine systems constitute the two main communication systems of the body, functioning in a closely co-ordinated manner such that each is dependent on the other for its proper operation The total behaviour of an organism is therefore integrated by a constant traffic of neural and hormonal signals which are received and responded to by appropriate tissues Moreover, the activities of the CNS and endocrine glands are themselves dependent on feedback control through neural and hormonal stimuli The ensuing sections will attempt to illustrate the significance of TRH within this neural and hormonal framework

1 3 2 1 The hypothalamic-pituitary-thyroid axis A neuroendocrine pathway for TRH

The central regulation of hormonal release from the anterior pituitary (adenohypophysis) is mediated by the hypothalamus The lack of nerve fibres connecting the hypothalamus to the secretory cells of the adenohypophysis meant however that, until the early 1970s, the means whereby the hypothalamus influenced the secretory cells remained a mystery It is now known that communication between the two is through a specialised vascular system called the hypophyseal portal system This blood supply runs from the median eminence of the ventral hypothalamus down the pituitary stalk to the capillaries of the adenohypophysis Hypophysiotrophic peptide hormones (releasing and release-inhibiting factors) are secreted into this portal system by hypothalamic neurons present in the arcuate and other nuclei of the median eminence (ME) in response to hormonal and neural stimuli These hormones are then carried in the blood stream of the portal system to the cells of the adenohypophysis where they act on their target cells to stimulate or inhibit the release of adenohypophyseal hormones This neuroendocrine route is referred to as the hypothalamic-pituitary axis and is one of many pathways which interface endocrine and neural events Table 1 3 lists the hypothalamic "releasing" hormones and their corresponding effects on the anterior pituitary (TRH and its effects are highlighted in bold face)

Table 1 3 Hypothalamic releasing hormones

Hypothalamic regulatory hormone	Released pituitary hormone	Main functions
Thyrotropin releasing hormone (TRH)	**Thyrotropin (TSH)**	**Maintains the thyroid gland and stimulates the synthesis and release of thyroid hormones**
	Prolactin *	**Promotes mammary development and lactation**
Corticotrophin releasing hormone (CRH)	Corticotrophin (ACTH)	Maintains the adrenal cortex and stimulates the synthesis and release of adrenocortical hormones, especially hydrocortisone
Follicle stimulating hormone-releasing hormone (FSH-RH)	Follicle stimulating hormone (FSH)	Stimulates the growth of the ovum in the female and the sperm in the male, acts with LH to stimulate the release of oestrogen
Luteinizing hormone releasing hormone (LHRH)	Luteinizing hormone (LH)	Stimulates the development of the corpus luteum, and acts with FSH to cause the release of progesterone, stimulates the release of testosterone in the male
Growth hormone releasing hormone (GHRH)	Growth hormone (GH)	Increases rate of growth of young animals, increases protein synthesis, increases blood glucose concentration, mobilizes free fatty acids from adipose tissue
Melanocyte stimulating hormone-releasing hormone (MSH-RH)	Melanocyte stimulating hormone (MSH)	Stimulates melanin synthesis by melanocytes

* At the hypothalamic level, the secretion of prolactin in mammals is also controlled by prolactin release-inhibiting hormone (PR-IH) Other release-inhibiting hormones not mentioned in the above table are growth hormone release-inhibiting hormone (GHR-IH or somatostatin) and melanocyte stimulating hormone release-inhibiting hormone (MSHR-IH)

TRH released into the hypophyseal portal system acts on the thyrotroph cells of the adenohypophysis causing the secretion of TSH (thyrotropin) In turn, TSH acts on the thyroid gland to stimulate the release of thyroid hormones (Fig 1 8 - the hypothalamic-pituitary-thyroid axis) Only TRH synthesised in the hypothalamic paraventricular nucleus (PVN) and transported to the median eminence can regulate adenohypophyseal secretion of TSH (Nikodemova and Strbak, 1995) At the level of the thyroid gland itself, it has been shown that TRH can directly stimulate the release of T4 (tetraiodothyronine or thyroxine) from perfused rat thyroid gland fragments *in vitro* (Attali *et al*, 1984) adding another complexity to the control of thyroid hormone secretion

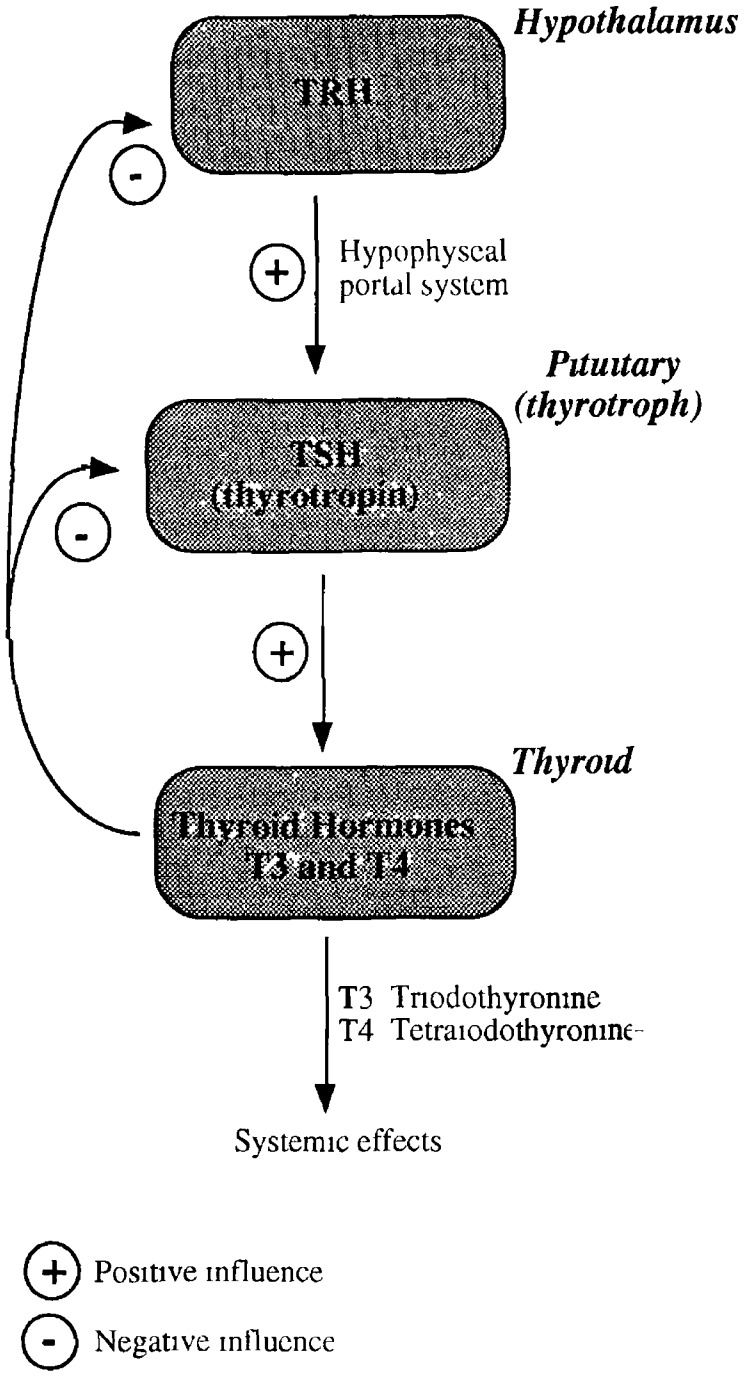
A parallel neuroendocrine role for TRH in the release of prolactin from anterior pituitary lactotrophs has also been convincingly demonstrated The TRH-induced release of prolactin has been observed both *in vivo* and *in vitro* in mammals including man (Hall, 1984) Roles for tyrosine kinase (Kanda *et al*, 1994), Ca^{2+} ions (Guerineau *et al*, 1995) and gonadal steroid hormones (Hu and Lawson, 1995) have also been implicated in this TRH-related process It is widely believed however, that the overall role of TRH in the regulation of prolactin secretion is a minor one (Gautvik *et al*, 1974, Harris *et al*, 1978)

1 3 2 2 TRH receptors

The action of TRH on target cells, such as those of the anterior pituitary, is mediated through its interaction with highly specific membrane-bound receptors Recent cloning and sequencing of the TRH receptor (TRH-R) from anterior pituitary cells of different species has revealed that it is a member of the seven-transmembrane-spanning, GTP-binding protein-coupled family of receptors or GPCRs The mouse pituitary TRH-R mRNA encodes for a 393 amino acid GPCR (Straub *et al*, 1990) which bears significant homology with sequences observed for the cloned TRH-R from humans (Duthie *et al*, 1993, Matre *et al*, 1993, 398 amino acids) and rats (de la Pena *et al*, 1992a and 1992b, 412 and 387 amino acids - two isoforms of this receptor, generated by alternative splicing, have been cloned and sequenced) The major sequence variation between species and isoforms was seen to occur within the intracellular carboxy-terminal tail of the TRH-R (Morrison *et al*, 1994), a portion of the receptor sequence known to contain several serine and threonine residues which are potential sites for regulatory phosphorylation by kinase enzymes, including protein kinase C (Kikkawa *et al*, 1989)

By activating a phospholipase C/inositol-1,4,5-triphosphate/Ca^{2+}/1,2-diacylglycerol signal transduction pathway, the TRH/TRH-R binding event is transduced into an intracellular chemical signal (Gershengorn, 1986, Drummond, 1986) The TRH-R is, in turn, coupled to phospholipase C via a guanine nucleotide-binding (G) protein (Oron *et al*, 1987a, 1987b, de la Pena *et al*, 1995) The binding of TRH to the TRH-R results in the G-protein-mediated activation of phospholipase C The "activated" enzyme subsequently hydrolyses phosphatidylinositol 4,5-biphosphate to yield 1,2-diacylglycerol (DG) and inositoltriphosphate ($InsP_3$), both DG and InsP3 serving as second messengers which transduce and amplify the binding signal, leading to stimulation of the physiological response (i e the release of TSH and prolactin)

Fig 1 8 The hypothalamic-pituitary-thyroid (HPT) axis



1.3 2 3 Regulation of TRH synthesis, secretion and actions

Hypothalamic neurons that secrete pituitary releasing or release-inhibiting hormones are in turn regulated by a combination of "classic" monoamine neurotransmitters (Wurtman, 1971) and other hormonal factors, a situation which applies to the regulation of TRH synthesis secretion and actions Norepinephrine (Grimm-Jorgensen and Reichlin, 1973, Hirooka *et al*, 1978), dopamine (Maeda and Frohman, 1980) and histamine (Joseph-Bravo *et al*, 1979) have each been reported to stimulate TRH release from hypothalamic tissues *in vitro* whilst Mannisto (1983) also reports that gamma-

ammobutyric acid (GABA) and serotonin (5-HT) may potentially influence the hypothalamic release of TRH Hormonal factors such as somatostatin (GHR-IH, see Table 1 3), growth hormone (GH), gonadal steroids and adrenocortical hormones have also been implicated in modulating TSH response to TRH (Edwardson and Bennett, 1977, Jackson, 1982, Manmsto, 1983, Huang *et al*, 1995, Vanhaasteren, G A C *et al*, 1995, Wang *et al*, 1994)

Thyroid hormones play a pivotal role in the maintenance of homeostatic mechanisms such as the hypothalamic-pituitary-thyroid axis It is well known that TSH synthesis by anterior pituitary thyrotrophs is directly regulated by thyroid hormones in a negative fashion (Hershman and Pekary, 1985, Morley, 1981) Using a mouse thyrotrophic tumour model *in vivo* and *in vitro*, Shupnik *et al* (1983, 1985) have demonstrated that thyroid hormone administration markedly and rapidly down-regulates TSH subunit gene expression at the transcriptional level

In contrast, the effect of thyroid hormones on the synthesis and secretion of hypothalamic TRH has been the subject of much controversy, with many conflicting reports as to whether or not they exert feedback inhibition at the hypothalamic level However, the advent of molecular approaches enabling researchers to assess gene activity, and the development of more sensitive techniques for direct monitoring of hypothalamic neurosecretory substances in hypophyseal portal blood (the portal blood collection technique - Caraty *et al*, 1994, Dahl *et al*, 1994, Thomas *et al*, 1988), subsequently enabled researchers to demonstrate convincingly that thyroid hormones exert negative feedback control on hypothalamic TRH neurons with respect to the biosynthesis and secretion of TRH and TRH precursor forms More recently, Hollenberg *et al*, 1995 have demonstrated that thyroid hormone, T3, can directly down-regulate TRH synthesis in the paraventricular nucleus of the human hypothalamus at the transcriptional level in a manner virtually identical to the down-regulation seen earlier for the TSH subunit genes in anterior pituitary thyrotrophs (Shupnik *et al*, 1986) Interestingly, this regulatory effect is specific for TRH-producing neurons located in the medial division of the paraventricular nucleus (PVN), whereas TRH neurons elsewhere in the hypothalamus or in extrahypothalamic locations are not affected by changes of thyroid status (Segerson *et al*, 1987, Dyess *et al*, 1988, Kakucska *et al*, 1992, Bruhn *et al*, 1991, Liao *et al*, 1989) This raises the possibility that thyroid hormones may differentially regulate TRH gene expression in various anatomical locations (Jackson *et al*, 1990)

Researchers have also examined the ability of thyroid hormones to regulate the TSH-releasing capacity of hypothalamic TRH in ways other than a change in its rate of secretion and/or synthesis Numerous reports have indicated that thyroid hormones can down-regulate the number of pituitary receptors for TRH (Hinkle *et al*, 1981, Gershengorn, 1978) and enhance its rate of enzymatic degradation in portal blood (Jackson *et al*, 1979) (see section 1 2 3 1)

1.3.3 Neuroregulatory role of TRH

TRH was originally classified as a hormone through its hypophysiotropic effects on the anterior pituitary This neuropeptide is however, widely distributed throughout the mammalian extrahypothalamic brain Indeed, over 70% of total brain TRH is located outside the hypothalamic-pituitary region, although concentrations are lower than those within the hypothalamus (Jackson and Reichlin, 1979) TRH has been observed in the brain stem and spinal cord (Johansson and Hokfelt, 1980), medulla oblongata (Hokfelt *et al*, 1980), cerebellum (Winters *et al*, 1974), thalamus and cerebral cortex (Koch and Okon, 1979) as well as in cerebrospinal fluid (Oliver *et al*, 1974a) Numerous studies have also reported the presence of TRH (or TRH-like immunoreactivity) in non-neural tissues such as retina (Martino *et al*, 1980), blood plasma and urine (Oliver *et al*, 1974b), pancreas (Engler *et al*, 1981), gastrointestinal tract (Furukawa *et al*, 1980) placenta (Shambaugh *et al*, 1979) and amniotic fluid (Morley *et al*, 1979)

As a consequence of this and other factors, many researchers now feel that TRH qualifies for serious consideration as a neurotransmitter/neuromodulator Its extrahypothalamic distribution in the brain combined with its localisation at the synaptic level, release at synaptic terminals, attachment to high-affinity receptors which show a remarkable degree of anatomical localisation, specific effects on neuronal activity, its stimulation of a wide range of centrally-mediated behavioural effects and the existence of brain peptidases capable of inactivating the tripeptide provide a formidable list of criteria consistent with such a neuroregulatory function (Jackson, 1982, Griffiths and Bennett, 1983)

The direct application of TRH to the brain has revealed many of its behavioural effects For example, the tripeptide can (1) stimulate locomotor activity in rats (Heal *et al*, 1983), (2) induce shaking behaviour, sometimes referred to as "wet dog shaking" seen in opiate withdrawal (Griffiths *et al*, 1982), (3) reverse narcotic-induced sedation via neuronal activation (Kalivas and Horita, 1983) and (4) antagonise muscular relaxation caused by neurotensin, suggesting a role in the control of skeletal muscle tone (Griffiths *et al*, 1983a) The central administration of TRH has been shown to have profound effects on the cardiovascular and respiratory systems such as increased blood pressure (Nemeroff *et al*, 1984) and respiratory rate (Hedner *et al*, 1983) TRH administered centrally will induce gastrointestinal motility and increase gastric secretion (Nemeroff *et al*, 1984) TRH has also been shown to potentiate the excitatory actions of acetylcholine on cerebral cortical neurones (Yarborough, 1976) and to enhance cerebral noradrenaline turnover (Keller *et al*, 1974) A role as a possible mediator of thermoregulation has also been implicated for this neuropeptide by a number of researchers (Metcalf, 1974, Prasad *et al*, 1980) In all of these actions, there is an apparent interaction with a variety of classical neurotransmitters and other neuropeptides The complexity of these interactions however, makes the definition of the exact physiological significance of TRH in the extrahypothalamic brain more difficult For a more complete review of this topic, the reader is directed to Griffiths (1985) and Morley (1979)

1 3 4 Biosynthesis of TRH

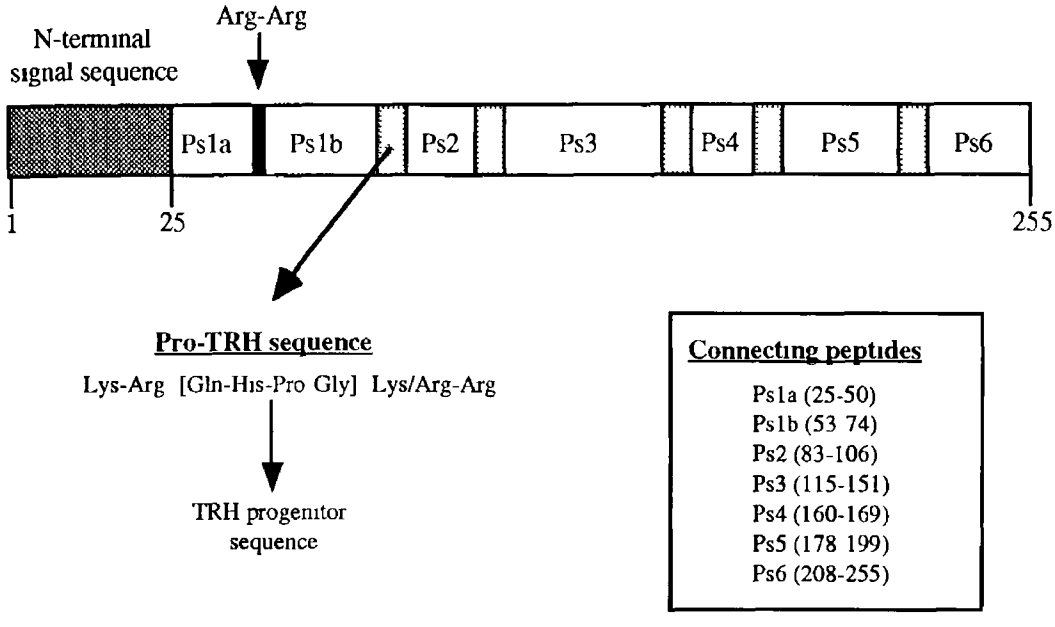
The mechanism of releasing hormone biosynthesis is of fundamental importance, since knowledge of this process must underlie any approach to rational studies on the regulation of synthesis An earlier review of hypothalamic peptide biosynthesis by McKelvy (1977) suggested three possible mechanisms for peptide bond formation for the hypophysiotrophic hormones (1) Ribosomal synthesis, either of the biologically active peptides themselves or of precursor polypeptides which are processed to yield the biologically active forms, (2) Synthesis via an RNA-independent protein template mechanism, a reasonably attractive possibility for TRH biosynthesis but, much less so for the larger peptide hormones such as LHRH and (3) Enzymatic synthesis by an RNA-dependent, ribosome-independent mechanism in which amino acid activation occurs via aminoacyl RNA species

Early studies of TRH biosynthesis focused on the possible existence of a non-ribosomal biosynthetic mechanism (Mitnick and Reichlin, 1971, 1972, Guillemin, 1971, McKelvy *et al*, 1975, Bauer and Lipmann, 1976) However, no conclusive evidence was found to verify this possibility It was not until 1979 that Rupnow *et al* confirmed that TRH arises from the post-translational cleavage of a large precursor protein and not by soluble, non-ribosomal enzymatic mechanisms (Rupnow *et al* 1979a) Indeed studies of peptide hormone biogenesis in higher animals have demonstrated that most peptide hormones are formed in this manner Notable examples include vasopressin (Sachs, 1969), angiotensin (Page and Bumpus, 1961), insulin (Steiner *et al*, 1969), gastrin (Yalow and Berson, 1971) and the hypothalamic hypophysiotrophic hormone LHRH (Seeburg and Adelman, 1984)

Molecular biological studies of the gene encoding the TRH prohormone were initiated by Richter *et al* (1984) This research group isolated messenger RNA from the skin of the frog *Xenopus laevis* (a tissue known to contain a great deal of TRH) and subsequently obtained a cDNA clone with an insert of 478 nucleotides coding for a portion of the preprohormone precursor of TRH (prepro-TRH) The deduced TRH precursor of 123 amino acids contained three copies of the sequence Lys-Arg-Gln-His-Pro-Gly-Lys/Arg-Arg and a fourth incomplete copy The paired basic amino acid residues flanking the TRH progenitor sequence, Gln-His-Pro-Gly, are potential cleavage sites in peptide biosynthesis (Docherty and Steiner, 1982) Subsequent studies (Kuchler *et al*, 1990) revealed that the entire prepro-TRH gene from this source encodes for a precursor polypeptide (prepro-TRH) containing seven TRH progenitor sequences (pro-TRH sequences) flanked by paired basic residues which act as prohormone processing signals The mature TRH tripeptide, pGlu-His-Pro-NH_2, is formed by excising the progenitor sequence at the paired basic residues (Griffiths *et al*, 1983b), trimming the basic residues with a carboxypeptidase B-like enzyme (Gainer *et al*, 1985), cyclizing the amino-terminal glutamine residue to pyroglutamic acid with a glutamine cyclase-like enzyme (Busby *et al*, 1987, Fischer and Speiss, 1987) and amidating the carboxy terminal proline residue (Bradbury *et al*, 1982, Eipper and Mains 1988) This final step utilises the glycine residue as an amide donor and is catalysed by the enzyme peptidylglycine alpha-amidating monooxygenase or PAM (Eipper and Mains, 1988)

The cloned cDNA of a TRH precursor has more recently been isolated from the brain of *Xenopus laevis* which differs from the skin prepro-TRH by approximately 16% (Bulant *et al*, 1992a) The cDNAs encoding the prepro-TRH precursors from the hypothalamus of rats (Lechan *et al*, 1986), humans (Yamada *et al*, 1990) and mice (Satoh *et al*, 1992) have also been sequenced and have demonstrated a significant degree of homology Fig 1 9 describes the predicted structure of the TRH preprohormone precursor from rat hypothalamus (approximately 30,000 daltons)

Fig 1 9 Structure of prepro-TRH from rat hypothalamus

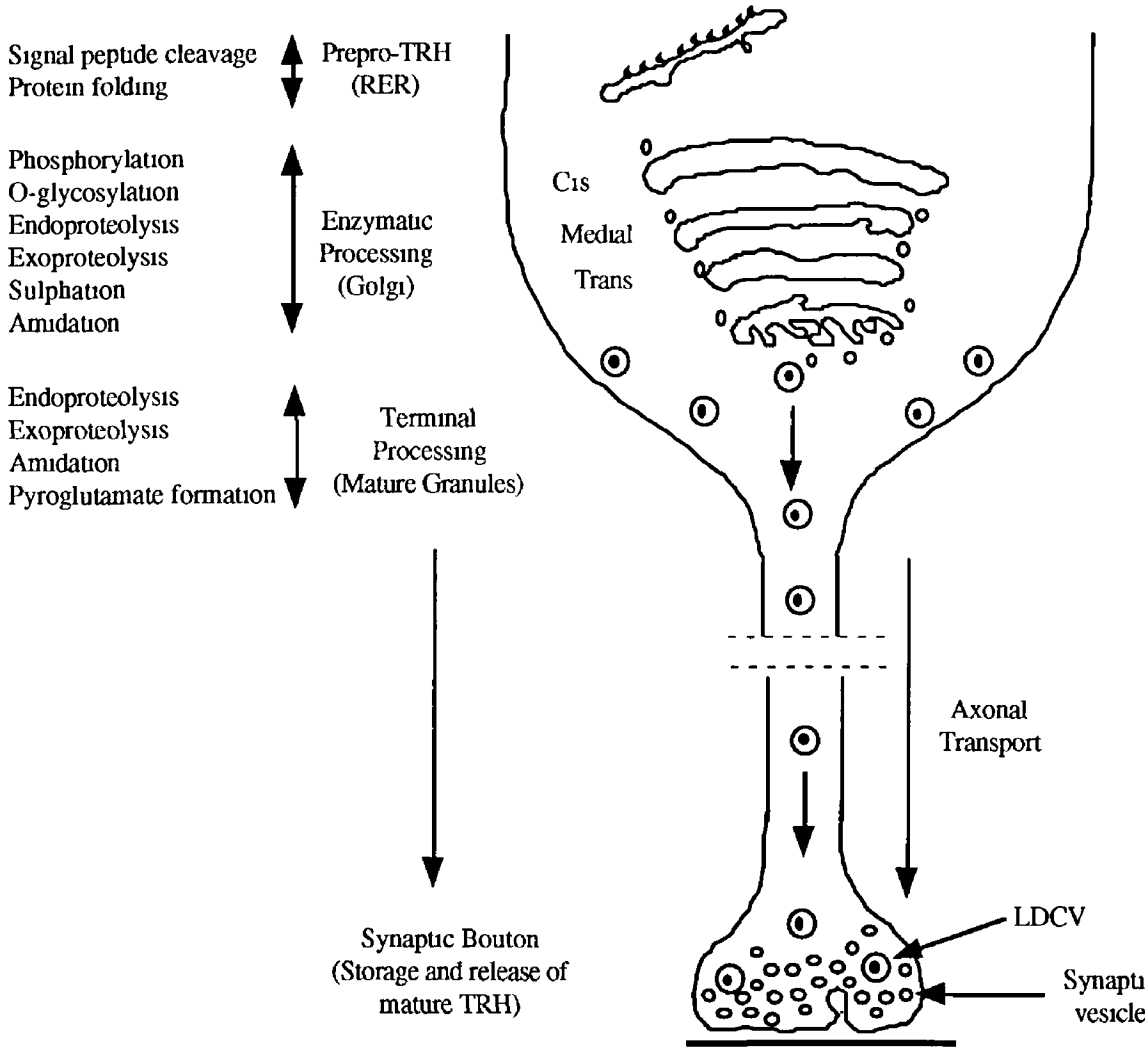


1 3 4 1 TRH precursor processing and secretion

TRH is expressed at early postmitotic stages of hypothalamic neuron development in the mouse and rat, as revealed by the presence of the mature tripeptide, of prepro-TRH mRNAs, and of pro-TRH precursor forms (Tixier-Vidal and Faivre-Bauman, 1992) This indicates a co-ordinate expression of several genes encoding, respectively, prepro-TRH, its processing enzymes, and the cell machinery necessary for intracellular transport, sorting and release of mature TRH Considerable effort has been devoted to elucidating the post-translational processing pattern of the TRH precursor molecule in various regions of the brain (and in peripheral tissues) Mains *et al* (1990) have suggested that the maturation of prepro-TRH *(i e prepro-TRH to pro-TRH to mature TRH)* consists of a cascade of chemical and enzymatic modifications that occur sequentially during transit of the precursor molecule, following cleavage of the signal sequence, from the rough endoplasmic reticulum to mature secretory granules via the golgi apparatus Fig 1 10, proposed by the aforementioned research group, describes a tentative model of prepro-TRH maturation in hypothalamic neurons in which large dense core vesicles (that is, secretory granules) are the site of terminal maturation of precursor forms to TRH The subsequent co-release of mature TRH and prohormone related peptides at the synapse has been shown

to involve the activation of voltage-sensitive Ca^{2+} channels and protein kinase C (Loudes *et al*, 1988, Ladram *et al*, 1994)

Fig 1 10 Processing of the prepro-TRH gene product to mature TRH



The above diagram describes the steps involved in prepro-TRH processing in the successive compartments of the secretory pathway in hypothalamic neurons (borrowed from Tixier-Vidal and Faivre-Bauman (1992) and inspired by Mains *et al* (1990)) The maturation of TRH is a sequential phenomenon starting in the rough endoplasmic reticulum (RER) after cleavage of the signal sequence Then, pro-TRH travels vectorially in the successive compartments (*Cis*, *Medial*, and *Trans*) of the Golgi complex, where it undergoes enzymatic modifications such as those listed in the above schematic The terminal step, that is, amidation, is believed to begin in the *Trans* Golgi compartment upon sorting of secretory granules (i e large dense-core vesicles or LDCVs) At the exit from this compartment, partially or completely processed pro-TRH would become associated with secretogrannin II in the dense matrix of the LDCV In embryonic neurons the final maturation continues during anterograde transport of LDCVs in neurites In mature neurons, the maturation is completed at the exit from perikaryon of LDCV-containing mature TRH

The most complete studies of prepro-TRH processing have, to date, been carried out on rat hypothalamus From Fig 1 9, proteolytic processing of rat prepro-TRH would be expected to produce both TRH and several other non-TRH peptides These peptides should correspond to pro-TRH peptide sequences, to connecting sequences (Ps1-6) and to various C-terminally and/or N-terminally extended forms of TRH produced by incomplete processing of the precursor molecule Antibodies have been raised against synthetic peptides corresponding to the connecting sequences of the rat precursor molecule and used to obtain a more precise picture of prepro-TRH processing in different neural tissues The extraction and chromatographic separation of immunoreactive material has demonstrated the presence of prepro-$TRH_{25\ 50}$ (Ps1a), prepro-$TRH_{115\ 152}$ (Ps3), prepro-$TRH_{160\ 169}$ (Ps4), prepro-$TRH_{178\ 199}$ (Ps5) and prepro-$TRH_{208\ 255}$ (Ps6) (Wu *et al*, 1987, Wu and Jackson, 1988, Bulant *et al*, 1988, Gkonos *et al*, 1989) These data provide convincing evidence that the major pathway of prepro-TRH processing in the hypothalamus involves nearly complete proteolytic cleavage at all the pairs of basic residues which flank the TRH progenitor sequences However some studies have shown the presence of incompletely processed portions of the prepro-TRH molecule in the hypothalamus Peptides extended at the N- and C-terminal of the TRH progenitor sequence were seen to occur in bovine hypothalamus (Cockle and Smyth, 1986) whilst in rat hypothalamus, only the C-terminally extended forms of the TRH progenitor sequence were observed (Cockle and Smyth, 1987) Bulant *et al* (1988) have also detected two of the connecting sequences, Ps4 and Ps5 in spinal cord extracts in equimolar amounts and have suggested that, in addition to TRH, they are the main storage forms of TRH precursor-related peptides in this tissue

The biological significance of these connecting peptides and TRH-extended peptides is currently a subject of intense investigation Recently, one of the connecting peptides produced *in vivo* has been chemically characterised This peptide was purified from bovine hypothalamus using a radioimmunoassay directed against Ps4 The amino acid sequence obtained for the decapeptide was Ser-Phe-Pro-Trp-Met-Glu-Ser-Asp-Val-Thr This represents the first direct chemical evidence for non-TRH peptides originating from the TRH precursor *in vivo* (Bulant *et al*, 1992b) Studies indicate that this connecting peptide can potentiate the neuroendocrine action of TRH on thyrotroph cells of the anterior pituitary, *in vitro* and *in vivo*, to cause the release of TSH (Bulant *et al*, 1990, Carr *et al*, 1992) Ps4, like TRH, interacts with specific pituitary cell receptors (distinct from TRH receptors), coupled to a Ca^{2+} channel mechanism (Roussel *et al*, 1991), causing dose-dependent increases in the steady-state levels of mRNAs of TSH and prolactin through stimulation of the respective promoter activities However, unlike TRH, Ps4 alone has no significant effect on TSH or prolactin secretion On the basis of this evidence, it would appear that TRH and Ps4, two peptides which originate from a single multifunctional biosynthetic precursor, can function on the same target tissue (the anterior pituitary) in a synergistic manner to promote hormonal secretion, suggesting that differential processing of the TRH precursor molecule may have the potential to modulate the biological activities of TRH

1 3.5 Inactivation of TRH

The controlled inactivation of neuropeptides almost certainly represents an important regulatory mechanism within homeostatic adaptation processes Degradative enzymes are believed to play a very important role in controlling neuropeptide action, initially by regulating the amount of neuropeptide available for release at its site of production or the amount actually reaching its site of action, then by controlling the duration of action through neuropeptide inactivation at the receptor sites in a particular target tissue, and finally, by removal of the neuropeptide from the general circulation so that an excessive response to this compound is prevented The neuropeptide can be transformed from its active form to either another bioactive compound or to inactive metabolites When applied to TRH these concepts suggest several potential locations for the enzymic inactivation of this tripeptide (hypothalamic, extra-hypothalamic, extra-CNS and blood) TRH-degrading peptidases would also be expected to show some degree of specificity, this specificity also being inferred from the unique structural features of TRH such as the blocked N- and C-termini combined with an internal proline residue

The rapid inactivation of TRH by blood and tissue enzymes was first observed prior to the elucidation of its chemical structure (Bowers *et al*, 1966, Guillemin, 1967, Redding and Schally, 1969) Much effort has since been devoted to developing a more complete understanding of these enzymic inactivation mechanisms TRH inactivation by the hypothalamus (its principle site of biosynthesis and release) was first observed by Bauer *et al* (1973) Several workers have since examined the degradation of TRH by hypothalamic and adenohypophyseal tissue extracts derived from different mammalian species (Griffiths *et al*, 1980, Bauer and Kleinkauf, 1980, Faivre-Bauman *et al*, 1981, Prasad and Peterkofsky, 1976, Fellows and Mudge, 1971b, Vargas *et al*, 1992b, Aratan-Spire *et al*, 1983) with a view to delineating the enzymic mechanisms responsible for modulating TRH neuroendocrine activity within the confines of the hypothalamic-pituitary-thyroid axis
The observation of TRH in extrahypothalamic brain as well as other mammalian organs suggests a potential neuroregulatory capacity for this tripeptide (see section 1 3 3) which one would expect to be coupled to specific peptidase inactivation mechanisms TRH-degrading enzymes have subsequently been reported in numerous tissues including whole brain (Hayes *et al*, 1979, Browne and O'Cuinn, 1983a, Garat *et al*, 1985, Prasad *et al* 1982a, Koivusalo, 1980), pancreas (Aratan-Spire *et al*, 1986, Koivusalo, 1980), liver (Scharfmann and Aratan-Spire, 1991, Aratan-Spire *et al*, 1983), cerebrospinal fluid (Prasad and Jayarman, 1986) and serum (Bauer and Nowak, 1979, Bauer *et al*, 1979, Aratan-Spire *et al*, 1983)

Based on the identification of its metabolic end products and by the demonstration that the individual enzymatic reactions can be preferentially blocked by specific enzyme inhibitors, workers have demonstrated that the fragmentation of TRH to free ammo acids by neural and non-neural tissue extracts involves the participation of several different enzyme activities (Browne and O'Cuinn, 1983a, Bauer and Kleinkauf 1980, Aratan-Spire *et al* 1986) TRH inactivation is known to occur in two

stages, primary catabolism in which neuropeptide breakdown is initiated by one of two different enzymes which are capable of hydrolysing the N- and C-terminal bonds of the tripeptide respectively, and secondary catabolism which involves the catabolism of peptide fragments resulting from the primary step by a number of different enzymic activities Fig 1 12 examines the different catabolic pathways which have been elucidated for TRH in both soluble and particulate tissue extracts, whilst the reader is directed to O'Cuinn *et al* (1990) and Bauer (1987b) for a more thorough review of this topic

1 3 5 1 Primary and secondary catabolism of TRH by soluble enzymes

Two soluble enzymes have been described which introduce primary cleavages into the TRH molecule, pyroglutamyl aminopeptidase type-1 (EC 3 4 19 3) and prolyl endopeptidase (EC 3 4 21 26) The former enzyme has been described in detail elsewhere in this report (section 1 2) and has been shown to hydrolytically remove the pGlu residue from the amino terminus of TRH *in vitro*, thereby generating free pGlu and His-Pro-NH_2 The latter enzyme, prolyl endopeptidase (PE), also frequently referred to as TRH-deamidase and post proline cleaving enzyme (PPCE), deamidates TRH to form acid TRH or pGlu-His-Pro This enzyme is a broad-specificity neuropeptidase capable of cleaving other neuropeptides (3-30 amino acids) at the Pro-X bond of a sequence of the form Y-Pro-X (where Y = peptide and X = peptide, aminoacylamide or amide other than proline) (Koida and Walter, 1976) In addition to TRH therefore, PE has been shown to cleave LHRH, angiotensin, neurotensin, bradykinin, substance P and insulin B chain on the carboxy side of the proline residue(s) in each of these peptides (Taylor and Dixon, 1980, Wilk and Orlowski, 1982, Wilk, 1983)

PE was originally discovered in human uterus as the activity which cleaved the Pro^7-Leu^8 bond of oxytocin (Walter *et al*, 1971) Similar activities (approx 65-75,000 daltons) have since been identified in lamb kidney (Yoshimoto *et al*, 1981), rat brain (Rupnow *et al*, 1979b), rabbit brain (Oliveira *et al*, 1976, Orlowski *et al*, 1979) and bovine brain (Hersh, 1981, Tate, 1981, Yoshimoto *et al*, 1983) This enzyme was identified as a serine protease by Yoshimoto *et al* (1977), although its sensitivity to sulphydryl-reactive agents indicates the necessity of an -SH group for the expression of enzyme activity (Browne and O'Cuinn, 1983a, Wilk, 1983) PE typically displays a sensitivity to bacitracin (Browne and O'Cuinn, 1983a) and is potently inhibited by Z-Pro-Prolinal (Fig 1 11), a transition state aldehyde inhibitor (Wilk and Orlowski, 1983, Friedman *et al*, 1984)

Fig 1 11 The chemical structure of Z-Pro-Prolinal

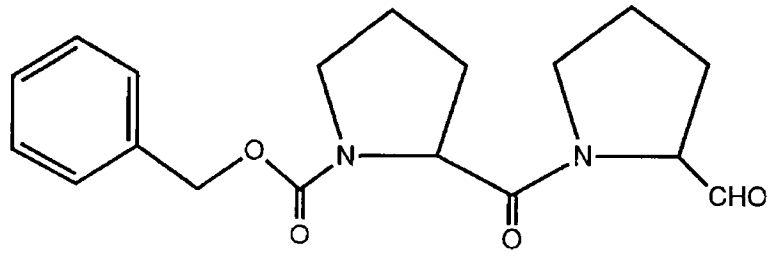
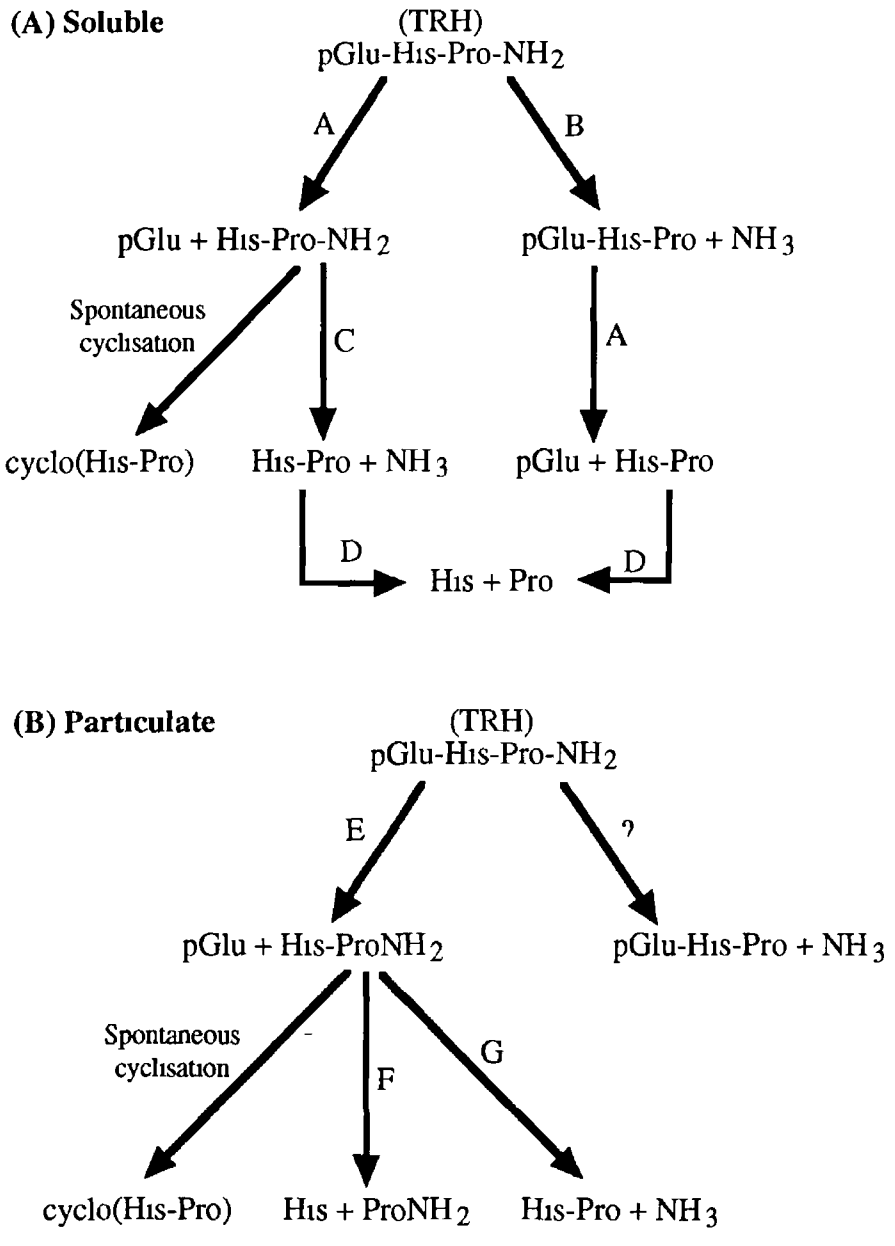


Fig 1 12 The enzymatic degradation of TRH



A = Pyroglutamyl aminopeptidase I
B = Prolyl endopeptidase
C = Post proline dipeptidyl aminopeptidase/DAP-II (puromycin-sensitive)
D = Proline dipeptidase
E = Pyroglutamyl aminopeptidase II
F = Imidopeptidase
G = Post proline dipeptidyl aminopeptidase/DAP-IV (bacitracin-sensitive)

The conversion of TRH to acid TRH by PE represents an example of peptide biotransformation, as acid TRH is reported to produce a "wet dog shaking" effect following intraventricular injection (Boschi *et al*, 1980) Acid TRH may be further metabolised (secondary catabolism) to free pGlu and His-Pro by PAP-I, whereas His-Pro may be further hydrolysed to histidine and proline by a proline dipeptidase (EC 3 4 13 9) (Browne and O'Cuinn, 1983b)

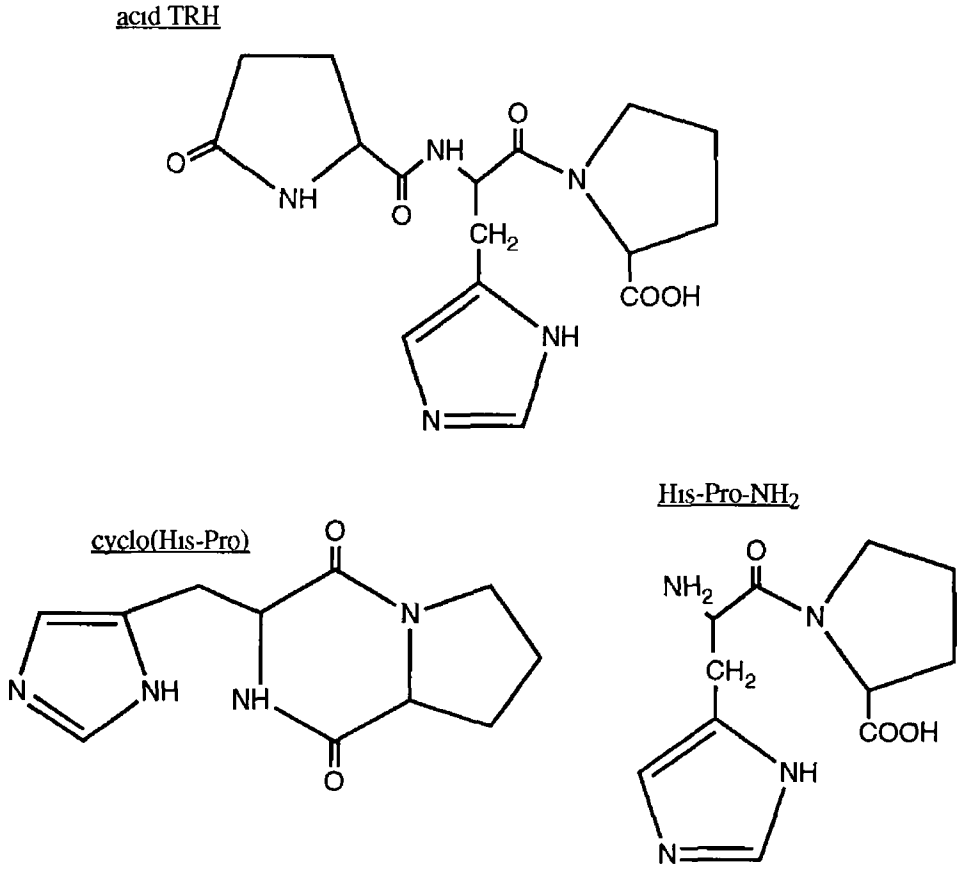
As indicated earlier, the primary inactivation of TRH by PAP-I generates His-ProNH_2, a metabolite which has been reported to cyclise spontaneously and nonenzymatically, at neutral and alkaline pH, to produce cyclo(His-Pro) (Peterkofsky *et al*, 1982, Prasad *et al*, 1982b) cyclo(His-Pro), which does not appear to be further degraded by any enzymatic mechanism, is itself reported to possess endocrine activity (Brabant *et al*, 1981, Melmed, *et al*, 1982) as well as numerous central nervous actions (Peterkofsky *et al*, 1982, Prasad *et al*, 1982b) As such, the conversion of TRH to cyclo(His-Pro) represents another instance of the biotransformation of a peptide (Griffiths and McDermott, 1984) Workers have also demonstrated that His-ProNH_2 is also susceptible to hydrolytic cleavage by a soluble post proline dipeptidyl ammopeptidase or PPDA (Bauer and Kleinkauf, 1980, Browne and O'Cuinn, 1983a) which is capable of converting this metabolite to His-Pro This enzyme activity (approx 200,000 daltons) displays a sensitivity to puromycin, suggesting that it should properly be classified as a dipeptidyl aminopeptidase II (EC 3 4 14 2) activity (McDonald and Barrett, 1986), an activity normally associated with lysosomes Noteworthy is the fact that by converting His-ProNH_2 to a compound other than cyclo(His-Pro), PPDA is competing with the biotransformation process and therefore should be considered a regulator of cyclo(His-Pro) formation (the chemical structures of acid TRH, His-ProNH_2 and cyclo(His-Pro) are illustrated in Fig 1 13)

1 3 5 2 The role of soluble peptidase activities in TRH metabolism *in vivo*

Despite the ability of the aforementioned soluble enzyme activities to hydrolyse TRH to its constituent amino acids *in vitro*, much doubt has been cast on their respective roles in this regard *in vivo* Two possible modes for the enzymic inactivation of TRH (and other neuropeptides) *in vivo* have been considered, (1) extracellular hydrolysis by surface ectoenzymes and (2) lysosomal hydrolysis after endocytosis Neither of these models support a role for cytosolic peptidase activities Indeed, several studies have suggested that the contribution of soluble enzymes to TRH metabolism *in vivo* is relatively insignificant (Bauer, 1987, Charli *et al*, 1987, Torres *et al*, 1986, Salers *et al*, 1991, 1992, Mendez *et al*, 1990) Conversely, some researchers have provided evidence in support of the notion that cytosolic enzymes may have some role, direct or otherwise, in regulating intracellular levels of TRH Faivre-Bauman *et al* (1986) have reported that the addition of specific inhibitors of PAP-I and PE to TRH-synthesising hypothalamic cells in primary culture results in a significant increase in their TRH content and especially a pronounced increase in the amount of TRH being released from these cells under basal or potassium-stimulated conditions Ramirez *et al* (1991) have also demonstrated that PAP-I activity in rat hypothalamus, intermediate-posterior pituitary and retina are subject to circadian variation and asymmetrical distribution, a finding which has also been reported for its potential endogenous substrate, TRH (Schaeffer *et al*, 1977, Kerdelhue *et al* 1981)

Workers have hypothesised that cytosolic enzymes may represent a mechanism for returning neuropeptides released from damaged or ageing vesicles to the cellular amino acid pool (O'Cuinn *et al*, 1990), or, in cases where secretion from neuropeptide-synthesising cells is suppressed, cytosolic degradation of neuropeptides might conceivably represent a security device system to ensure the degradation of neuropeptides which are produced in excess (Bauer, 1987) More recently, evidence has been put forward suggesting that following the interaction and subsequent internalisation of the TRH receptor and its ligand, both receptor and ligand are recycled dissociated from one another (Petrou and Tashjian, 1995, Ashworth *et al*, 1995) Since this recycling event proceeds intracellularly, it is tempting therefore to speculate that cytoplasmic peptidases may participate in modulating this process in some manner

Fig 1 13 Chemical structures of TRH metabolites



1 3 5 3 Primary and secondary catabolism of TRH by particulate enzymes

The primary inactivation of TRH at the external cell surface level is known to be initiated by the apparently TRH-specific PAP-II (EC 3 4 19 -) This enzyme has been dealt with in detail elsewhere in this report (section 1 2 3 2) As in the case of soluble PAP-I, the products of action of PAP-II on TRH are free pGlu and His-ProNH_2 In the absence of further enzyme activity, the latter metabolite will cyclise spontaneously and non-enzymatically to yield cyclo(His-Pro) A bacitracin-sensitive PPDA however, has been demonstrated in synaptosomal membrane preparations of rat (Torres *et al*, 1986)

and guinea-pig (O'Connor and O'Cuinn, 1986) brain, which, because of its synaptosomal location, can directly compete with the biotransformation of His-ProNH$_2$ arising as a result of PAP-II action on TRH The bacitracin sensitivity of this particulate PPDA distinguishes it from the soluble puromycin-sensitive PPDA examined earlier, suggesting that it should properly be classified as a dipeptidyl aminopeptidase IV (EC 3 4 14 5) activity (McDonald and Barrett, 1986)

Torres *et al* (1986) have also demonstrated the production of ProNH$_2$ from His-ProNH$_2$ in rat brain membrane preparations incubated with TRH, presumably through the action of an imidopeptidase, as originally reported by Matsui *et al* (1979) Unfortunately these researchers were unable to localise this enzyme activity to any specific subcellular fraction Also worthy of mention is the possible existence of a membrane-bound PE activity The importance of such a particulate enzyme resides in the fact that it is a primary inactivator of TRH and, as such, would be expected to function in a coordinate fashion with PAP-II at the membrane level to regulate the physiological actions of this tripeptide Noteworthy in this respect is the recent localisation of a broad-specificity PE activity within a synaptosomal membrane preparation from bovine brain (O'Leary and O'Connor, 1995b)

2. MATERIALS AND METHODS

2.1 Materials

Sigma Chemical Company (Poole, Dorset, England)

AG-25 Silver Stain Kit
7-Amino-4-MethylCoumarin (MCA)
Ammonium Persulphate
Bacitracin
Benzamidine
Bestatin
Blue Dextran
Bovine Serum Albumin (BSA)
Coomassie Brilliant Blue G
Dimethylformamide (DMF)
2,2'-Dithiopyridine
Dithiothreitol (DTT)
E-64
EDTA
Eledoisin
N-Ethylmaleimide
Glycine
p-Hydroxymercuribenzoate (PHMB)
8-Hydroxyquinoline
2-Iodoacetamide
Iodoacetate
Lauryl Sulphate (SDS)
Luliberin (LHRH)
Lithium Chloride
Lys-Ala-MCA
2-Mercaptoethanol
MES
N,N'-Methylene-Bisacrylamide
MW-GF-200 Marker Kit
MW-SDS-200 Marker Kit
Neurotensin
pGlu-ßNA
pGlu-His-Gly
pGlu-His-Gly-NH_2
pGlu-His-Pro
pGlu-pNA
1,10-Phenanthroline
Phenylmethylsulphonylfluoride(PMSF)
Potassium Phosphate (Monobasic)
Potassium Phosphate (Dibasic)
Puromycin
Pyroglutamic Acid
Sodium Acetate ($NaCH_3CO_2$)
Sodium Chloride (NaCl)
Trizma Base
TEMED

Bachem Feinchemikalein AG (Bubendorf, Switzerland)

Bombesin
Cyclo(His-Pro)
Gly-Pro-MCA
pGlu-Ala
pGlu-His-Pro-MCA
pGlu-MCA
pGlu-Val
Thyroliberin (TRH)
Z-Gly-Pro-MCA

BDH Chemicals Ltd (Poole, Dorset, England).

Acetic Acid

Acrylamide

Biuret Reagent

Bromophenol Blue

Cadmium Acetate ($Cd(CH_3CO_2)_2\ 2H_2O$)

Calcium Chloride ($CaCl_2$)

Citric Acid

Cobalt (II) Chloride ($CoCl_2\ 6H_2O$)

Copper Sulphate ($CuSO_4$)

Dimethylsulphoxide (DMSO)

Glacial Acetic Acid

Glycerol

Hydrochloric Acid

Iron (III) Chloride ($FeCl_3\ 6H_2O$)

Magnesium Chloride ($MgCl_2\ 6H_2O$)

Methanol

Zinc Sulphate ($ZnSO_4$)

Merck Chemical Company (Frankfurt, Germany)

Iron (II) Sulphate ($FeSO_4\ 7H_2O$)

Manganese Sulphate ($MnSO_4$)

Polyethylene Glycol (PEG)

Sodium Hydroxide

Aldrich Chemical Company (Poole, Dorset, England):

Mercury (II) Chloride ($HgCL_2$)

1,7-Phenanthroline

4,7-Phenanthroline

2-Pyrrolidone

Trifluoroacetic Acid (TFA)

Pharmacia Fine Chemical Company (Uppsala, Sweden):

Activated Thiol Sepharose 4B

DEAE Sepharose Fast Flow

Sephacryl S-200 HR

Pierce Chemical Company (Illinois, USA):

BCA Reagent

Riedel de Haen AG (Germany)

Potassium Chloride (KCl)
Silver Nitrate ($AgNO_3$)

Romil Chemicals (Loughborough, Leicestershire, England)

Acetonitrile (Super High Purity grade)

Calbiochem-Novabiochem (UK) Ltd (Nottingham, England)

pGlu-Pro-NH_2

Penninsula Laboratories (Belmont, CA , USA)

pGlu-His

Mount Sinai School of Medicine (New York - Courtesy of Dr. S Wilk)

Fmoc-Pro-Pro-Nitrile
Z-Pro-Prolinal

2.2 Determination of enzyme activities

2 2 1 Cytosolic pyroglutamyl aminopeptidase

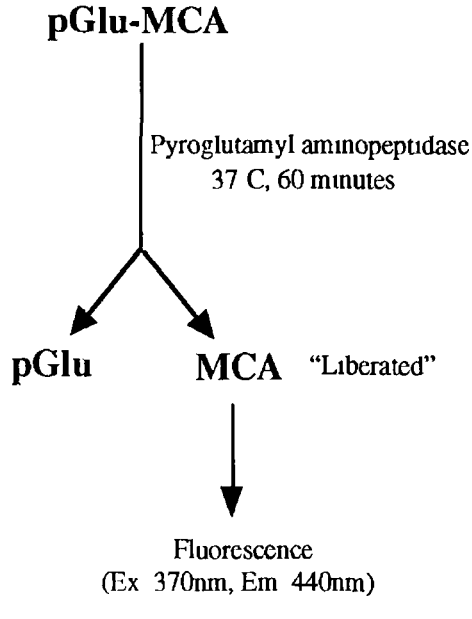
Cytosolic pyroglutamyl aminopeptidase (PAP) activity was determined according to the method of Fujiwara and Tsuru (1978) as modified by Browne and O'Cuinn (1983a) (see Fig 2 1 below) 0 1mM pGlu-MCA was prepared in 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT, 2mM EDTA and 2%v/v DMSO 100µL of sample was incubated with 400µL of substrate at 37 C for 60 minutes after which the reaction was terminated by the addition of 1ml of 1 5M acetic acid A suitable negative control was also included in each assay by adding acetic acid to the enzyme prior to the substrate Liberated MCA was determined using a Perkin-Elmer LS-50 fluorescence spectrophotometer with excitation and emission wavelengths set at 370 and 440nm respectively Excitation and emission slit widths were adjusted for different sample types Fluorescence readings could be converted into nanomoles of MCA released by using a standard curve prepared with the latter compound under corresponding assay conditions and read at the corresponding slit widths Any samples containing particulate material (crude homogenates, pellets etc) were centrifuged at 10,000rpm for 10 minutes in a microfuge prior to fluorescence reading

Fig 2 1 Assay of cytosolic PAP activity using the specific substrate pGlu-MCA

Assay mechanism

1 100µL of sample + 400µL of 0 1mM pGlu-MCA
2 React at 37 C for 60 minutes
3 Stop reaction with 1ml of 1 5M acetic acid
4 Read fluorescence at Ex 370nm and Em 440nm

Schematic



In addition to the above assay, a non-quantitative microplate assay technique was devised in this laboratory to monitor cytosolic PAP activity within eluted post column fractions generated by column chromatography 50µL of sample was incubated with 100µL of substrate (0 1mM pGlu-MCA + 2mM DTT + 2mM EDTA as above) at 37 C for 30 minutes after which the reaction was terminated by the addition of 100µl of 1 5M acetic acid A suitable negative control was also included on each plate by replacing the enzyme sample with buffer Liberated MCA was detected in the same manner as the above assay using a Perkin-Elmer LS-50 fluorescence spectrophotometer fitted with a microplate reader Excitation and emission slit widths varied with each chromatographic column used

2 2 2 Prolyl endopeptidase

Prolyl endopeptidase (PE) activity was determined according to a modification of the original procedure of Yoshimoto *et al* (1979) using the specific substrate, Z-Gly-Pro-MCA This substrate was prepared at a concentration of 0 1mM in 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT, 2mM EDTA and 4%v/v DMSO 100µL of sample was incubated with 400µL of substrate at 37 C for 60 minutes after which the reaction was terminated by the addition of 1ml of 1 5M acetic acid A suitable negative control was also included in each assay by adding acetic acid to the enzyme prior to the substrate Liberated MCA was determined as described in section 2 2 1 above Excitation and emission slit widths were adjusted for different sample types

Using the microplate assay technique (as described in section 2 2 1), PE activity within eluted post column fractions generated by column chromatography could also be monitored using the specific substrate, Z-Gly-Pro-MCA (0 1mM Z-Gly-Pro-MCA + 2mM DTT + 2mM EDTA) Excitation and emission slit widths were set at 10 and 5nm respectively

2 2 3 Dipeptidyl aminopeptidase type-II

Dipeptidyl aminopeptidase type-II (DAP-II) activity was determined using the dipeptide substrate, Lys-Ala-MCA 0 1mM Lys-Ala-MCA was prepared in 50mM potassium phosphate buffer at pH 7 4 100µL of sample was incubated with 400µL of substrate for 60 minutes at 37°C after which the assay was terminated by the addition of 1ml of 1 5M acetic acid A suitable negative control was also included in each assay by adding acetic acid to the enzyme prior to the substrate Liberated MCA was determined as described in section 2 2 1 Excitation and emission slit widths were set at 10 and 5nm respectively

Using the microplate assay technique (as described in section 2 2 1), DAP-II activity within eluted post column fractions generated by column chromatography could also be monitored using the substrate Lys-Ala-MCA (0 1mM) Excitation and emission slit widths were set at 10 and 5nm respectively

2 2 4 Dipeptidyl aminopeptidase type-IV

Dipeptidyl aminopeptidase type-IV (DAP-IV) activity was determined according to a modification of the original procedure of Kato *et al* (1978) using the dipeptide substrate, Gly-Pro-MCA 0 1mM Gly-Pro-MCA was prepared in 50mM potassium phosphate buffer at pH 7 4 100µL of sample was

incubated with 400µL of substrate for 60 minutes at 37°C after which the assay was terminated by the addition of 1ml of 1 5M acetic acid A suitable negative control was also included in each assay by adding acetic acid to the enzyme prior to the substrate Liberated MCA was determined as described in section 2 2 1 Excitation and emission slit widths were set at 10 and 5nm respectively

Using the microplate assay technique (as described in section 2 2 1), DAP-IV activity within eluted post column fractions generated by column chromatography could also be monitored using the substrate Gly-Pro-MCA (0 1mM) Excitation and emission slit widths were set at 10 and 5nm respectively

2.3 Protein Determination

Three methods were employed for the determination of protein concentration in samples as no single method was suitable for all samples

2 3 1 Biuret protein assay

The Biuret assay was used for the determination of protein concentration in crude homogenate, supernatant (S_2) and pooled post anion-exchange samples Prior to assay, each of these samples was dialysed for over 12 hours against 2L of distilled water at 4 C in order to remove interfering substances such as tris buffer, DTT and EDTA 50µL of sample was incubated with 200µL of Biuret reagent at room temperature for 30 minutes after which, the absorbance at 540nm was read on a TitreTek TwinReader Plus spectrophotometric plate reader In the case of the crude homogenate, this assay was scaled up 4 fold in a test tube and, after 30 minutes, the reaction mixture was centrifuged @ 10,000rpm for 10 minutes in a microfuge to remove particulate material prior to measuring the absorbance at 540nm A 0-10mg/ml BSA standard curve was prepared in parallel with the assay each time it was performed

2 3.2 Enhanced BCA protein assay

The Enhanced BCA protein assay, based on the method of Smith *et al* (1985), was used to determine protein concentration in pooled post gel-filtration samples Prior to assay, samples were dialysed for over 12 hours against 2L of distilled water at 4 C in order to remove interfering substances, particularly thiol based compounds such as DTT 10µL of sample was incubated with 200µL of BCA working reagent at 60 C for 30 minutes after which, the absorbance at 540nm was read on a TitreTek TwinReader Plus spectrophotometric plate reader A 0-250µg/ml BSA standard curve was prepared in parallel with the assay each time it was performed

2 3 3 Biorad protein assay

The Biorad protein assay, based on the method of Bradford (1976), was used to determine protein concentration in purified PAP preparations (pooled post affinity chromatography) Prior to assay, samples were dialysed for 3-4 hours against 500mL of distilled water at 4 C in order to remove

interfering substances, particularly thiol based compounds such as DTT and 2-thiopyridone which is released from the Activated Thiol Sepharose 4B column during protein binding and elution 0 8mL of sample was incubated with 0 2mL of Biorad working reagent at room temperature for 5 minutes after which, the absorbance at 595nm was read on a Shimadzu UV 160-A absorbance spectrophotmeter A 0-8μg/ml BSA standard curve was prepared in parallel with the assay each time it was performed

2.4 Subcellular localisation of PAP in bovine brain

2 4 1 Examination of pGlu-MCA hydrolysing PAP activity in the soluble and particulate fractions of bovine brain

25g of bovine brain was homogenised, using a Sorvall Omni Mixer, in 100mL of 50mM tris/HCl buffer at pH 8 0 containing 2mM DTT and 2mM EDTA Tissue was disrupted by three 5 second pulses at speed setting four 10mL of the crude homogenate was then centrifuged for 30 minutes at 27,000g in a Sorvall RC-5B refrigerated superspeed centrifuge The supernatant (S_1) was retained, whilst the pellet (P_1) was gently redissolved in 10mL of the homogenisation buffer and re-spun as above The new supernatant was combined with S_1 to form S_2 whilst the new pellet (P_2) was redissolved in 10ml of homogenisation buffer All operations proceeded at 4 C

The crude homogenate, S_2 and P_2 fractions were then assayed for cytosolic PAP activity as outlined in section 2 2 1

2 4 2 Effect of salt washing on the release of pGlu-MCA hydrolysing PAP activity from the particulate fraction of bovine brain

The homogenisation and centrifugation procedures were performed exactly as above (section 2 4 1) However, four 20mL aliquots of crude homogenate were centrifuged, the supernatants (S_1^{1-4}) were retained and the pellets (P_1^{1-4}) gently redissolved in 10mL of the homogenisation buffer (section 2 4 1) containing 0, 1, 2 and 3M NaCl respectively The redissolved pellets were then re-spun as above The new supernatants were combined with the corresponding S_1 supernatants to form the S_2^{1-4} supernatants Pellets were discarded All operations proceeded at 4°C

The crude homogenate and S_2^{1-4} supernatants were then assayed for cytosolic PAP activity as outlined in section 2 2 1

2.5 Purification of PAP from bovine brain cytosol

2 5 1 Tissue preparation and centrifugation

25g of bovine brain was homogenised, using a Sorvall Omni Mixer, in 100mL of 50mM tris/HCl buffer at pH 8 0 containing 2mM DTT and 2mM EDTA Tissue was disrupted by three 5 second pulses at speed setting four 80mL of the crude homogenate was then centrifuged for 30 minutes at 27 000g in a Sorvall RC-5B refrigerated superspeed centrifuge The supernatant (S_1) was retained whilst the pellet (P_1) was gently redissolved in 40mL of the homogenisation buffer and re-spun as above The new

supernatant was combined with S_1 to form S_2 whilst the new pellet (P_2) was discarded All operations proceeded at 4 C

2 5 2 DEAE Sepharose Fast Flow anion-exchange chromatography

A 15ml DEAE Sepharose Fast Flow anion-exchange column (2 5cm x 3 1cm) was equilibrated with 100mL of 50mM tris/HCl buffer at pH 8 0 containing 2mM DTT and 2mM EDTA 40mL of supernatant (S_2) was applied to the column after which, the column was washed with 40mL of equilibration buffer The column was then eluted with a linear NaCl gradient (0-0 75M NaCl, 60mL total volume) in equilibration buffer 3mL fractions were collected from the point of sample application through to gradient elution A flow rate of 1mL/min was maintained throughout this procedure All operations proceeded at 4 C

The fractions were assayed for cytosolic PAP activity using the microplate assay technique outlined in section 2 2 1 Fractions were also monitored for protein by measuring the absorbance of the samples at 280nm in a quartz cuvette using a Shimadzu UV 160-A absorbance spectrophotometer Those fractions with the highest PAP activities were pooled and stored on ice

2 5 3 Sephacryl S-200 HR gel-filtration chromatography

2 5 3 1 Void volume determination

A 230mL Sephacryl S-200 HR gel-filtration column (2 5cm x 46 4cm) was equilibrated with 350mL of distilled water 5mL of a 2mg/mL solution of blue dextran was applied to the column The column was then eluted with 120mL of distilled water during which time 3mL fractions were collected The absorbance of these fractions at 620nm was monitored on a Titretek TwinReader Plus spectrophotometric plate reader in order to calculate the void volume of the column A flow rate of 1mL/min was maintained throughout this procedure All operations proceeded at 4°C

2 5 3 2 Gel-filtration

The 230mL gel-filtration column outlined above was equilibrated with 350mL of 50mM tris/HCl buffer at pH 8 0 containing 2mM DTT, 2mM EDTA and 0 15M KCl 5mL of the pooled post anion-exchange PAP was applied to the column immediately after which, the column was eluted with 220mL of equilibration buffer After 75mL of equilibration buffer had been run through the column, 3mL fractions were collected for the remaining 145mL of buffer A flow rate of 1mL/min was maintained throughout this procedure All operations proceeded at 4 C

The fractions were assayed for cytosolic PAP activity using the microplate assay technique outlined in section 2 2 1 Fractions were also monitored for protein by measuring the absorbance of the samples at 280nm in a quartz cuvette using a Shimadzu UV 160-A absorbance spectrophotometer Those fractions with the highest PAP activities were pooled and stored on ice

2 5 4 Activated Thiol Sepharose 4B affinity chromatography

A 7mL Activated Thiol-Sepharose 4B column (1 5cm x 3 8cm) was equilibrated with 100mL of 50mM tris/HCl buffer at pH 8 0 containing 2mM EDTA and 0 3M NaCl The pooled post gel-filtration PAP (14-16mL approx) was dialysed for 3 hours against 1L of 50mM tris/HCl buffer at pH 8 0 containing 2mM EDTA in order to remove DTT (prevents binding of thiol enzymes to the column), before being applied to the column The column was washed with 40mL of equilibration buffer and then eluted with a single step-up concentration of DTT (0-5mM DTT, 45mL total volume) in equilibration buffer 3mL fractions were collected from the point of sample application through to gradient elution A flow rate of 0 5mL/min was maintained during the equilibration and elution stages In order to facilitate complete binding of PAP however, a slower flow rate of 0 25mL/min was employed during the sample application and wash stages All operations proceeded at 4 C

The fractions were assayed for cytosolic PAP activity using the microplate assay technique outlined in section 2 2 1 Due to extremely low levels of protein eluting from this column however, fractions were monitored for protein using the Enhanced BCA protein assay For this purpose, 200µL of each fraction was dialysed, prior to the BCA assay, against 3L of distilled water at room temperature for 6 hours to remove interfering substances, particularly thiol based compounds such as DTT and 2-thiopyridone which is released from the Activated Thiol Sepharose 4B column during protein binding and elution Using a Shimadzu UV 160-A absorbance spectrophotometer, the release of 2-thiopyridone from the column could be monitored at 343nm

Those fractions with the highest PAP activities were pooled The protein content of the pooled enzyme was brought to 0 5%w/v (5mg/mL) with BSA, the enzyme subsequently being stored at -80°C *Unless otherwise specifically indicated, post affinity PAP (+0.5%w/v BSA) was the form of purified enzyme used for the majority of characterisation studies (see section 2 7)*

2.6 Polyacrylamide gel electrophoresis

SDS PAGE was performed on various fractions generated throughout the purification procedure, from crude cytosol (S_2) to purified PAP (post affinity chromatography), both to assess the efficiency of purification at each individual stage and, to estimate the molecular mass of the purified enzyme under non-native or denaturing conditions A non-native discontinuous system based on the method of Laemmli (1970) was used

2 6 1 Sample preparation

A suitable sample solubilisation buffer was prepared which consisted of 0 0625M tris/HCl buffer at pH 6 8, 20%v/v glycerol, 8%w/v SDS, 10%v/v 2-mercaptoethanol and 0 01%w/v bromophenol blue

Small aliquots of crude cytosol (S_2) post anion-exchange PAP, post gel-filtration PAP and purified PAP (post affinity chromatography) were dialysed extensively over a 24 hour period at room temperature against 2L of 0 0625M tris/HCl buffer at pH 6 8 The dialysis buffer was changed after 1

3, 6 and 22 hours Dialysed samples were then mixed with an equal volume of sample solubilisation buffer Protein concentrations within the final samples ranged from approximately 3mg/mL (crude cytosol) to 5µg/mL (purified PAP)

Six known molecular mass markers, provided by a Sigma MW-SDS-200 marker kit, were prepared individually by dissolution in 0 0625M tris/HCl buffer at pH 6 8 and subsequently combined to form a molecular mass marker cocktail The marker cocktail was mixed with an equal volume of sample solubilisation buffer The marker concentrations within the final cocktail can be seen in Table 2 1

All prepared samples and markers were placed in a boiling water bath for 2 minutes and then allowed to cool to room temperature before being applied to the gel In addition, it should also be noted that prior to performing SDS PAGE with silver staining, the molecular mass marker cocktail and all of the above samples, with the exception of the purifed PAP preparation were significantly diluted in 0 0625M tris/HCl buffer at pH 6 8 before being mixed with an equal volume of sample solubilisation buffer

2 6 2 SDS PAGE

A range of stock solutions were made up with distilled/deionized water and used to prepare both the resolving gel and stacking gel These include (1) Resolving gel buffer [3M tris/HCl, pH 8 8], (2) Stacking gel buffer [0 5M tris/HCl, pH 6 8], (3) Bisacryl stock [30%w/v acrylamide, 0 8%w/v bisacrylamide], (4) 1 5%w/v ammonium persulphate and (5) 10%w/v SDS

Table 2 2 highlights the volumes required for the preparation of a 10% resolving gel overlayed with a 3 75% stacking gel Gels were prepared in an Atto vertical electrophoresis system (Midi, 16cm x 16cm x 1mm) 20µL of sample and 10µL of marker cocktail were loaded onto the gels which were then electrophoresed in a suitable electrode buffer (0 025M tris, 0 192M glycine, 0 1%w/v SDS, pH 8 3) at 25mA per gel for approximately 3 hours

Table 2 1 *Composition of the molecular mass marker cocktail for SDS PAGE*

Molecular mass marker	Molecular mass (daltons)	Concentration (mg/mL)
Carbonic anhydrase	29,000	0 17
Ovalbumin	45,000	0 25
Bovine Serum Albumin	66,000	0 25
Phosphorylase B	97,400	0 08
ß-Galactosidase	116,000	0 17
Myosin	205,000	0 14

Table 2 2 *Volumes required for SDS PAGE resolving/stacking gels*

Solution	10% Resolving gel (mL)	3 75% Stacking gel (mL)
Bisacryl stock	10	2 5
0 5M tris/HCl, pH 6 8	-	5
3M tris/HCl, pH 8 8	3 75	-
10%w/v SDS	0 3	0 2
Water	14 45	11 3
1 5%w/v ammonium persulphate	1 5	1
TEMED	0 015	0 015

2 6 3 Staining with Coomassie Brilliant Blue G

Immediately following electrophoresis, gels were fixed for approximately 45 minutes in a solution of 40% methanol/7% glacial acetic acid Gels were then stained for 45 minutes in a solution of Coomassie Brilliant Blue G (0 1%w/v Coomassie Brilliant Blue G, 25%v/v methanol and 5%v/v acetic acid) after which, they were destained for over 12 hours in a solution of 25% methanol/10% glacial acetic acid

2 6.4 Silver staining

A Sigma AG-25 silver stain kit was employed to perform the silver staining of SDS PAGE gels according to the method of Heukeshoven and Dernick (1985) Unlike the Coomassie Brilliant Blue G staining procedure outlined above, silver staining is a much more sensitive and labour-intensive technique Table 2 3 highlights the steps involved in the silver staining process

Table 2 3 Silver staining procedure for SDS PAGE

Silver stain step	Solvent/Reagent	Duration
1 Fixing	30%v/v Ethanol/10%v/v Glacial Acetic Acid	60 minutes
2 Rinsing	Distilled/Deionised Water	30 minutes
3 Silver staining	Silver Nitrate	30 minutes
4 Rinsing	Distilled/Deionised Water	20 seconds
5 Developing	Sodium Carbonate/Formaldehyde	30 minutes
6 Development stop	1%v/v Glacial Acetic Acid	5 minutes
7 Rinsing	Distilled/Deionised Water	30 minutes
8 Reducing	Sodium Thiosulphate/Sodium Carbonate	30 seconds
9 Rinsing	Distilled/Deionised Water	24 hours

2.7 Characterisation of cytosolic PAP

2 7 1 Relative molecular mass determination

The relative molecular mass of cytosolic PAP was estimated under both native, non-denaturing conditions (gel-filtration chromatography) and under non-native, denaturing conditions (SDS PAGE) This not only provides a value for the molecular mass of the enzyme, but also whether or not the enzyme has a subunit structure

2.7.1 1 Determination of relative molecular mass under native, non-denaturing conditions via gel-filtration chromatography

Five known molecular mass markers, provided by a Sigma MW-GF-200 marker kit, were prepared individually by dissolution in 50mM potassium phosphate buffer at pH 7 4 containing 0 15M KCl The marker concentrations prepared can be seen in Table 2 4 The Sephacryl S-200 HR gel-filtration column outlined in section 2 5 3 was equilibrated with 250mL of the above buffer The molecular mass markers were then applied separately to the column in a 2mL volume immediately after which, the column was eluted with approximately 200mL of equilibration buffer A flow rate of 2mL/min was maintained throughout this procedure All operations proceeded at 4°C

Fractions were monitored for absorbance at 280nm using a Phillips UV/VIS spectrophotometer and a plot of Log_{10} of molecular mass versus elution volume/void volume (Ve/Vo) was subsequently prepared Using this graph, it is possible to estimate the molecular mass of cytosolic PAP since the elution volume (Ve) of the enzyme from this column is already known (from section 2 5 3 2)

Table 2 4 Gel-filtration molecular mass markers

Molecular mass marker	Molecular mass (daltons)	Concentration (mg/mL)
Cytochrome C	12,400	2
Carbonic Anhydrase	29,000	3
Bovine Serum Albumin	66,000	10
Alcohol Dehydrogenase	150,000	5
ß-Amylase	200,000	4

2 7 1 2 Determination of relative molecular mass under non-native, denaturing conditions via SDS PAGE

The molecular mass markers used for this procedure are highlighted in Table 2 1 (section 2 6 1), whilst the procedure itself is outlined in section 2 6 2 A plot of Log_{10} of molecular mass versus band migration distance/dye front migration distance (R_f, relative mobility) was subsequently prepared Using this graph, it is possible to estimate the molecular mass of cytosolic PAP which was also run on the same SDS gel

2 7 2 Linearity studies with the pGlu-MCA - based assay

The linearity of the PAP assay, using pGlu-MCA as substrate (section 2 2 1), was examined with respect to assay time and enzyme concentration using both crude (S_2) and pure (post affinity chromatography) preparations of cytosolic PAP These studies were essential in order to ensure that the assay could be used in a quantitative manner

2 7.2 1 Linearity with respect to time

Crude cytosol (S_2) and purified PAP (stored in both the presence and absence of 0 5%w/v BSA) were assayed as outlined in section 2 2 1 Reactions however, were stopped with 1 5M acetic acid after 15, 30, 45, 60, 75 and 90 minutes The progress curves (nanomoles of MCA formed versus time) were plotted for each sample to enable Initial Rate determination

2 7 2 2 Linearity with respect to enzyme concentration

A range of dilutions (2x, 4x, 6x, 8x and 10x) of crude cytosol (S_2) and purified PAP were prepared using a suitable diluant for each sample (in the case of the purified PAP, the diluant contained 0 5%w/v BSA) These sample dilutions were then assayed for cytosolic PAP activity as outlined in section 2 2 1 Plots of Initial Rate versus enzyme concentration were then prepared for each sample

2 7 2 3 Effect of BSA concentration on the stability of a purified preparation of cytosolic PAP during assay

The protein concentration of a purified preparation of cytosolic PAP (stored in the absence of BSA) was adjusted to a range of concentrations (0 0, 0 05, 0 1, 0 25, 0 5, 1 0, 2 5 and 5 0mg/mL) using BSA This range of samples was then assayed for PAP activity as outlined in section 2 2 1 A plot of enzyme activity versus BSA concentration was then prepared

2 7 3 Storage of cytosolic PAP under different conditions

Purified PAP was stored in both the presence and absence of 0 5%w/v BSA at room temperature, 4°C and -80 C One aliquot of enzyme (+/-BSA) at each storage temperature was removed and assayed for cytosolic PAP activity after 6, 14, 22 and 28 days as outlined in section 2 2 1 Purified PAP (+/-BSA) was also assayed, as normal, prior to being stored at any of the above temperatures Plots of enzyme activity versus time were then prepared for each sample storage condition

2 7.4 Effects of DTT and EDTA

For this study, 6mL of purified PAP was dialysed at 4°C for 3 hours against 1L of 50mM potassium phosphate buffer at pH 7 4 This ensured the removal of any DTT or EDTA present in the purified enzyme sample which would interfere with the study

2 7 4 1 Effect of DTT on cytosolic PAP activity

50µL of the above enzyme was preincubated, for 10 minutes at 37 C, with an equal volume of 50mM potassium phosphate buffer at pH 7 4 containing 0, 2, 4, 8, 12, 16 and 20mM DTT The final volume of sample preincubating in each test tube was therefore 100µL After 10 minutes, the preincubating samples were assayed for cytosolic PAP activity as outlined in section 2 2 1 It should be noted that the samples were assayed with substrate containing the corresponding concentrations of DTT (0-10mM) EDTA was absent from the substrate A plot of enzyme activity versus DTT concentration was then prepared

2 7 4 2 Effect of EDTA on cytosolic PAP activity

50µL of the above enzyme was preincubated, for 10 minutes at 37 C, with an equal volume of 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT and 0, 2, 4, 8, 12, 16 and 20mM EDTA The final volume of sample preincubating in each test tube was therefore 100µL After 10 minutes, the preincubating samples were assayed for cytosolic PAP activity as outlined in section 2 2 1 It should be noted that the samples were assayed with substrate containing the corresponding concentrations of EDTA (0-10mM) DTT was present in the substrate at a concentration of 2mM A plot of enzyme activity versus EDTA concentration was then prepared

2 7.5 Effect of pH on cytosolic PAP activity

5mL of purified PAP was dialysed at 4°C for 3 hours against 500mL of distilled water containing 2mM DTT 50µL of the dialysed enzyme was preincubated, for 10 minutes at 37 C, with an equal volume of 0 2M buffer at different pH units The buffers used were citric acid/NaOH (pH 4 5-5 5), MES/NaOH (pH 5 5-6 5), potassium phosphate (pH 6 5-8 0), tris/HCl (pH 7 0-9 0) and glycine/NaOH (pH 9 0-10 5) The final volume of sample preincubating in each test tube was therefore 100µL After 10 minutes, the preincubating samples were assayed for cytosolic PAP activity as outlined in section 2 2 1 It should also be noted that the samples were assayed with substrate prepared in the corresponding buffers (at the corresponding pH values) A plot of enzyme activity versus pH was then prepared

2 7.6 Thermostability studies

2.7.6 1 Effect of preincubating cytosolic PAP for various times at different temperatures

A 1 5mL aliquot of purified PAP was preincubated at 37, 40, 50 and 60°C Samples were subsequently removed from each temperature after 15, 30 and 45 minutes and assayed for cytosolic PAP activity as outlined in section 2 2 1 A sample of the purified PAP was also assayed, as normal, prior to being incubated at any of the above temperatures A plot of enzyme activity versus preincubation time was subsequently prepared for each preincubation temperature

2 7 6 2 Effect of performing the assay for cytosolic PAP activity at different temperatures

A 0 5mL aliquot of purified PAP was preincubated for 10 minutes at 30, 37, 40, 45, 50 and 60°C Each aliquot was then assayed for cytosolic PAP activity, as outlined in section 2 2 1 , at the corresponding temperature of preincubation A plot of enzyme activity versus assay temperature was subsequently prepared

2 7 7 Metal ion studies

4mL of purified PAP was dialysed at 4 C for 3 hours against 500mL of 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT 50µL of dialysed enzyme was then preincubated, at 37°C for 10 minutes, with an equal volume of metal ion (prepared from the corresponding metal salt in either 50mM potassium phosphate at pH 7 4 or distilled water) to give a final metal ion concentration of 1mM The final volume of sample preincubating in each test tube was therefore 100µL After 10 minutes, the premcubating samples were assayed for cytosolic PAP activity as outlined in section 2 2 1 It should also be noted that the samples were assayed with substrate prepared in the absence of 2mM EDTA in order to avoid chelating of the metal ions under study A suitable positive control in which metal ions were absent was also prepared and then preincubated and assayed as above Results were tabulated

In the case of three of the metal ions tested above, Hg^{2+}, Cu^{2+} and Zn^{2+}, this experiment was repeated This time however, the samples were assayed for cytosolic PAP activity, as normal, with substrate containing 2mM EDTA, in an attempt to determine if the inhibitory effect of these metal ions could be reversed by the presence of a chelating agent in the substrate Results were tabulated

In addition, a separate control was prepared for each metal ion, under assay conditions, in which the substrate was replaced with a standard dilution of MCA and the enzyme replaced with 50mM potassium phosphate buffer at pH 7 4 in order to determine if the metal ion under investigation has any effect on MCA fluorescence

2.7 8 Inhibitor studies

For these studies, a small aliquot of purified PAP was dialysed at 4°C for 3 hours against 500mL of a suitable buffer The buffer used in the dialysis was determined by the inhibitor to be tested For sulphydryl-blocking reagents, the enzyme was dialysed against 50mM potassium phosphate buffer at pH 7 4 containing 20µM DTT, whilst for EDTA, this buffer contained 2mM DTT For all other inhibitors tested, 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT and 2mM EDTA was used to dialyse the enzyme

2 7 8 1 Effects of various functional reagents on the activity of cytosolic PAP

50µL of dialysed enzyme was preincubated, at 37 C for 10 minutes, with an equal volume of inhibitor (prepared at two concentrations in 50mM potassium phosphate, pH 7 4) The final volume of sample preincubating in each test tube was therefore 100µL After 10 minutes, the premcubating samples were assayed for cytosolic PAP activity as outlined in section 2 2 1 It should also be noted that the samples were assayed with substrate containing the corresponding concentration of DTT and EDTA used to initially dialyse the enzyme Suitable positive controls in which inhibitors were absent were also prepared and then preincubated and assayed as above Results were tabulated

In addition a separate control was prepared for each inhibitor (highest test concentration), under assay conditions, in which the substrate was replaced with a standard dilution of MCA and the enzyme replaced with 50mM potassium phosphate buffer at pH 7 4 in order to determine if the inhibitor under investigation has any effect on MCA fluorescence

2 7 8 2 Demonstration of the reversible nature of 2-pyrrolidone inhibition

4mL of purified PAP was dialysed at 4 C for 3 hours against 500mL of 50mM tris/HCl buffer at pH 8 0 containing 2mM DTT and 2mM EDTA A 3 5mL aliquot of the dialysed enzyme was treated with 2-pyrrolidone to give a final concentration of 0 1M The dialysed enzyme (+/- 0 1M 2-pyrrolidone) was then assayed for cytosolic PAP activity as outlined in section 2 2 1

In an attempt to reverse the observed inhibition, a 3mL aliquot of the "inhibited" enzyme was then re-dialysed at 4 C for 7 hours against 500mL of the above buffer The dialysis buffer was changed after 1, 2 and 4 hours and a sample of the dialysing enzyme was taken after 4 hours and finally after 7 hours The re-dialysed enzyme samples were then assayed for cytosolic PAP activity as above A plot of enzyme activity versus dialysis time was subsequently prepared

2 7 8 3 Timecourse inhibition studies

In an attempt to examine the rapidity with which certain inhibitors of cytosolic PAP act on the enzyme, a continuous, real-time fluorimetric assay, using pGlu-MCA as the substrate, was devised

2mL of purified PAP was dialysed at 4°C for 3 hours against 500mL of 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT and 2mM EDTA 250µL of the dialysed enzyme was then aliquoted into a cuvette preincubated at 37 C within the heated cuvette block of the Perkin-Elmer LS-50 fluorescence spectrophotometer The reaction was initiated by the addition of 25 5µL of inhibitor and 1mL of 0 1mM pGlu-MCA, prepared as described in section 2 2 1 The contents of the cuvette were mixed and the release of MCA was monitored continuously over a 30 minute period at excitation and emission wavelengths of 370 and 440nm respectively Excitation and emission slit widths were both set at 10nm The final inhibitor concentrations tested were 10mM and 0 5mM 2-pyrrohdone and 1mM and 0 2mM 1,10-phenanthroline A suitable positive control in which inhibitors were absent and a suitable negative control in which both enzyme and inhibitors were absent were also prepared and assayed continuously as above Plots of fluorescence intensity versus time were subsequently prepared

2.7 9 Kinetic studies

2 7 9 1 K_m determination for pGlu-MCA and pGlu-ßNA

The Michaelis-Menten Constant (K_m) values of purified PAP for the fluorimetric substrates pGlu-MCA and pGlu-ßNA were determined Both substrates were prepared at a stock concentration of 0 5mM in 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT, 2mM EDTA and 2%v/v DMSO Both substrates were diluted in the above buffer (-DMSO) to give a range of concentrations

from 0 005-0 5mM Purified PAP was then assayed with both substrate concentration ranges, as outlined in section 2 2 1 In the case of the pGlu-ßNA substrate, the excitation and emission wavelengths used were 315 and 425nm respectively (Fusc *et al* , 1990) and the reaction was terminated by the addition of 1M sodium acetate buffer at pH 4 2 It should also be noted that for this particular substrate, a ßNA standard was not available Using various kinetic models, the data for both substrates was subsequently plotted and the K_m values calculated

2.7 9 2 K_m determination for pGlu-His-Pro-MCA

The Michaelis-Menten Constant (K_m) of purified PAP for the TRH analog, pGlu-His-Pro-MCA, was determined The substrate was prepared at a stock concentration of 0 25mM in 50mM potassium phosphate buffer at pH 7 4 containing 2mM DTT and 2mM EDTA The substrate was diluted in the above buffer to give a range of concentrations from 0 005-0 25mM Purified PAP was then assayed across this substrate concentration range The assay used is based on a modification of the normal assay outlined in section 2 2 1 and is described in Fig 2 2 overleaf Using various kinetic models, the data for this substrate was subsequently plotted and the K_m value calculated

2 7 9 3 K_I determination for pyroglutamyl peptides

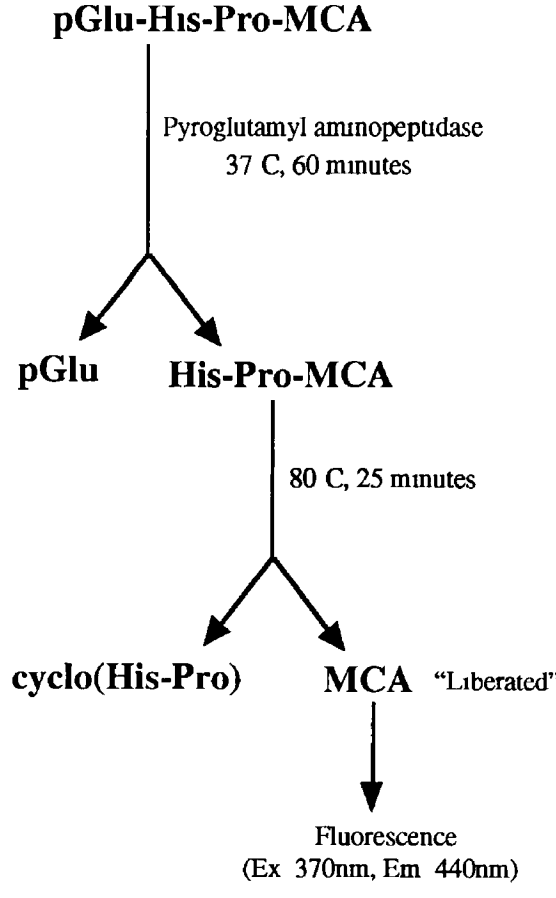
The inhibitor Constant (K_I) values of purified PAP for a range of pyroglutamyl peptides was determined using pGlu-MCA as a substrate A 0 2mM pGlu-MCA stock solution was prepared in 50mM potassium phosphate buffer at pH 7 4 containing 4mM DTT, 4mM EDTA and 2%v/v DMSO The substrate was then diluted out in the above buffer (-DMSO) to give a concentration range from 0 02-0 2mM 1mL of each substrate dilution was subsequently added to an equal volume of pyroglutamyl peptide inhibitor (prepared to a specific concentration in 50mM potassium phosphate, pH 7 4) to give a final pGlu-MCA concentration range of 0 01-0 1mM containing a constant inhibitor concentration across this range Purified PAP was then assayed across this substrate concentration range as outlined in section 2 2 1 Using various kinetic models, the data obtained was subsequently plotted and the K_I values calculated for each inhibitor This type of analysis also enabled the nature of the inhibition (competitive or non-competitive) of each inhibitor to be determined

Fig 2 2 Assay of cytosolic PAP activity using the TRH analog, pGlu-His-Pro-MCA

Assay mechanism

1 100µL of sample + 20µL of 0 026mM Fmoc-Pro-Pro-Nitrile or 0 1mM Z-Pro-Prolinal *
2 Preincubate at 37 C for 10 minutes
3 +380µL of 0 01-0 25mM pGlu-His-Pro-MCA
4 React at 37 C for 60 minutes
5 Stop reaction with 1ml of 1 5M acetic acid
6 Heat samples at 80 C for 25 minutes
7 Read fluorescence at Ex 370nm and Em 440nm

Schematic



* Fmoc-Pro-Pro-Nitrile and Z-Pro-Prolinal are highly specific inhibitors of PE which can hydrolyse this substrate at the Pro-MCA bond

2 7 10 Investigation of the substrate specificity of cytosolic PAP by HPLC

The substrate specificity of post anion-exchange PAP was examined using a number of synthetic pyroglutamyl substrates A HPLC system was used to examine the cleavage products resulting from the incubation of cytosolic PAP with these substrates

2 7 10 1 Sample preparation

5mL of the partially purified enzyme was dialysed at 4°C for 3 hours against 500mL of 50mM potassium phosphate buffer at pH 7 4 containing 4mM DTT and 4mM EDTA To a mixture of 150µL of the potential substrate (prepared to a specific concentration in 50mM potassium phosphate, pH 7 4), 20µL of 1mM bestatin (a general aminopeptidase inhibitor) and 20µL of 0 1mM Z-Pro-Prolinal (a highly specific PE inhibitor) was added 150µL of the dialysed enzyme The enzyme reaction was allowed to proceed lor 24 hours after which, 20µL of 40mM DTT was added to each reaction After a further 16 hours at 37 C, reactions were terminated by the addition of 100µL of 0 2%v/v TFA at pH 1 4 Suitable negative controls were also prepared for each substrate in which the enzyme was omitted from the reaction until after the 40 hour incubation at 37°C

2.7 10 2 HPLC

The HPLC system used consisted of a Beckman System Gold™ Programmable Solvent Module 126 (dual pump), Diode Array Detector Module 168 and Autosampler 507 A Beckman Ultrasphere™ C-8 (octyl) reverse phase HPLC column (4 6mm x 25cm) was used with the above system The system was operated at a flow rate of 1mL/min All solvents were filtered and degassed before use **Solvent A 0.2%v/v TFA, Solvent B 0 2%v/v TFA + 70%v/v Acetonitrile**

Prior to sample application, the column was equilibrated in Solvent-A–Following sample application, the elution procedure adopted depended on the substrate/metabolite under investigation With weakly hydrophobic substrates (TRH, acid TRH, pGlu-Ala, pGlu-His, pGlu-Val, pGlu-His-Gly and pGlu-His-Gly-NH_2) and metabolites (cyclo(His-Pro) and pyroglutamic acid), the column was eluted with Solvent A for 5 minutes followed by a linear gradient from 0-50% of Solvent B in Solvent A over a 10 minute period (0-35% acetonitrile) The absorbance was monitored at 207nm (the observed absorbance maxima for pyroglutamic acid, the cleavage product of cytosolic PAP) With more strongly hydrophobic substrates (LHRH, Bombesin Neurotensin, Eledoisin and pGlu-Pro-NH_2), more hydrophobic elution conditions were required The column was eluted with Solvent A for 5 minutes followed by a linear gradient from 0-85% of Solvent B in Solvent A over a 12 minute period (0-60% acetonitrile) At the end of this gradient, elution was continued in 85% B for a further 3 minutes The absorbance was monitored at 207nm

It should be noted that standard preparations of all of the above substrates/metabolites were run under the above elution conditions beforehand, thus enabling the elution times of each of the standards to be recorded Pyroglutamic acid was run under both sets of elution conditions These elution times

facilitated peak identification when examining the metabolites resulting from the cleavage of the substrates by cytosolic PAP (section 2 7 10 1)

3. RESULTS

3.1 Preparation of MCA standard curves

As outlined in section 2 2 1, the specific fluorimetric substrate, pGlu-MCA was used to assay for cytosolic PAP activity The enzymatic release of MCA was monitored fluorimetrically at excitation and emission wavelengths of 370 and 440nm respectively MCA release could then be quantitated by using a standard curve prepared with the latter compound under corresponding assay conditions and read at the corresponding slit widths

Two sets of MCA standard curves were prepared for quantitative purposes One set was prepared in the absence of enzyme, whilst one set incoporated crude enzyme samples (i e a standard curve incorporating crude homogenate and a standard curve incorporating crude cytosol) in order to correct for the fluorimetric "quenching" effect observed with crude samples Quenching, or to be more precise, the "inner-filter effect", results from the absorbance of electromagnetic radiation, at both the excitation and emission wavelengths, by proteins and other contaminants present in crude samples, the net effect of which is the lowering of the fluorescent signal Unless corrected for, this quenching phenomenon can result in falsely low cytosolic PAP activity levels following the assay of crude enzyme samples

3 1 1 MCA standard curve preparation in the absence of crude enzyme samples

Four MCA standard curves were prepared under standard assay conditions in the absence of enzyme (0 5mL of MCA standard dilution + 1mL of 1 5M acetic acid)

Table 3 1 MCA standard curves

MCA concentration range (µM)	Excitation slit width (nm)	Emission slit width (nm)
0-45	10	2 5
0-6	10	5
0-1 4	10	10
0-0 5	10	15

The 0-45µM and 0-6µM standard curves can be seen in Fig 3 1 whilst the 0-1 4µM and 0-0 5µM standard curves can be seen in Fig 3 2 These standard curves were used for the quantitation of enzymatically released MCA during the assay of cytosolic PAP activity in pooled post column fractions, including purified cytosolic PAP activity

3 1 2 MCA standard curve preparation in the presence of crude enzyme samples (demonstration of the quenching effect on MCA fluorescence)

0-48µM MCA standard curves, which incorporated either crude homogenate or crude cytosol (see section 2 2 1) were prepared under standard assay conditions These curves were both read at

excitation and emission slit widths of 10 and 2 5nm respectively and, can be seen in Fig 3 3 where they are compared to the corresponding 0-45μM MCA standard curve prepared above From this plot it can be seen that in the presence of crude homogenate, the MCA standard curve is quenched by up to 17 2%, whilst, in the presence of crude cytosol, this figure is as high as 27 5%

3.2 Preparation of BSA standard curves

A BSA standard curve was prepared for each of the protein assays used For the Biuret protein assay, a 0-10mg/mL BSA standard curve was constructed whilst, for the more sensitive Enhanced BCA method, a 0-250μg/mL BSA standard curve was constructed A 0-8μg/mL BSA standard curve was constructed for the Biorad protein assay It was noted that above a BSA concentration of 8μg/mL, the Biorad standard curve rapidly became non-linear Figs 3 4 and 3 5 illustrate the standard curves obtained for the two more sensitive assays, the Enhanced BCA method and the Biorad method, respectively

Fig 3 1 MCA standard curves (Ex 10nm, Em 2 5 and 5nm)

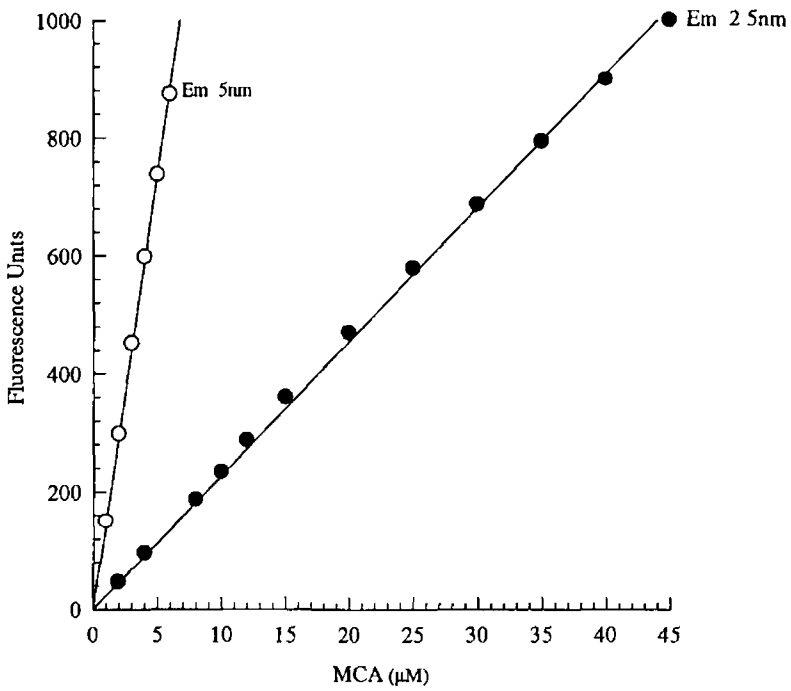


Fig 3 2 MCA standard curves (Ex 10nm, Em 10 and 15nm)

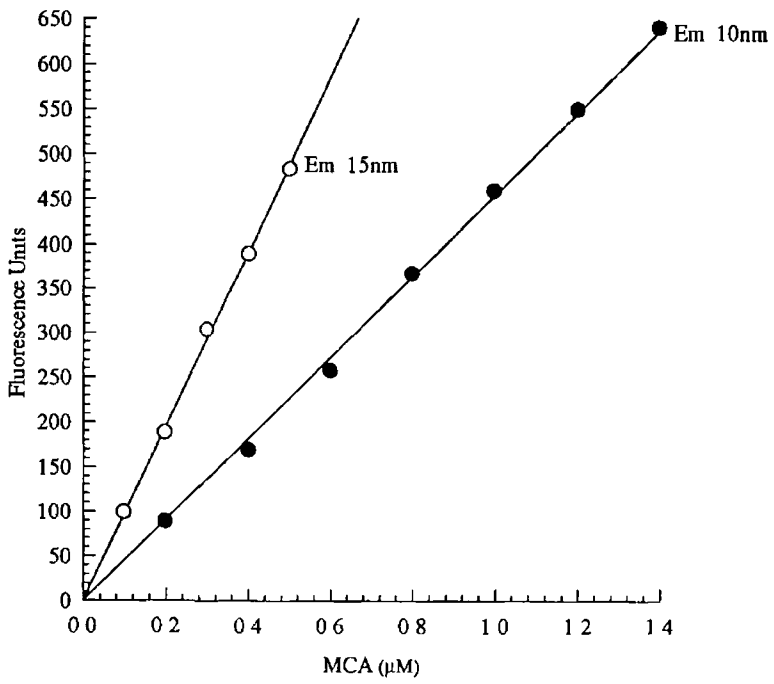
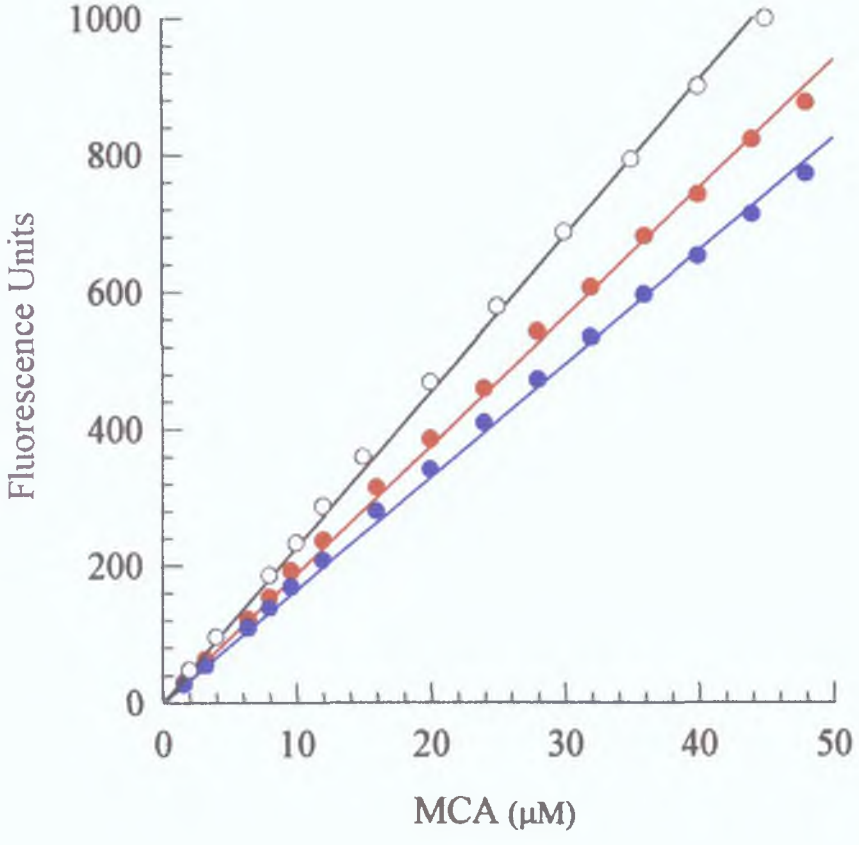


Fig. 3.3 *MCA standard curves incorporating enzyme*



○ No sample
● Homogenate (17.2% quenching)
● S_2 (27.5% quenching)

Excitation slit width = 10nm
Emission slit width = 2.5nm

Fig 3 4 BSA standard curve (Enhanced BCA method)

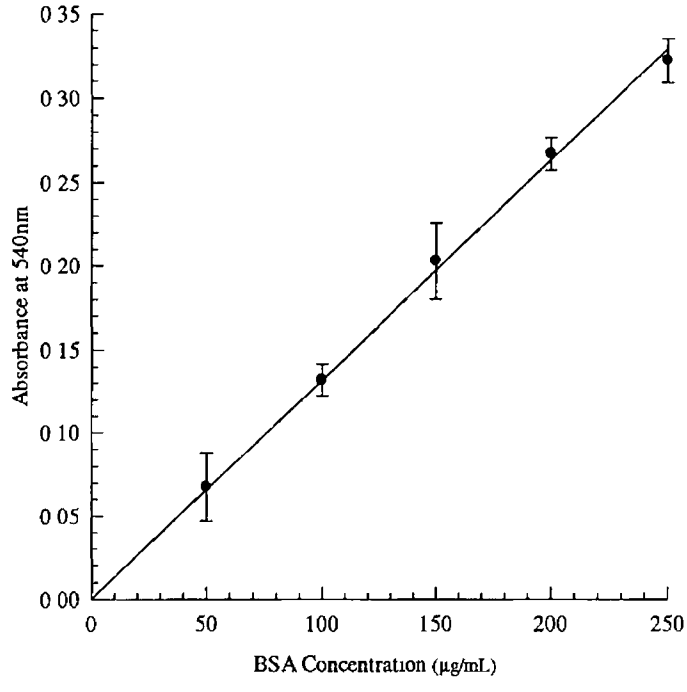
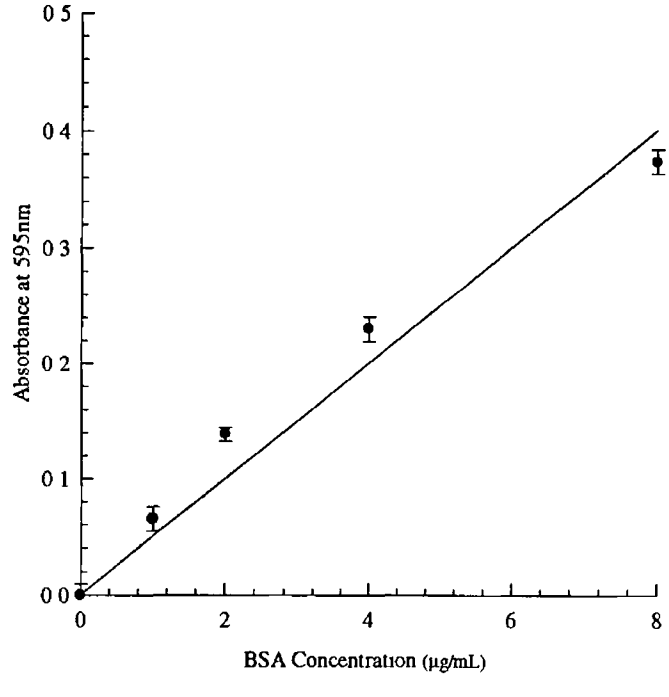


Fig 3 5 BSA standard curve (Biorad method)

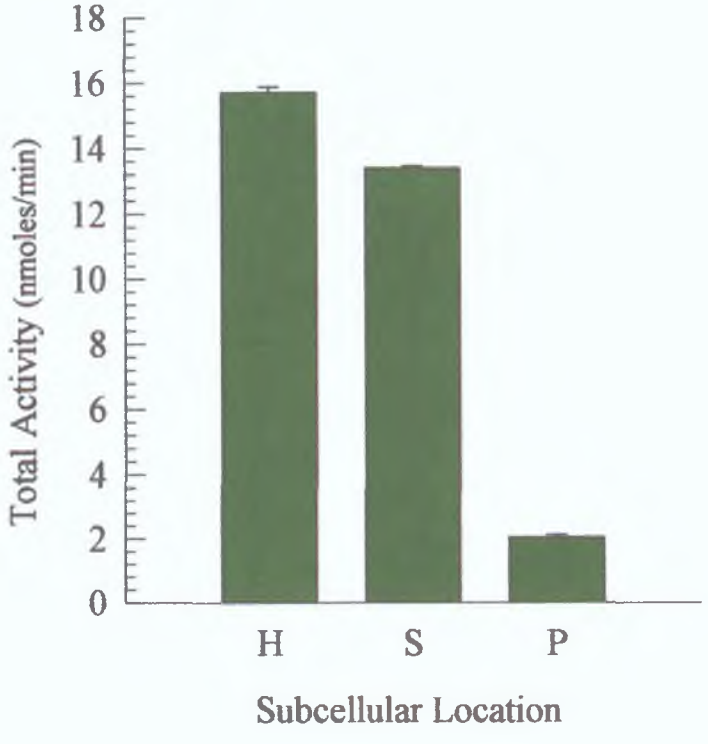


3.3 Subcellular localisation of the pGlu-MCA hydrolysing PAP activity in bovine brain

The subcellular localisation of the pGlu-MCA hydrolysing PAP activity in bovine brain was investigated as described in sections 2.4.1 and 2.4.2. From Fig. 3.6 it can be seen that over 85% of the pGlu-MCA hydrolysing PAP activity was found to be located in the soluble fraction of bovine brain. A significantly smaller proportion of PAP activity (13%) was found to be associated with the particulate fraction. 1.9% of the total PAP activity initially observed in the crude homogenate remained unaccounted for following the centrifugation process.

When the particulate fraction was subjected to salt washes of up to 3M NaCl, little or no pGlu-MCA hydrolysing PAP activity was found to be released from the membranes.

Fig. 3.6 *Subcellular Localisation of pGlu-MCA hydrolysing PAP*



Homogenate (H) = 100%
27,000g Supernatant (S) = 85.1%
Pellet (P) = 13%

3.4 Purification of PAP from the cytosolic fraction of bovine brain

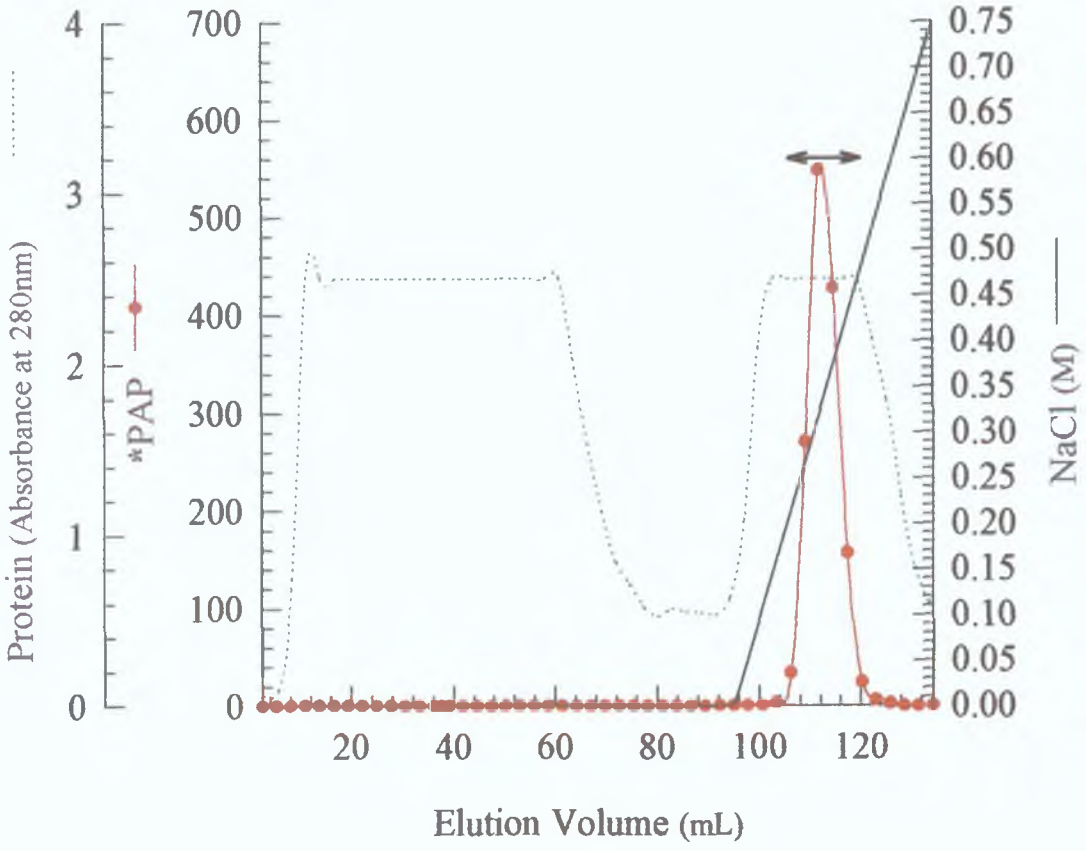
3 4 1 Partial purification of cytosolic PAP by anion-exchange chromatography

As outlined in section 2 5 2, a DEAE Sepharose Fast Flow anion-exchange column was equilibrated with 6-7 column volumes of running buffer Under these conditions, cytosolic PAP bound to the column whilst unbound contaminants were washed through the column with approximately 3 column volumes of running buffer Following the application of a linear NaCl gradient (0-0 75M), bound PAP activity eluted at approximately 0 3M NaCl, separating away from the bulk of protein and emerging between fractions 39-43 to give a final volume of 14mL when pooled The active recovery of applied PAP activity was approximately 85 8% A wash in 3 column volumes of 2M NaCl was sufficient to regenerate the anion-exchanger The elution of cytosolic PAP from this column is illustrated in Fig 3 7

3 4 2 Further purification of cytosolic PAP by gel-filtration chromatography

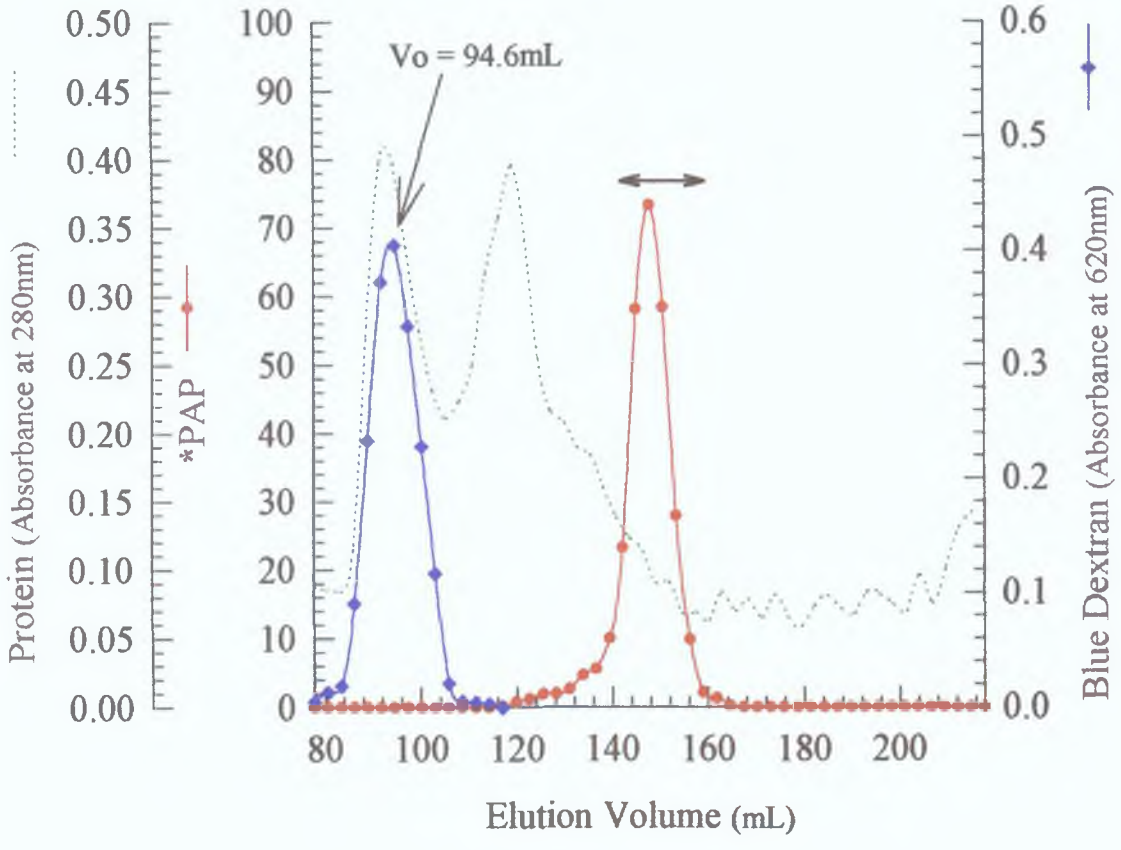
Following application of a 5mL aliquot of post anion-exchange cytosolic PAP to an equilibrated Sephacryl S-200 HR column, as outlined in section 2 5 3 2, the PAP activity eluted well after the void volume (Ve^{PAP} = 147 8mL) and well away from the two main protein peaks observed PAP activity eluted between fractions 23-29 to give a final volume of 19 6mL The active recovery of applied PAP activity was approximately 17 7% The elution profile can be seen in Fig 3 8

Fig. 3.7 *Elution profile of cytosolic PAP from DEAE Sepharose*



*Enzyme activity in Fluorescence Units
Excitation slit width = 10nm
Emission slit width = 5nm
⟷ Pooled fractions

Fig. 3.8 *Elution profile of cytosolic PAP from Sephacryl S-200 HR*



*Enzyme activity in Fluorescence Units
Excitation slit width = 10nm
Emission slit width = 5nm

Void volume (Vo) is indicated

⟷ Pooled fractions

3 4 3 Affinity chromatography of cytosolic PAP on Activated Thiol Sepharose 4B

An Activated Thiol Sepharose 4B column was equilibrated with approximately 15 column volumes of running buffer, as outlined in section 2 5 4 All of the pooled post gel-filtration PAP activity was subsequently applied to this column at a flow rate of 0 25mL/min Flowrates in excess of this prevented a significant proportion of the enzyme activity from binding PAP bound to this column under the conditions of equilibration and was subsequently eluted by the inclusion of 5mM DTT in the running buffer, emerging between fractions 23-28 to give a final volume of 16 8mL when pooled The active recovery of applied PAP activity was approximately 54% Following elution, the column could be regenerated with a wash in 4 column volumes of distilled water followed by 4 column volumes of 1 5mM 2,2-dithiopyridine

The release of 2-thiopyridone from the column was also monitored by absorbance spectrophotometry at 343nm Two distinct peaks of 2-thiopyridone were observed withm the elution profile The first peak (the smaller of the two peaks) emerged from the column prior to elution, and results from the binding of thiol enzymes to the column functional groups (step (A) in Fig 3 9) A second peak (significantly larger than the first) emerged immediately after elution commenced

The exact mechanism of affinity chromatography using Activated Thiol Sepharose 4B is briefly illustrated in Fig 3 9 For a more in-depth review of this technique, see Brocklehurst *et al* (1974) The elution profile obtained can be seen in Fig 3 10

Table 3 2 summarises the overall purification scheme

Fig 3 9 Mechanism of operation for Activated Thiol Sepharose 4B

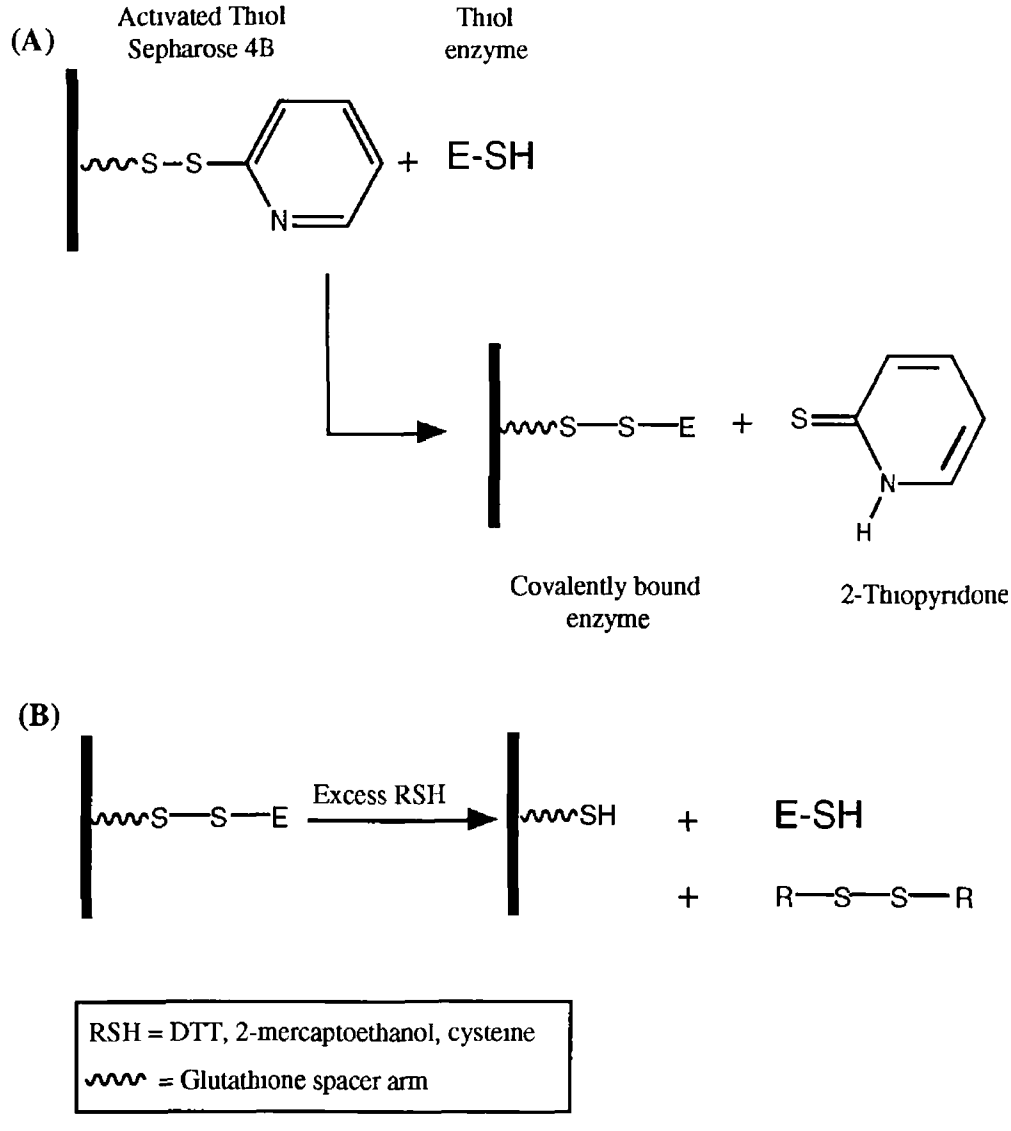
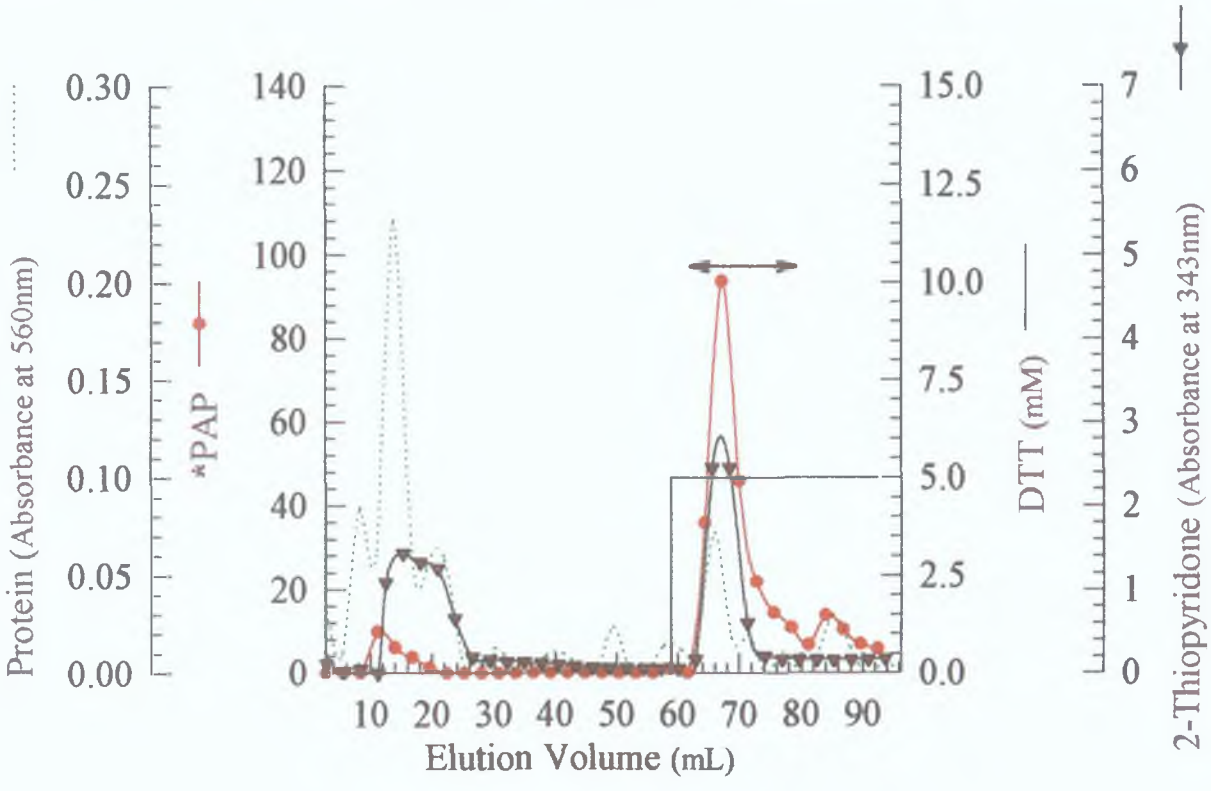


Fig. 3.10 *Elution profile of cytosolic PAP from Activated Thiol Sepharose 4B*



*Enzyme activity in Fluorescence Units
Excitation slit width = 10nm
Emission slit width = 10nm

Protein was monitored using an "Enhanced BCA" assay

⟷ Pooled fractions

Table 3 2 Purification of pyroglutamyl aminopeptidase from bovine brain cytosol

Purification stage	Total protein (mg)	Total activity (units*)	Specific activity (units/mg)	Purification factor	Recovery (%)
Homogenate	1993 6	103 2	0 052	1	100
27,000g Supernatant (S_2)	538 47	82 77	0 154	2 96	80 2
DEAE Sepharose	122 45	70 99	0 58	11 15	68 8
Sephacryl S-200 HR	6 805	12 52	1 84	35 39	12 15
Activated Thiol Sepharose 4B	1 42	6 72	4 73	91 01	6 56

* 1 unit of enzyme activity can be defined as that amount of enzyme which releases 1 nanomole of MCA per minute at 37 C

3.5 Monitoring of other TRH-degrading enzymes during the purification of PAP from bovine brain cytosol

In addition to cytosolic PAP, at least two other soluble enzymes known to participate in the degradation of TRH, *in vitro*, were observed within bovine brain cytosol Using specific fluorimetric substrates, prolyl endopeptidase (PE), an enzyme responsible for the deamidation of TRH (a "primary" TRH-degrading step) and dipeptidyl aminopeptidase (DAP), an enzyme which converts His-Pro-NH_2, a TRH metabolite, to cyclo(His-Pro) (a "secondary" TRH-degrading step), were both detected in bovine brain cytosol (the reader is referred to section 1 3 for a more detailed review of these enzymes)

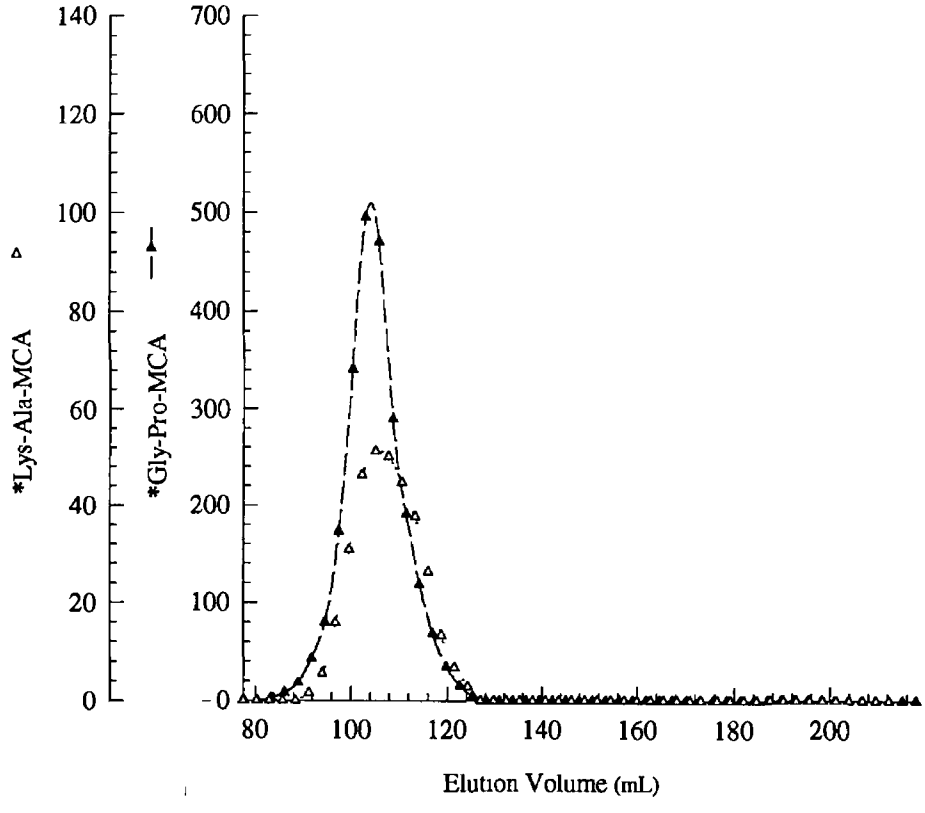
3 5 1 Identification of Gly-Pro-MCA and Lys-Ala-MCA hydrolysing DAP activity(s) in the soluble fraction of bovine brain

Whilst assaying for cytosolic PAP activity in the post gel-filtration fractions (section 2 5 3 2), specific fluorimetric assays for DAP-II and DAP-IV enzyme activities were also performed on these fractions as outlined in sections 2 2 3 and 2 2 4 respectively Both substrates were hydrolysed by a high molecular mass enzyme activity(s) (190,600 daltons) which eluted near the void volume of the Sephacryl S-200 HR column Fractions comprising the highest enzyme activity(s) were pooled for further analyses (below) The elution profile obtained can be seen in Fig 3 11

As described by Smyth and O'Cuinn (1994a, 1994b), the pooled sample was assayed with both substrates in the presence and absence of 0 1mM bestatin in order to determine if sequential release of amino acids from the dipeptidyl methyl coumarin substrates by cytosolic aminopeptidases was occurring Results indicated however, that levels of Gly-Pro-MCA and Lys-Ala-MCA hydrolysing activities within the sample were completely unaffected by the inclusion of bestatin in the assays It was also observed that the Gly-Pro-MCA substrate was hydrolysed approximately 2 4 times more rapidly than the Lys-Ala-MCA substrate

The pooled sample was also assayed with both substrates in the presence and absence of 0 1mM bacitracin and 0 1mM puromycin Results indicated that the hydrolysis of both substrates was very strongly inhibited by puromycin whilst the hydrolysis of Gly-Pro-MCA was mildly inhibited by bacitracin (approximately 20% inhibition)

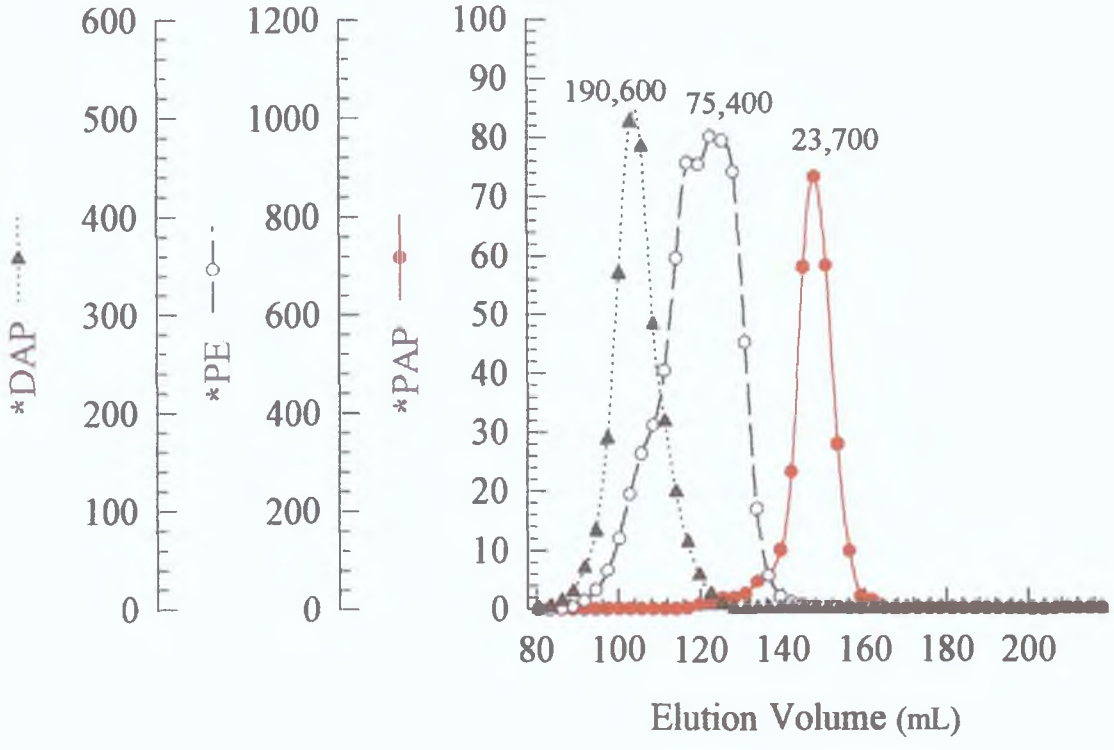
Fig. 3 11 Elution profile of Lys-Ala-MCA and Gly-Pro-MCA hydrolysing DAP activity(s) from Sephacryl S-200 HR



3.5.2 Separation of TRH-degrading enzymes by gel-filtration chromatography

Using the microplate assay technique, the post gel-filtration fractions (from section 2.5.3.2) were assayed for cytosolic PAP and PE, as well as the high molecular mass Gly-Pro-MCA hydrolysing DAP activity, as outlined in sections 2.2.1, 2.2.2 and 2.2.4 respectively. Fig. 3.12 illustrates the separation of these enzyme activities on the basis of differences in their relative molecular masses, reported in section 3.7.1.

Fig. 3.12 *Elution profile of cytosolic PAP, PE and DAP from Sephacryl S-200 HR*



*Enzyme activity in Fluorescence Units
Excitation slit width = 10nm
Emission slit width = 5nm

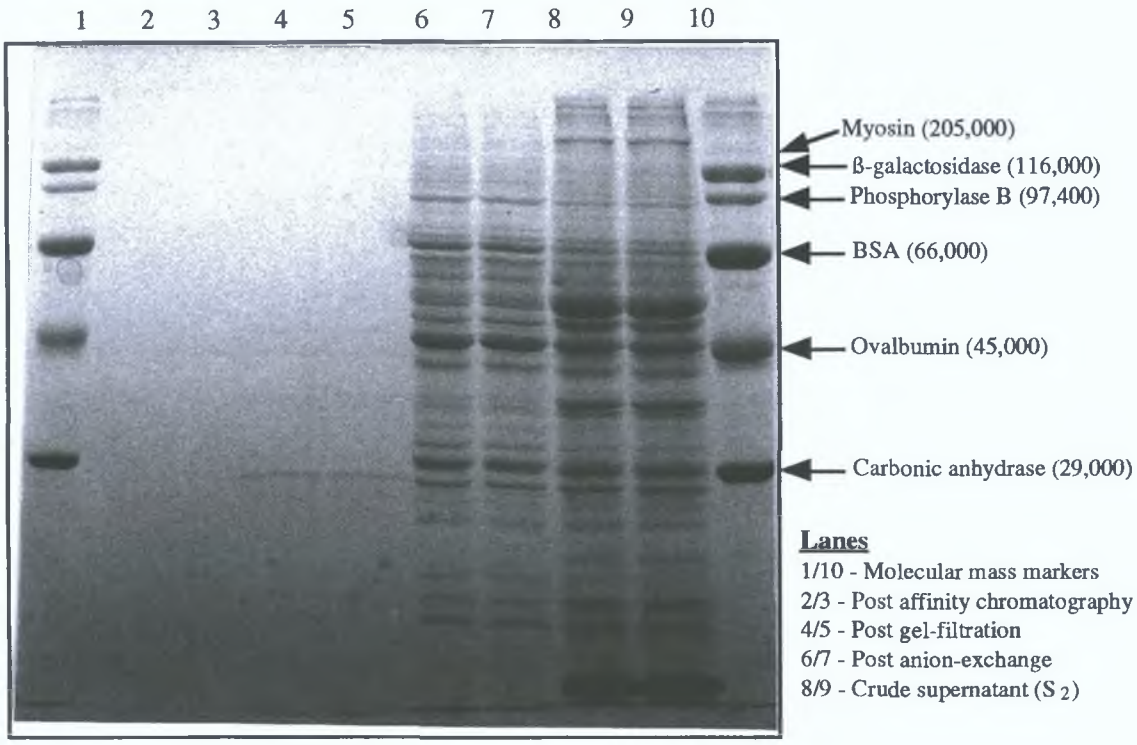
Molecular masses (daltons) are indicated
DAP monitored with Gly-Pro-MCA

3.6 SDS PAGE - Assessing the efficiency of the cytosolic PAP purification scheme

3.6.1 Staining with Coomassie Brilliant Blue G

The efficiency of each purification step on the separation of PAP from other cytosolic proteins can be clearly seen from the photographic evidence presented in Fig. 3.13. The gel-filtration and affinity chromatography steps in particular, had a significant effect on the removal of contaminating protein. Due to the extremely low levels of protein in the purified PAP preparation, no protein bands were observed in these lanes (2 and 3) using this staining procedure.

Fig. 3.13 SDS PAGE study using Coomassie Brilliant Blue stain



3.6.2 Silver staining

Fig. 3.14a displays an SDS gel which has been electrophoresed, as outlined in section 2.6, with molecular mass markers and a sample taken from each stage of the purification scheme. Fig. 3.14b displays an SDS mini-gel which has been prepared and electrophoresed, as outlined in section 2.6, with a 3.6 fold concentrate of purified PAP (concentrated using PEG 6000). In both cases, protein bands were subsequently visualized by silver staining (section 2.6.3).

Fig. 3.14a *SDS PAGE study using Silver stain*

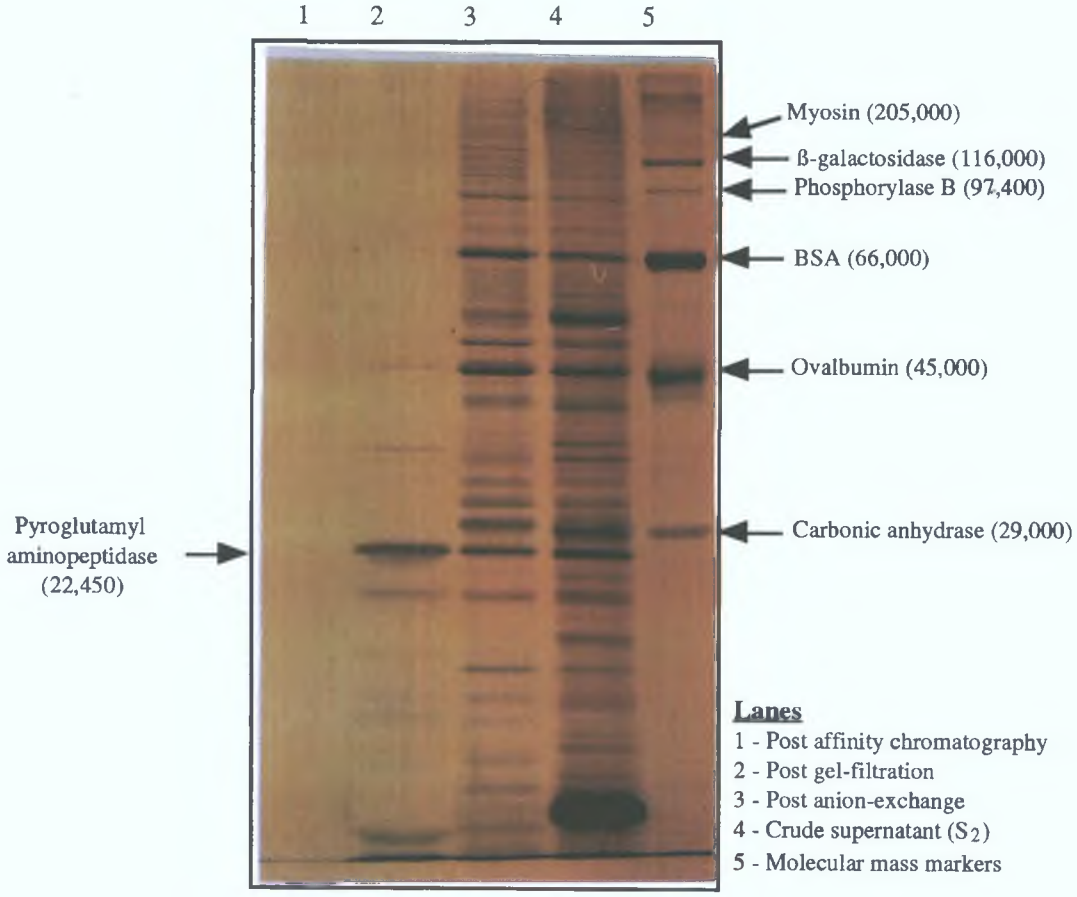
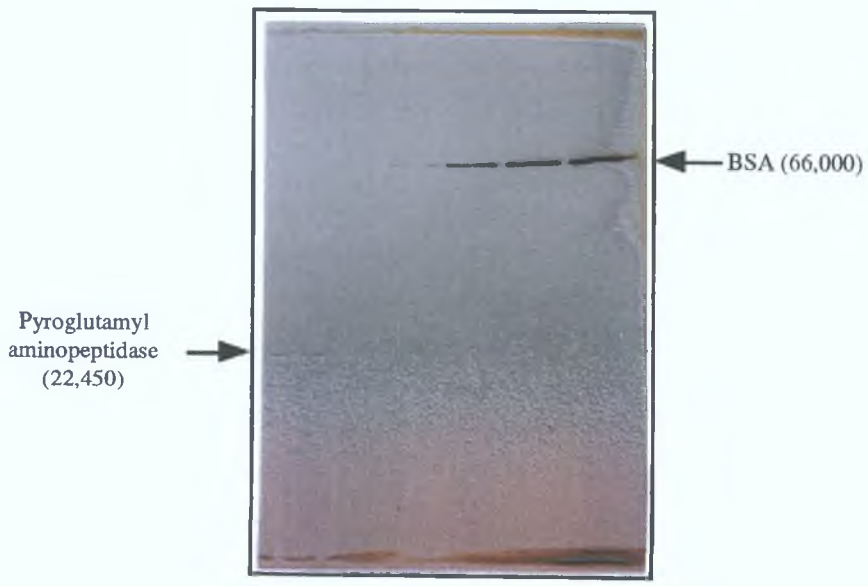


Fig. 3.14b *SDS PAGE study using Silver stain*

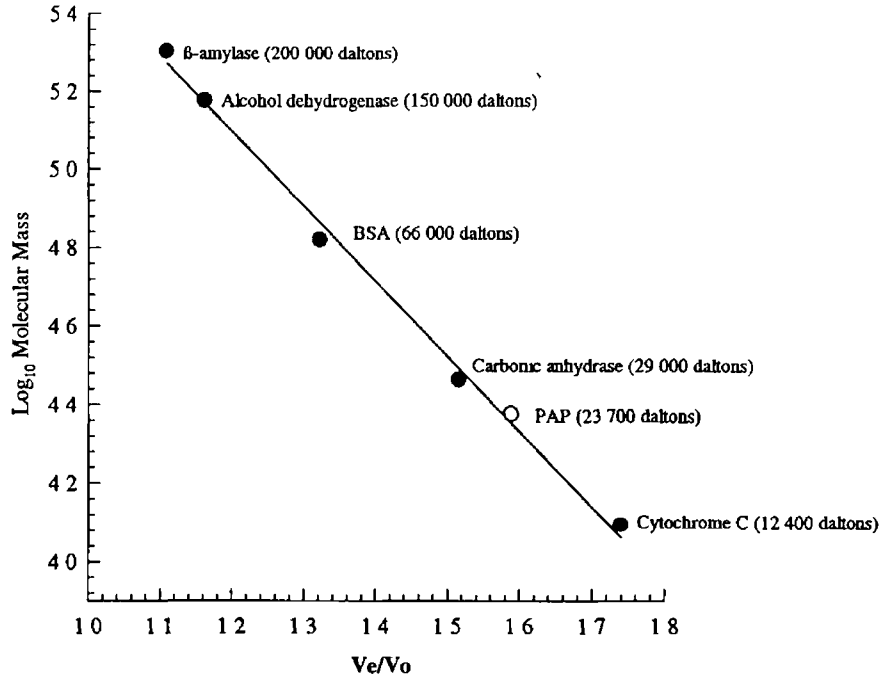


3.7 Characterisation studies performed on cytosolic PAP

3.7 1 Relative molecular mass determination via gel-filtration chromatography

As outlined in section 2 7 1 1, a range of standard proteins of known relative molecular mass were eluted from a Sephacryl S-200 HR gel-filtration column, and a molecular mass calibration curve (Fig 3 15) was subsequently constructed (Log_{10} of molecular mass versus Ve/Vo) From this curve, the native molecular mass of cytosolic PAP was found to be approximately 23,700 daltons

Fig 3 15 Molecular mass calibration curve (gel-filtration)



3 7 2 Linearity studies performed on the standard pGlu-MCA assay

3 7 2 1 Linearity with respect to time

Crude cytosol and purified PAP (stored in the presence and absence of 0 5%w/v BSA) were assayed for various times ranging from 0-90 minutes, as outlined in section 2 7 2 1 Figs 3 16 and 3 17 show the "progress curves" (nanomoles of MCA released versus time) obtained for each sample type From these curves it can be seen that both the crude cytosolic PAP and the purified PAP preparation, stored in the presence of 0 5%w/v BSA, react with pGlu-MCA in a perfectly linear fashion, over a 90 minute period, under standard assay conditions Conversely, the purified PAP preparation stored in the absence of 0 5%w/v BSA, reacted with the substrate in a linear manner for up to 10 minutes, after which the enzyme behaved in a completely non-linear fashion Maximum substrate hydrolysis was found to be 19 8% for the crude cytosolic PAP and 1 75% for purified PAP (+0 5%w/v BSA) after 90 minutes of reaction time

The Initial Rate, or steady-state reaction rate, for each sample type was obtained from the slope of the respective progress curve (in the case of the non-linear curve, the slope of the tangent to the linear portion of the curve) Crude cytosolic PAP demonstrated an Initial Rate of 0 09 nmoles/min whilst purified cytosolic PAP (+/- BSA) demonstrated an Initial Rate of 0 0078 nmoles/min These rates can also be expressed as specific values (Initial Rate/mg protein) For the crude cytosolic PAP, this value was 0 16 nmoles/min/mg, whilst for purified PAP, it was found to be 12 0 nmoles/min/mg

3 7 2 2 Linearity with respect to enzyme concentration

The effect of enzyme concentration on the linearity of the standard assay was investigated using crude cytosolic and purified PAP preparations, as outlined in section 2 7 2 2 Figs -3 18 and-3-19 (Initial Rate versus enzyme concentration) demonstrate the relatively linear rate of pGlu-MCA hydrolysis by both sample types with respect to PAP concentration

3.7 2 3 Effect of BSA concentration on the stability of a purified preparation of cytosolic PAP during assay

The effect of storing purified PAP in different concentrations of BSA (0-5mg/mL) was examined, as described in section 2 7 2 3 Fig 3 20 clearly illustrates the stabilising effect of BSA on purified PAP reactivity during the enzyme assay From this plot it can be seen that a minimum protein concentration of approximately 1mg/mL was required for optimum pGlu-MCA hydrolysis during the standard assay A negative control was used to eliminate the possibility of any direct effect of BSA on the pGlu-MCA substrate

Fig 3 16 Assay linearity w r t time when assaying with crude cytosol (27,000g supernatant, S_2)

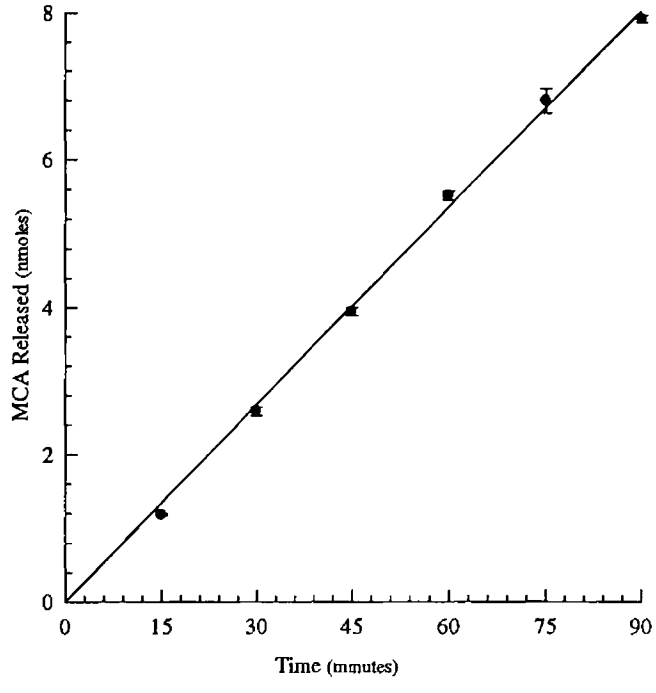


Fig 3 17 Linearity w r t time when assaying purified PAP (post affinity chromatography)

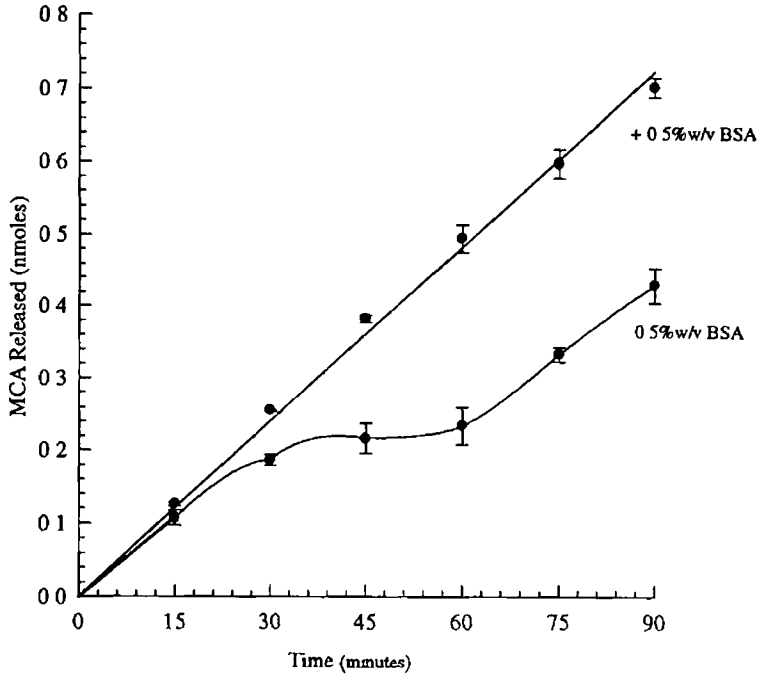


Fig 3 18 Assay linearity w r t enzyme concentration when assaying crude cytosol (27,000g supernatant, S_2)

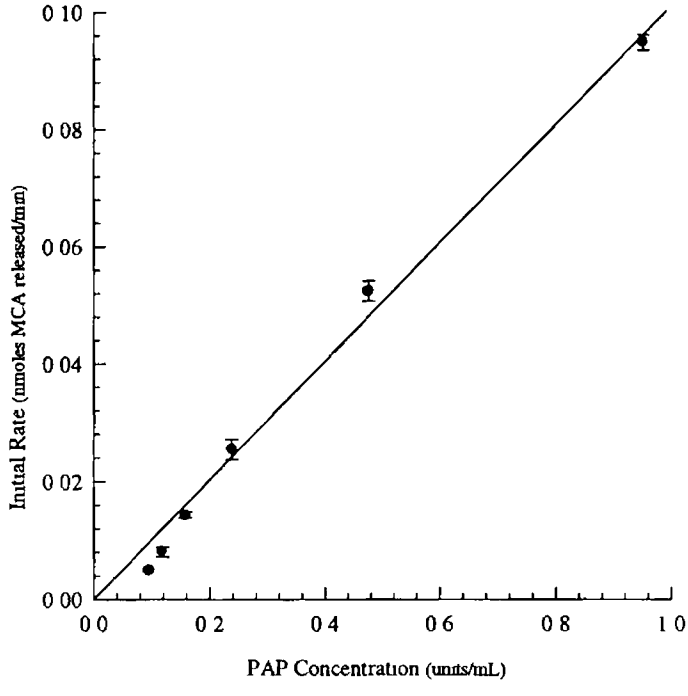


Fig 3 19 Assay linearity w r t enzyme concentration when assaying purified PAP (post affinity chromatography)

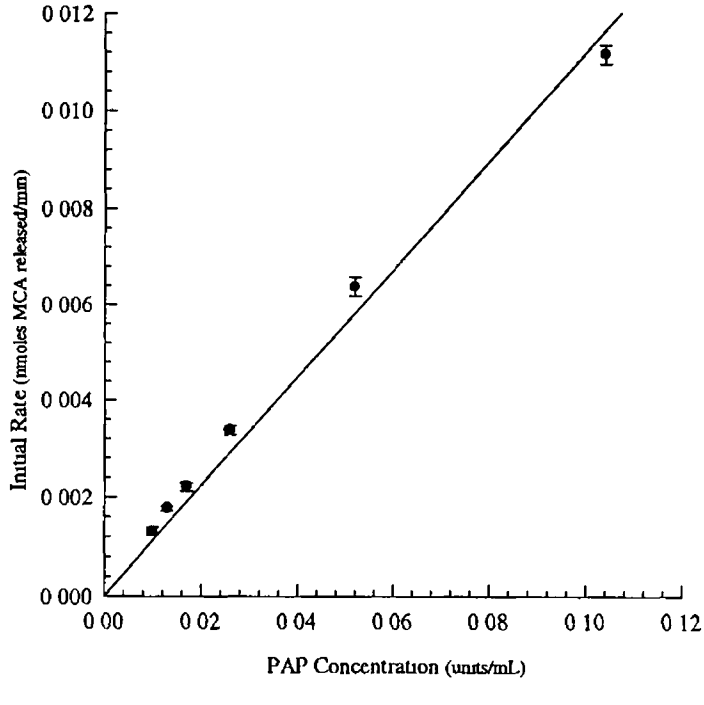
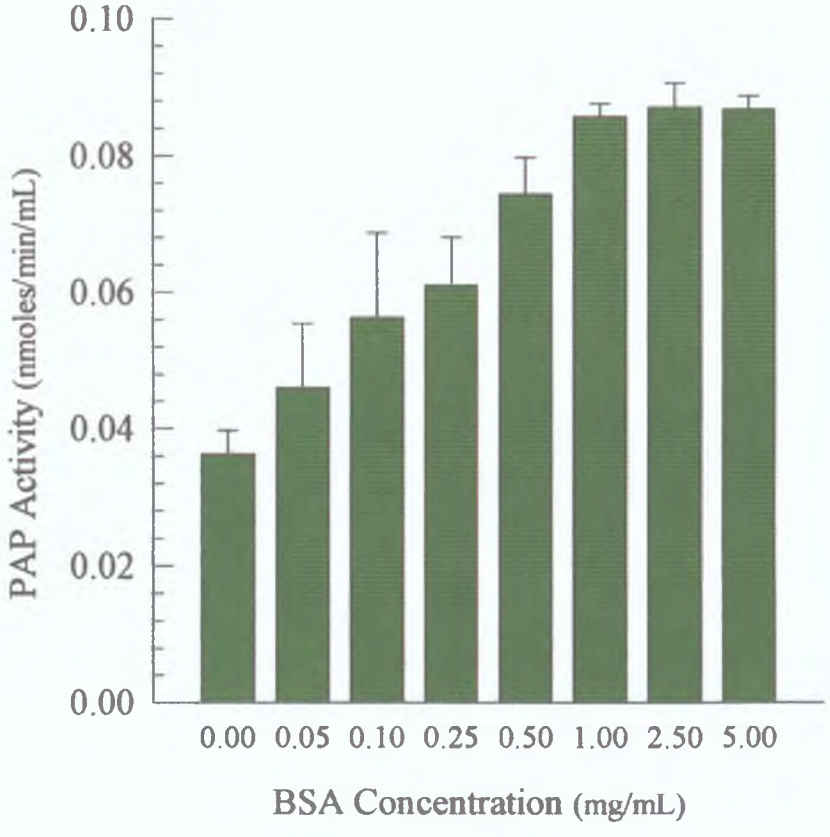


Fig. 3.20 *Effect of BSA concentration on the stability of purified PAP during the standard assay*



3 7 3 Storage of cytosolic PAP under different conditions

The effect of storing purified PAP at different temperatures over a four week period in the presence and absence of 0 5%w/v BSA was investigated, as outlined in section 2 7 3 Fig 3 21 describes the subsequent effect of each storage temperature (room temperature, 4 C and -80 C) on the stability of purified PAP stored in the presence of 0 5%w/v BSA over this time period The enzyme displays significant stability at both 4 C and room temperature, retaining a good deal of its original activity after 4 weeks of storage (65% and 81% respectively) Overall however, the rate of activity loss was slower at -80°C, particularly during the first 2 weeks, with the loss of 17% overall PAP activity after 4 weeks storage

Fig 3 22 describes the effect of each of the above temperatures on the stability of purified PAP stored over a period of 4 weeks in the absence of 0 5%w/v BSA In contrast to the above results, storage of the purified enzyme in the absence of BSA at either 4 C or room temperature for a period of 1 week results in a decrease of cytosolic PAP activity to 4% and 31% respectively No apparent decrease in cytosolic PAP activity was observed however, when samples were stored at -80°C over the 4 week period Indeed it would appear that cytosolic PAP activity increases over time when stored at this temperature

Fig. 3.21 *Storage of purified PAP at different temperatures (+0.5%w/v BSA)*

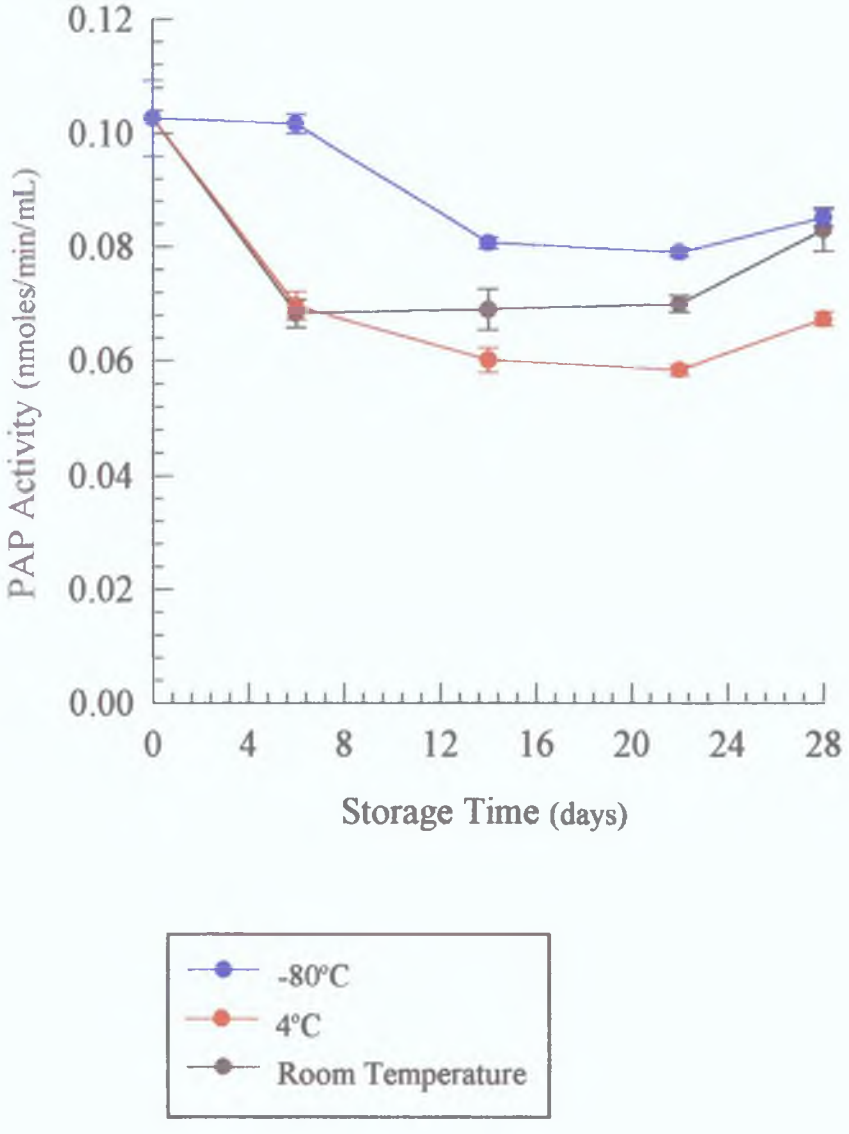
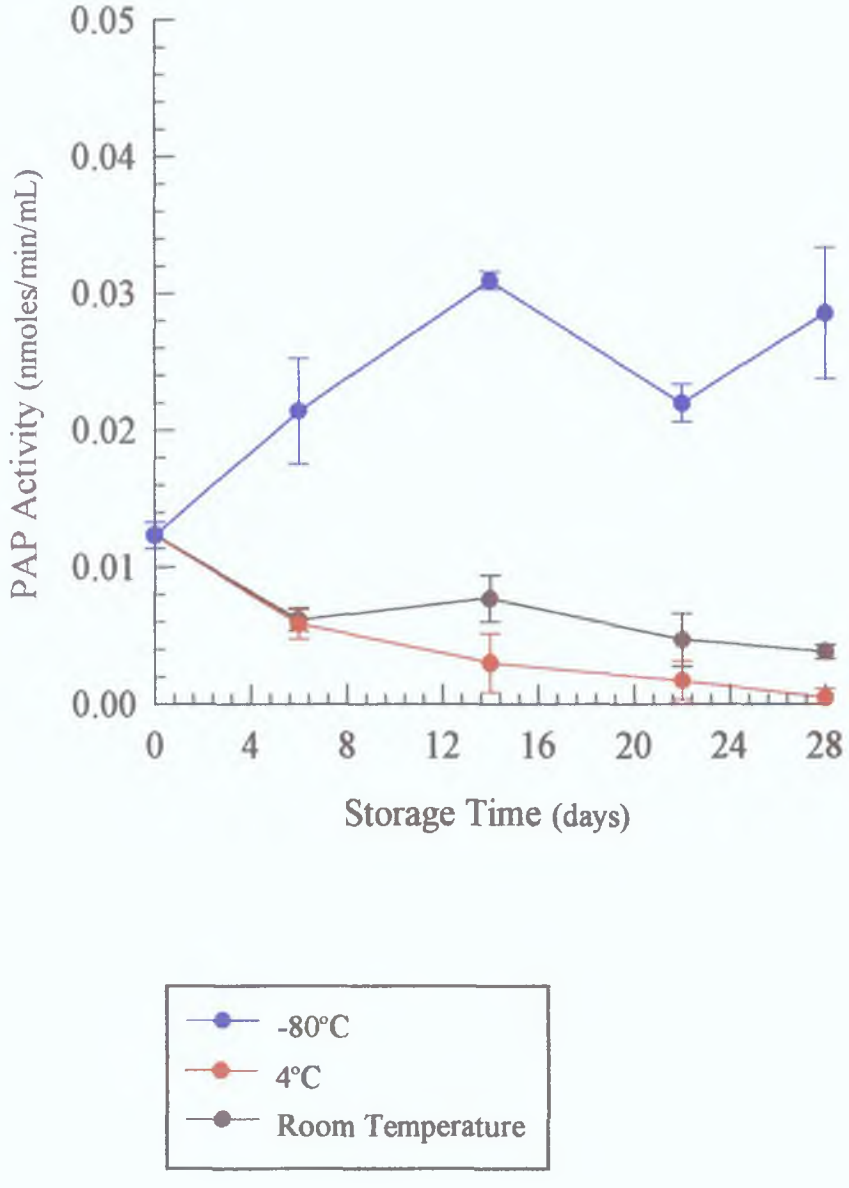


Fig. 3.22 *Storage of purified PAP at different temperatures (-0.5%w/v BSA)*



3 7 4 Effects of DTT and EDTA on cytosolic PAP activity

3 7 4 1 Effect of different DTT concentrations on cytosolic PAP activity

as outlined in section 2 7 4 1 the effect of preincubating and assaying purified PAP in the presence of a range of different concentrations (0-10mM) of DTT, a disulphide bond-reducing agent, was investigated From Fig 3 23 it can be seen that DTT enhances PAP activity, with stimulation of activity continuing up to 8mM DTT (up to 30% more activity than at the standard assay concentration of 2mM DTT) In the absence of D TT, cytosolic PAP activity was almost totally abolished but could be completely restored by the re-introduction of DTT (approximately 2mM for most studies) to the purified enzyme

3 7.4.2 Effect of different EDTA concentrations in the presence of 2mM DTT on cytosolic PAP activity

The effect of preincubating and assaying purified PAP in the presence of a range of different concentrations (0-10mM) of EDTA combined with a single concentration of DTT (2mM) was investigated, as described in section 2 7 4 2 As can be seen from Fig 3 24 EDTA has a relatively negligible effect on purified PAP activity over this concentration range

3 7.5 Effect of pH on cytosolic PAP activity

Using a selection of five different buffers, the activity of cytosolic PAP over the pH range 4 5-10 5 was examined (section 2 7 5) The purified enzyme displays optimum activity between pH 8 5-9 0 with the maximum activity observed in glycine/NaOH buffer at pH 9 0 (approximately 1 5 fold more PAP activity than that observed in potassium phosphate buffer at pH 7 4) cytosolic PAP activity declined rapidly below pH 6 5 and above pH 9 0 It was also noted that cytosolic PAP activity varied as a function of the buffer "type" used, with different levels of PAP activity observed in different buffers at similar pH values Fig 3 25 describes the results of this study

Fig. 3.23 *Effect of DTT on purified PAP activity*

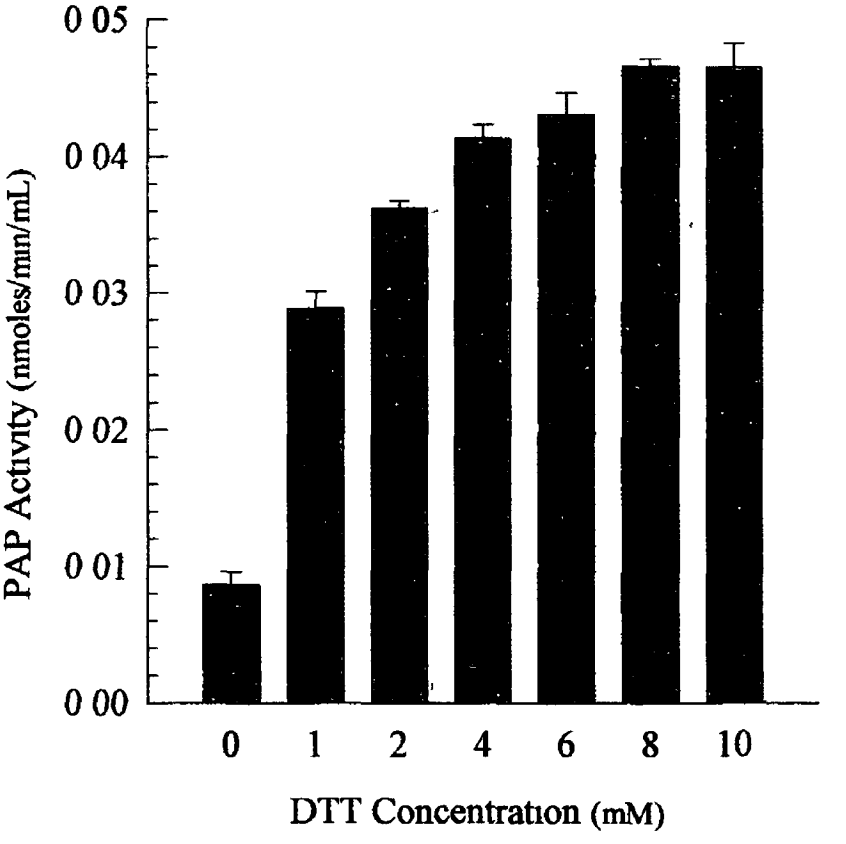


Fig. 3.24 *Effect of EDTA on purified PAP activity*

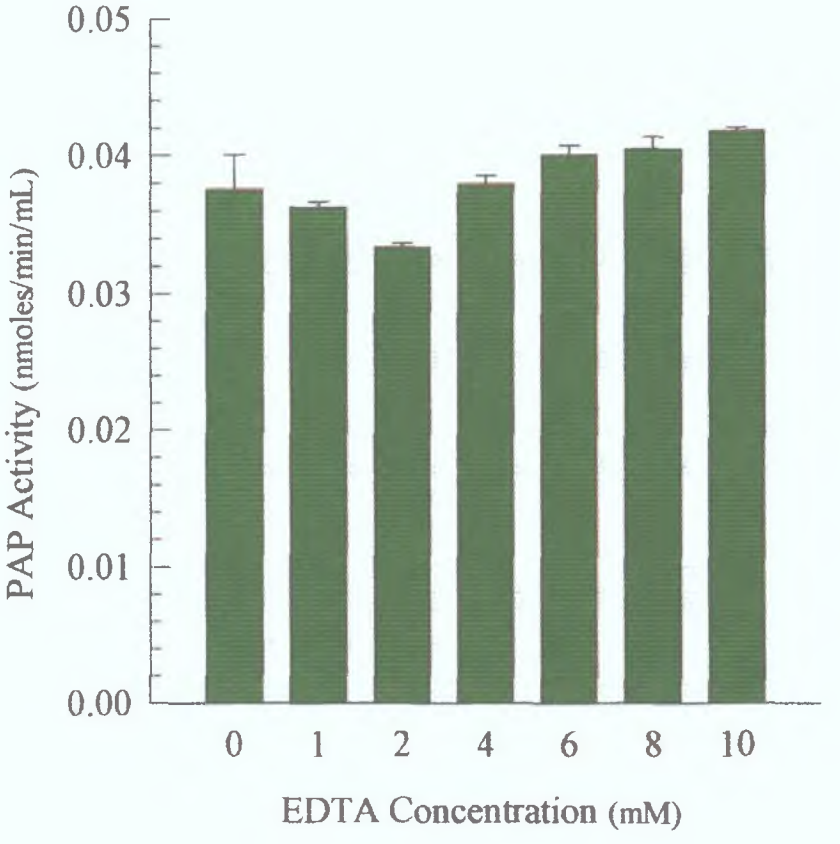
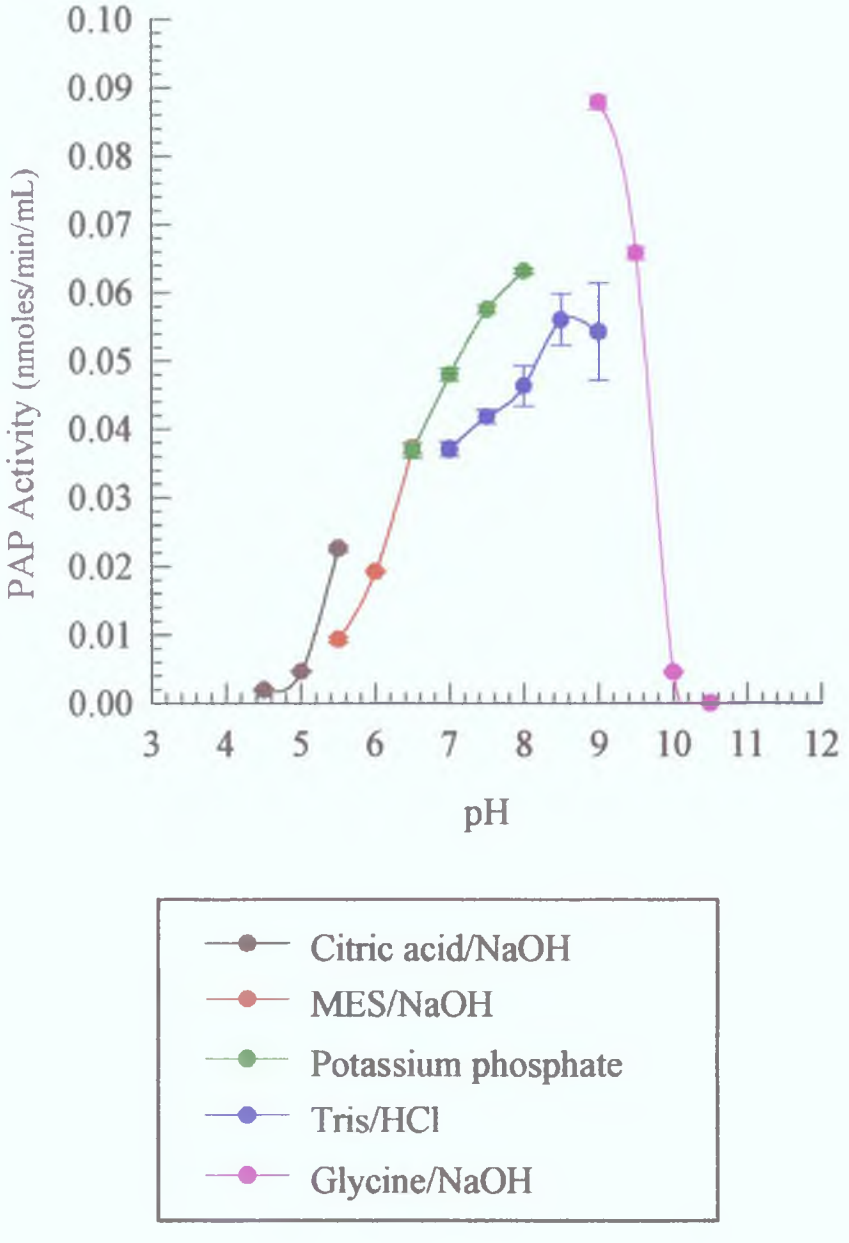


Fig. 3.25 *Effect of pH on purified PAP activity*



3.7.6 Thermostability studies

As outlined in section 2 7 6 1, the effect of incubating purified PAP for various times at different temperatures was investigated in order to assess the thermal stability of the purified enzyme Incubating purified PAP for up to 45 minutes at 37 C, prior to assay, had virtually no effect on enzyme activity, whilst at 40°C approximately 17% of activity was lost after the same period of incubation At 50°C, the enzyme activity is reduced by 29% after just 15 minutes of incubation and 65% after 45 minutes of incubation, whilst at 60 C 100% of the cytosolic PAP activity is lost after just 15 minutes of incubation The results obtained are highlighted in Fig 3 26

An investigation to examine the effect of varying the standard assay temperature was performed, as described in section 2 7 6 2, to determine if this assay proceeds more optimally at a temperature other than 37 C Purified PAP was assayed at various temperatures ranging from 30-60°C, as outlined in section 2 2 1 As seen in Fig 3 27, the highest PAP activity was observed after a 60 minute assay at 45°C (1 07 fold more activity than at 37°C)

Fig. 3.26 *Effect of preincubating purified PAP at different temperatures*

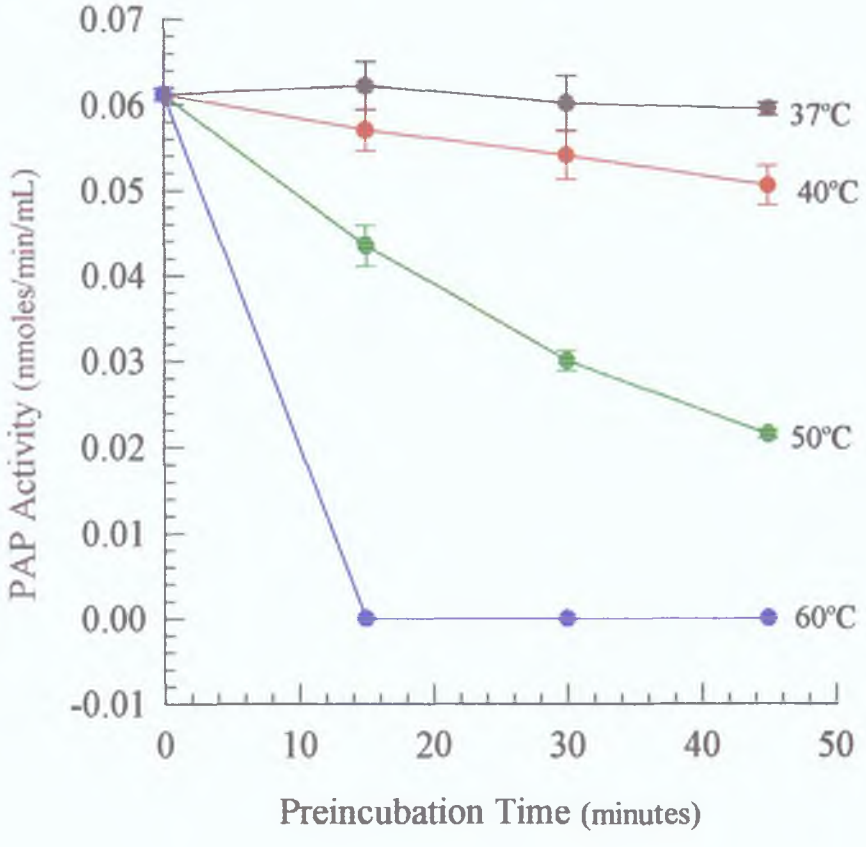
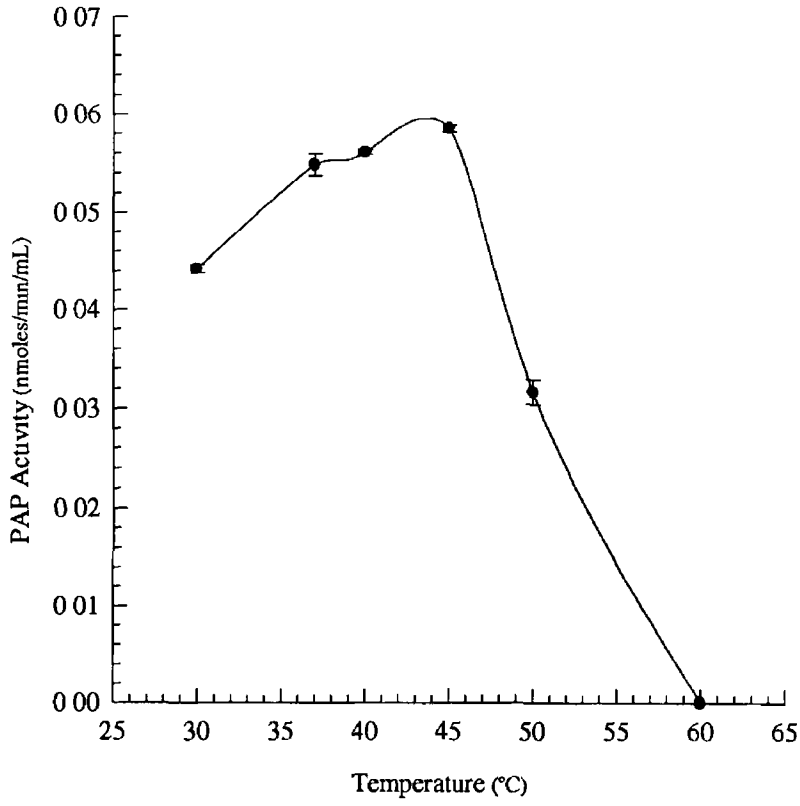


Fig 3.27 Effect of performing the cytosolic PAP assay at different temperatures



3.7 7 Effect of metal ions on cytosolic PAP activity

In section 2 7 7, the effects of a range of monovalent and divalent metal ions on the activity of purified PAP were investigated From Table 3 3 it can be seen that Hg^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} were all found to be very strong inhibitors (over 80% inhibition) of cytosolic PAP activity at a concentration of 1mM (during preincubation), whilst Ag^{2+} and Li^{+} were found to be mildly inhibitory (11% and 9% inhibition respectively) Of the other metal ions tested (Mg^{2+}, Na^{+}, Fe^{2+}, Fe^{3+} and Mn^{2+}), negligible levels of cytosolic PAP inhibition were observed None of the metal ions tested stimulated cytosolic PAP activity above control levels, nor did any have a direct or indirect effect on MCA fluorescence

In addition, three of the most inhibitory heavy metal ions, Hg^{2+}, Cu^{2+} and Zn^{2+}, were re-tested and assayed in the presence of 2mM EDTA (excluded from the above study), to determine if the inclusion of a metal chelating agent in the enzyme assay would reverse the inhibitory effect of the metal ion The inhibitory effects of Hg^{2+} and Cu^{2+} were unaffected by the presence of EDTA In contrast to this result, the inhibitory effect of Zn^{2+} was almost completely reversed (from 96% inhibition in the absence of EDTA to 7% inhibition in the presence of EDTA)

***Table 3 3** Effect of metal ions on the activity of purified PAP*

Metal	Concentration (mM)	†Relative activity (%)
Li^{+}	1	92 +/- 1 3
Hg^{2+}	1	18 +/- 0 6 (22 +/- 2 3)*
Mg^{2+}	1	96 +/- 1 3
Na^{+}	1	99 +/- 5 7
Cu^{2+}	1	19 +/- 4 9 (11 +/- 0 9)*
Fe^{2+}	1	100 +/- 0 7
Fe^{3+}	1	95 +/- 4 1
Zn^{2+}	1	5 +/- 0 (93 +/- 2 7)*
Ca^{2+}	1	95 +/- 0 8
Mn^{2+}	1	98 +/- 2 9
Ag^{2+}	1	89 +/- 4 5
Cd^{2+}	1	27 +/- 0 7

The enzyme was dialysed against 50mM potassium phosphate (pH 7 4) containing 2mM DTT and then preincubated at 37 C with the 1mM metal ions (above) After 10 minutes, residual PAP activities were assayed in the presence (*) and absence of 2mM EDTA

† Activities are the mean values of three individual determinations (+/- SD) and are expressed as a % of the control activity (100%)

3.7.8 Inhibitor studies

3 7 8 1 Effects of various functional reagents on the activity of cytosolic PAP

The effects of a wide range of functional reagents on purified PAP activity were examined in section 2 7 8 1 Compounds tested ranged from sulphydryl-blocking agents and serine protease inhibitors to metal chelators and general, non-specific protease inhibitors

Sulphydryl-blocking agents had a very profound effect on cytosolic PAP activity (Table 3 4) Iodoacetate (0 1mM), 2-iodoacetamide (0 1mM), N-ethylmaleimide (1 0mM) and PHMB (1 0mM) completely inhibited the enzymes activity At lower concentrations, N-ethylmaleimide (0 1mM) and PHMB (0 1mM) were less effective (60% and 48% inhibition respectively) E-64 (0 25mM and 0 05mM) had a significantly milder inhibitory effect (18% and 11% respectively)

Serine protease inhibitors, PMSF and benzamidine (Table 3 5), had negligible inhibitory effects on cytosolic PAP activity at the highest concentrations (1mM) tested

Of the metal chelating agents tested (8-hydroxyquinoline, 1,10-phenanthroline, 1,7-phenanthroline, 4,7-phenanthroline and EDTA), only 1,10-phenanthroline (1mM) had any inhibitory effect on cytosolic PAP activity (28% inhibition) (see Table 3 5)

Other inhibitors tested for inhibitory effects on purified PAP activity include bacitracin and puromycin (microbial protease inhibitors), N-acetylimidazole, bestatin and 2-pyrrolidone (Table 3 5) Of these inhibitors, only 2-pyrrolidone (100mM and 10mM) had any significant inhibitory effect (95% inhibition and 67% inhibition respectively) Puromycin (10mM and 1mM) had a slightly stimulatory effect

With the exception of 8-hydroxyquinoline, none of the functional reagents investigated had any effect on MCA fluorescence 1mM 8-hydroxyquinoline, under the conditions of assay, "quenched" MCA fluorescence by 8 5%

Table 3 4 *Effect of sulphydryl-blocking reagents on the activity of purified PAP*

Inhibitor	Concentration (mM)	†Relative activity (%)
2-Iodoacetamide	1 0	0
	0 1	0
Iodoacetate	1 0	0
	0 1	0
N-Ethylmaleimide	1 0	0
	0 1	40 +/- 4 5
E-64	0 25	82 +/- 6 9
	0 05	89 +/- 2 6
PHMB	1 0	5 +/- 4 8
	0 1	52 +/- 13 9

The enzyme was dialysed against 50mM potassium phosphate (pH 7 4) containing 20μM DTT and then preincubated at 37°C with the sulphydryl reagents indicated above After 10 minutes, residual PAP activities were assayed in the presence of 20μM DTT

† Activities are the mean values of three individual determinations (+/- SD) and are expressed as a % of the control activity (100%)

Table 3 5 Effect of inhibitors on the activity of purified PAP

Inhibitor	Concentration (mM)	†Relative activity (%)
2-Pyrrolidone[a]	100 0	5 +/- 0
	10 0	23 +/- 0
Bestatin[a]	1 0	99 +/- 1 8
	0 1	106 +/- 1 5
8-Hydroxyquinoline[a]	1 0	96 +/- 1 7
EDTA[b]	10 0	94 +/- 2 1
	1 0	98 +/- 2 8
1,10-Phenanthroline[a]	1 0	72 +/- 3 1
	0 1	97 +/- 1 7
1,7-Phenanthroline[a]	1 0	94 +/- 1 5
	0 1	94 +/- 1 5
4,7-Phenanthroline[a]	1 0	94 +/- 1 5
	0 1	100 +/- 3 7
Benzamidine[a]	1 0	102 +/- 2 3
	0 1	103 +/- 1 7
PMSF[a]	1 0	95 +/- 4 1
	0 1	98 +/- 2 9
N-Acetylimidazole[a]	1 0	100 +/- 1 7
	0 1	104 +/- 2 3
Bacitracin[a]	1 0	94 +/- 1 5
	0 1	101 +/- 4 5
Puromycin[a]	10 0	106 +/- 2 3
	1 0	108 +/- 0 9

The enzyme was dialysed against 50mM potassium phosphate (pH 7 4) containing DTT and EDTA (concentrations in a and b below) and then preincubated at 37°C with the inhibitors (above) After 10 minutes, residual PAP activities were assayed

[a] Assayed in the presence of 2mM DTT and 2mM EDTA

[b] Assayed in the presence of 2mM DTT

† Activities are the mean values of three individual determinations (+/- SD) and are expressed as a % of the control activity (100%)

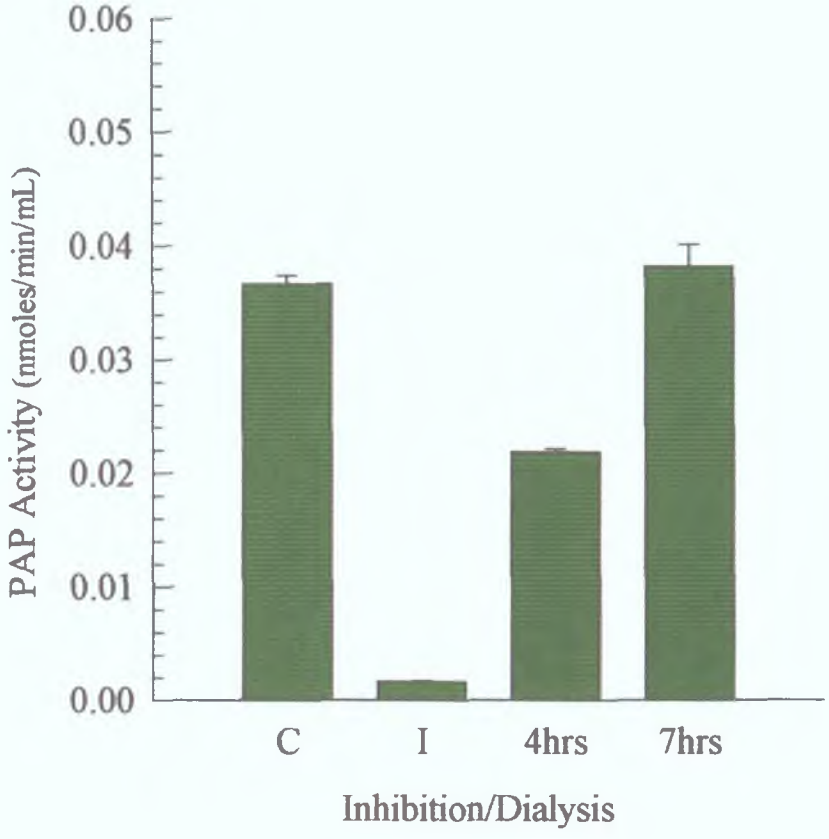
3 7.8 2 Demonstration of the reversible nature of 2-pyrrolidone inhibition

As can be seen from Table 3 5, bovine brain cytosolic PAP activity is almost completely inhibited by 0 1M 2-pyrrolidone (95% inhibition) In an attempt to determine if this inhibition could be completely reversed, the enzyme was incubated with 0 1M 2-pyrrolidone and then dialysed for up to 7 hours with buffer changes after 1, 2 and 4 hours, as outlined in section 2 7 8 2 As seen in Fig 3 28, after 4 hours dialysis, 60% of the inhibited PAP activity was recovered After a further 3 hours dialysis, cytosolic PAP activity was completely restored to normal control levels

3 7 8 3 Use of a continuous, real-time assay to examine the nature of cytosolic PAP inhibition by 2-pyrrolidone and 1,10-phenanthrohne

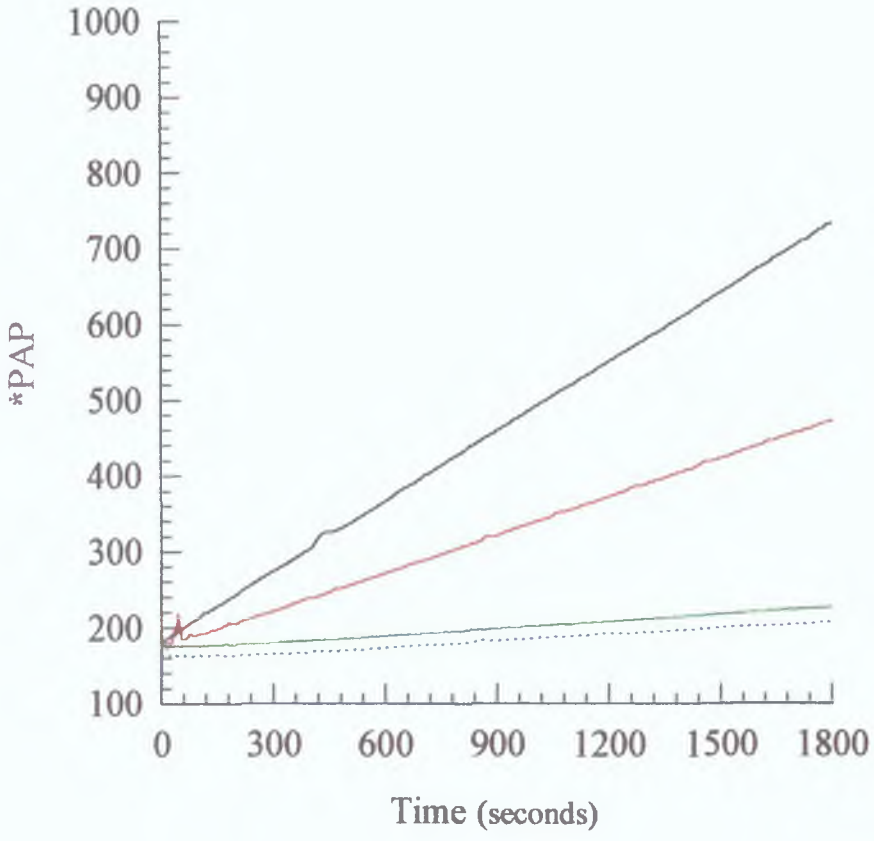
As outlined in section 2 7 8 3, a continuous, real-time fluorimetric assay, using pGlu-MCA as the substrate, was devised in an attempt to examine the rapidity with which certain inhibitors of cytosolic PAP act on the enzyme Fig 3 29 describes the continuous assay of purified cytosolic PAP, over a 30 minute period, in the presence of 10mM and 0 5mM 2-pyrrolidone Suitable positive and negative controls can also be seen on this plot Fig 3 30 describes the continuous assay of purified cytosolic PAP, over a 30 minute period, in the presence of 1mM and 0 2mM 1,10-phenanthroline with suitable positive and negative controls also included In both cases, the enzyme hydrolysed the substrate in a perfectly linear fashion, both in the absence and presence of inhibitor, regardless of inhibitor concentration

Fig. 3.28 *Reversibility of PAP inhibition by 2-pyrrolidone*



Control (C) = 100%
Inhibited (I) = 4.4%
4 hour dialysis (4hrs) = 59.7%
7 hour dialysis (7hrs) = 104%

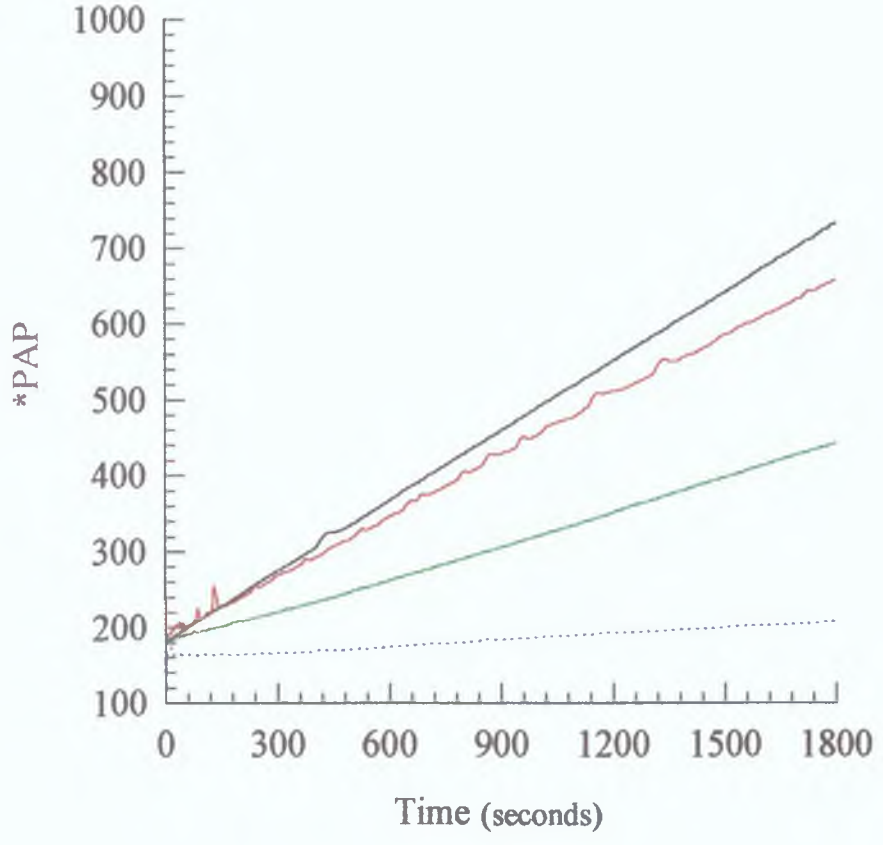
Fig. 3.29 *PAP inhibition by 2-Pyrrolidone (real-time)*



*Enzyme activities in Fluorescence Units
Excitation slit width = 10nm
Emission slit width = 10nm

Positive control (substrate + enzyme + buffer)
0.5mM 2-Pyrrolidone
10mM 2-Pyrrolidone
Negative control (substrate + buffer)

Fig. 3.30 *PAP inhibition by 1,10-Phenanthroline (real-time)*



*Enzyme activities in Fluorescence Units
Excitation slit width = 10nm
Emission slit width = 10nm

——— Positive control (substrate + enzyme + buffer)
——— 0.2mM 1,10-Phenanthroline
——— 1mM 1,10-Phenanthroline
····· Negative control (substrate + buffer)

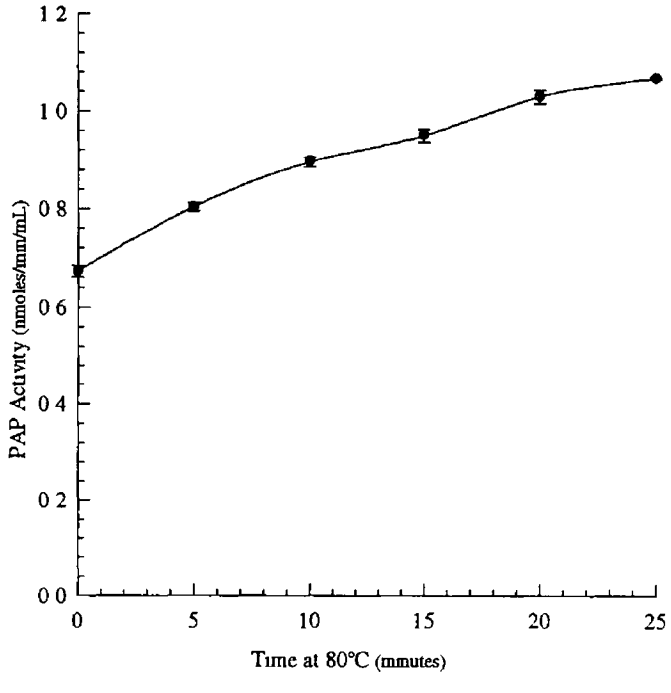
3 7 9 Kinetic studies

The reader is directed to the short appendix at the end of this report for an overview of the equations describing the kinetic models used in this study

3 7 9 1 Validation of the cytosolic PAP assay using pGlu-His-Pro-MCA as a substrate

As outlined in section 2 7 9 2, a modification of the standard assay outlined in section 2 2 1 was used when assaying cytosolic PAP activity with the fluorimetric TRH analog, pGlu-His-Pro-MCA (required for K_m determination below) This assay, described in Fig 2 2, focuses upon the release of MCA from the His-Pro-MCA fragment generated by the action of cytosolic PAP on the substrate The rapid and complete release of MCA from all of the His-Pro-MCA produced during the assay therefore, is essential if the assay is to have a sound quantitative basis To facilitate MCA release after termination of the assay, heating of samples at 80 C to increase the cyclization rate of His-Pro-MCA was employed (Prasad *et al*, 1983, Prasad and Jayaraman, 1986, Prasad, 1987) Fig 3 31 describes the effect of heating crude (S_2) cytosolic PAP samples, stopped with 1 5M acetic acid after a 1 hour assay with pGlu-His-Pro-MCA, for up to 25 minutes at 80 C in order to examine the rate of MCA release From this graph it can be seen that MCA release is virtually complete after 25 minutes

Fig 3 31 The cyclisation of His-Pro-MCA at 80°C



3.7 9 2 K_m determinations for various fluorimetric substrates

The hydrolysis of the synthetic pyroglutamyl substrates pGlu-MCA, pGlu-His-Pro-MCA (TRH analog) and pGlu-ßNA by cytosolic PAP, as outlined in sections 2 7 9 1 and 2 7 9 2, were found to obey Michaelis-Menten kinetics (Michaelis and Menten, 1913) The data obtained for each of the substrates was plotted using five different kinetic models, all of which are based upon the steady-state assumption made by the Michaelis-Menten model These kinetic models include, (1) Michaelis-Menten, (2) Lineweaver-Burk, (3) Eadie-Hofstee, (4) Hanes-Woolf and (5) Direct Linear plots The Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plots are all based upon reciprocal transformations of the Michaelis-Menten equation (Lineweaver and Burk, 1934, Hofstee *et al*, 1959, Hanes, 1932), whilst the Direct Linear plot (Eisenthal and Cornish-Bowden, 1974), widely regarded as the most accurate kinetic model for the evaluation of kinetic constants, is a graphical method which does not rely upon any such transformation

From each plot, the Michaelis-Menten constant or K_m of purified PAP for the substrate was evaluated It was observed that for each of the substrates, the K_m obtained from all five plots was almost identical Figs 3 32-35 and Figs 3 36-39 display the first four of the above plots for the substrates pGlu-MCA and pGlu-His-Pro-MCA respectively, whilst Table 3 6 displays the K_m values obtained via the Direct Linear plot method

Table 3 6 *Hydrolysis of fluorimetric and chromogenic substrates by purified PAP*

Substrate	Hydrolysis of substrate	K_m (µM)
pGlu-MCA	+	15 36
pGlu-His-Pro-MCA	+	13 64
pGlu-ßNA	+	20 76
pGlu-pNA	+	N D

K_m values were determined by the Direct Linear plot method of Eisenthal and Cornish-Bowden (1974)

+ Indicates hydrolysis N D Not determined

Fig 3 32 Determination of cytosolic PAP K_m for pGlu-MCA (Michaelis-Menten)

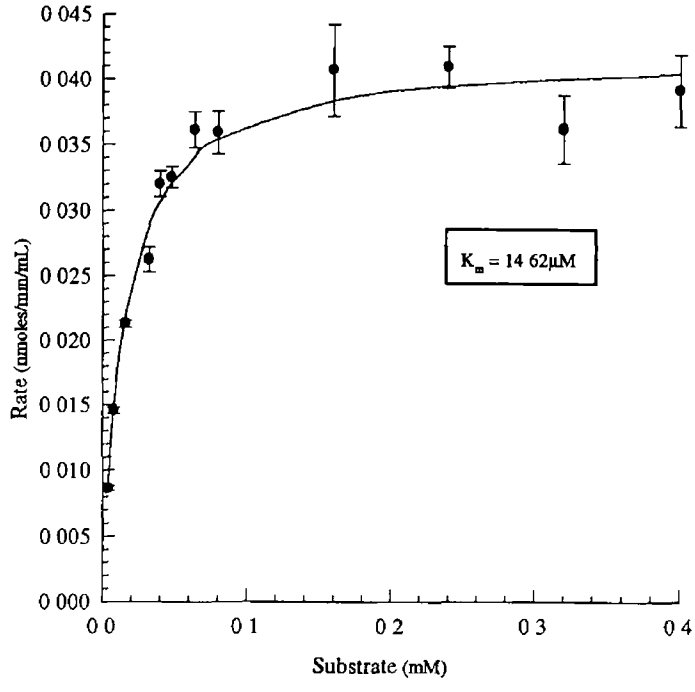


Fig 3 33 Determination of cytosolic PAP K_m for pGlu-MCA (Lineweaver-Burk)

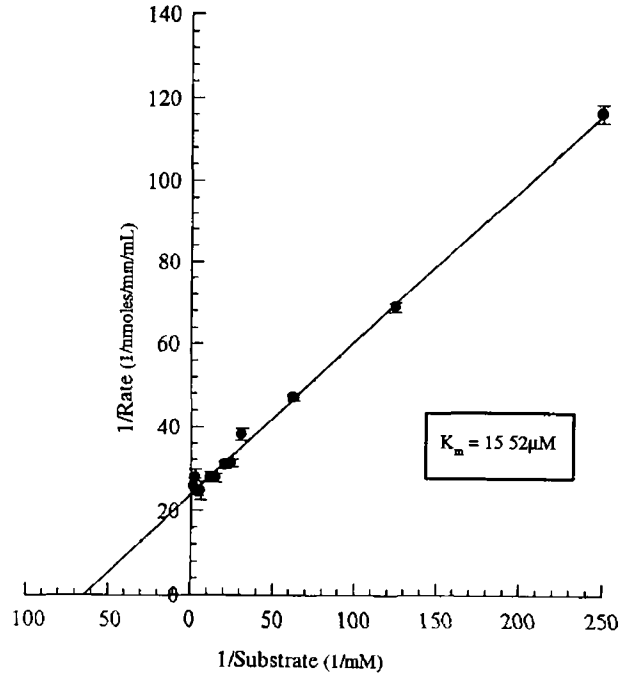


Fig 3 34 Determination of cytosolic PAP K_m for pGlu-MCA (Eadie-Hofstee)

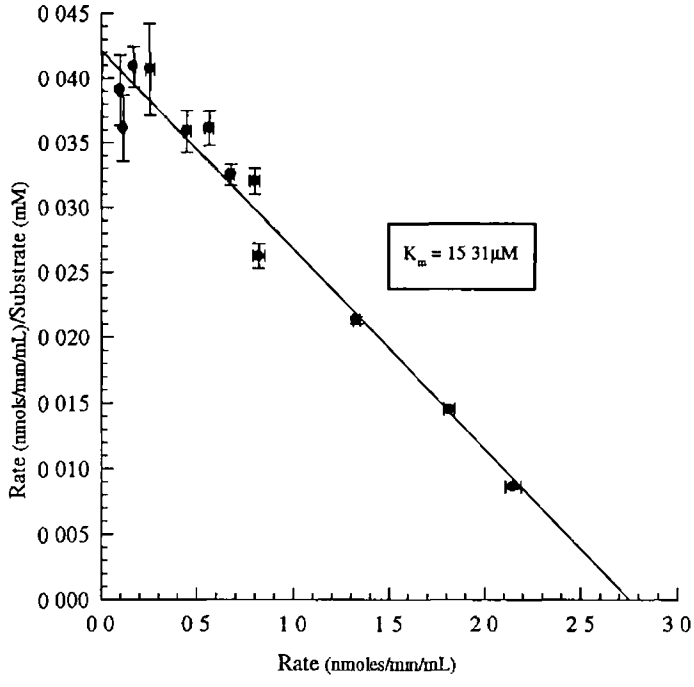


Fig 3 35 Determination of cytosolic PAP K_m for pGlu-MCA (Hanes-Woolf)

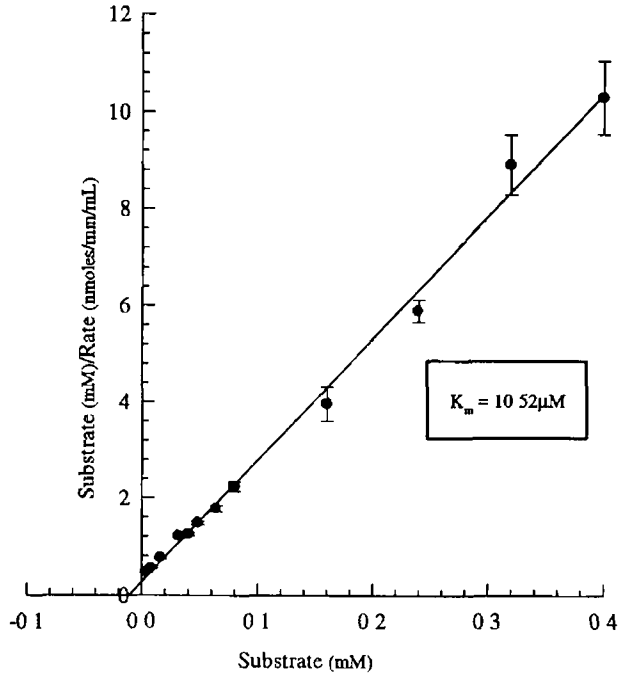


Fig 3 36 Determination of cytosolic PAP K_m for pGlu-His-Pro-MCA (Michaelis-Menten)

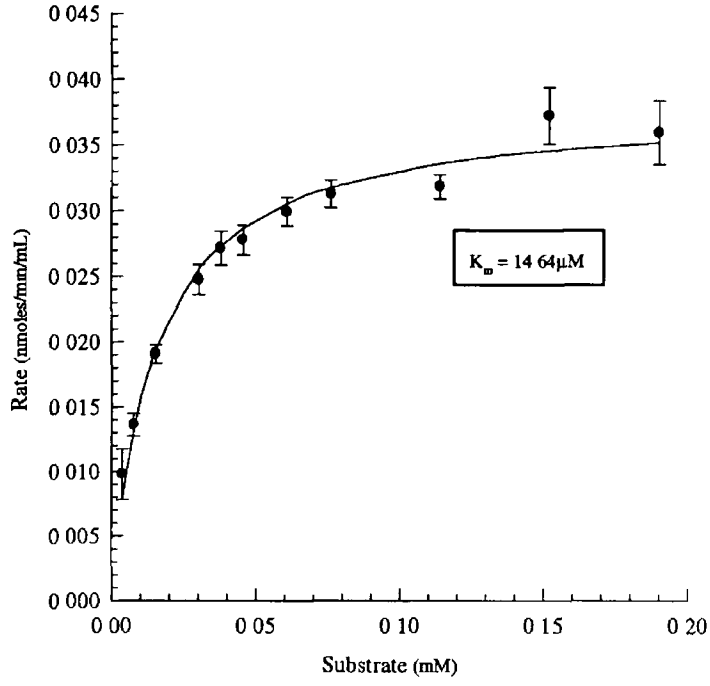


Fig 3 37 Determination of cytosolic PAP K_m for pGlu-His-Pro-MCA (Lineweaver-Burk)

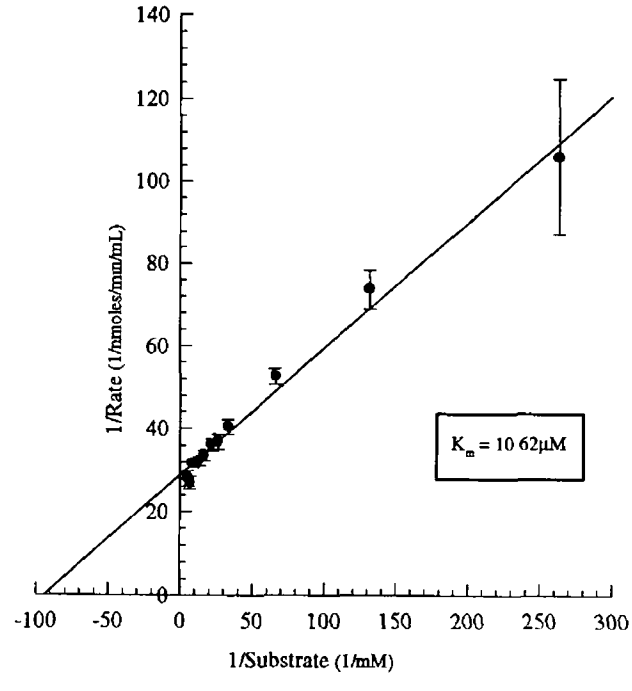


Fig 3 38 Determination of cytosolic PAP K_m for pGlu-His-Pro-MCA (Eadie-Hofstee)

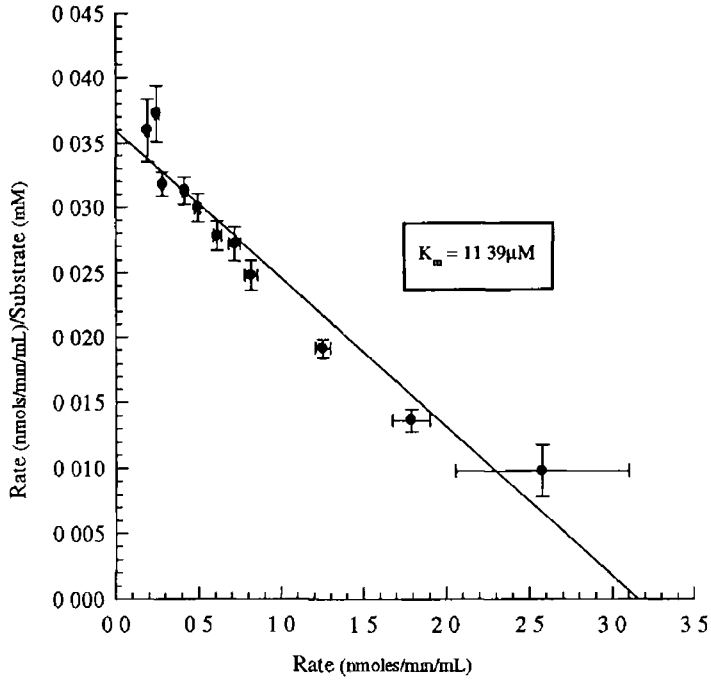
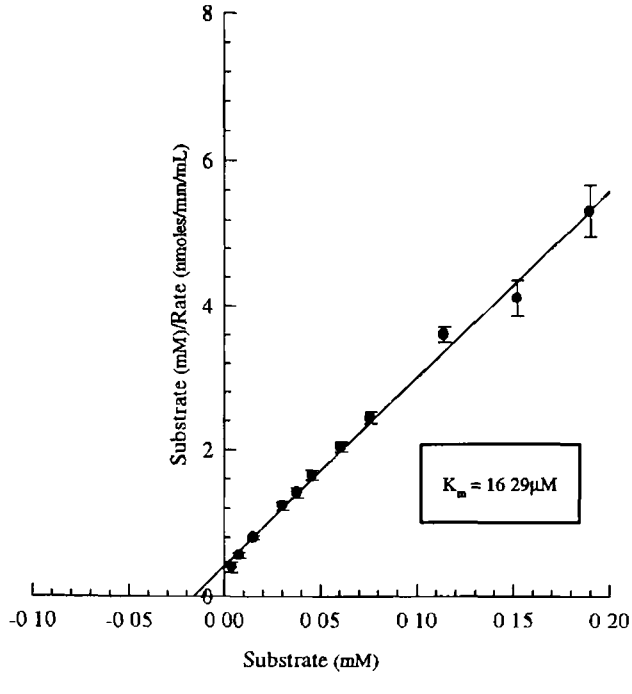


Fig 3 39 Determination of cytosolic PAP K_m for pGlu-His-Pro-MCA (Hanes-Woolf)



3 7 9.3 K_i determinations for various pyroglutamyl peptides

The inhibitor Constant or K_1 of purified PAP for a range of pyroglutamyl peptides was investigated using pGlu-MCA as a substrate (section 2 7 9 3) The data obtained for each of the peptides was plotted using three of the aforementioned kinetic models, namely the (1) Lineweaver-Burk, (2) Eadie-Hofstee and (3) Hanes-Woolf models Figs 3 40-42 display the Lineweaver-Burk plots obtained for purified cytosolic PAP assayed in the absence and presence of some of the peptides tested, whilst Table 3 7 lists the K_1 values obtained for all of the peptides tested using all three of the above models All three kinetic models generated a similar K_1 for any given peptide In addition, all of the peptides tested were found to be competitive inhibitors The physiological peptides LHRH, TRH, acid TRH, bombesin and pGlu-His-Gly (anorexogenic peptide) all proved to be very effective competitive inhibitors of the purified enzyme Conversely, the synthetic peptides pGlu-Ala, pGlu-His and, in particular, pGlu-Val and pGlu-Pro-NH_2 were far less effective as competitive inhibitors of the enzyme

Table 3 7 *Cytosolic PAP K_I values for a range of pyroglutamyl peptides*

Peptide	Inhibition type	[1]K_1 (μM)	[2]K_1 (μM)	[3]K_1 (μM)
LHRH	Competitive	20 6	24 2	25 1
Acid TRH	Competitive	23 1	17 8	18 2
TRH	Competitive	24 9	23 9	25 4
pGlu-His-Gly	Competitive	58 8	38 7	42 8
Bombesin	Competitive	64 2	69 8	73 4
pGlu-Ala	Competitive	101 8	85 3	94 5
pGlu-His	Competitive	255 4	277 5	296 7
pGlu-Val	Competitive	820 8	643 5	895 3
pGlu-Pro-NH_2	Competitive	827 6	898 8	977 3

[1]Lineweaver-Burk method

[2]Hanes-Woolf method

[3]Eadie-Hofstee method

Fig 3 40 Determination of cytosolic PAP K_i for TRH

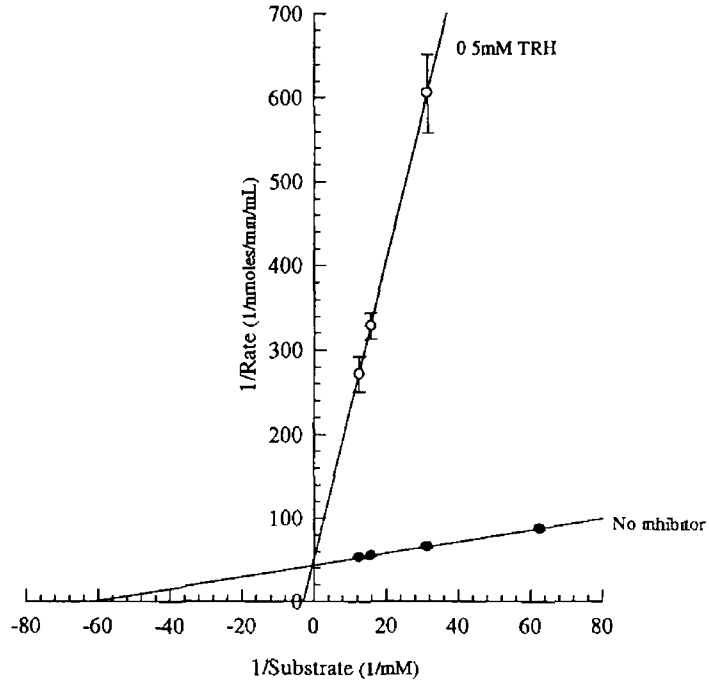


Fig 3 41 Determination of cytosolic PAP K_i for acid TRH

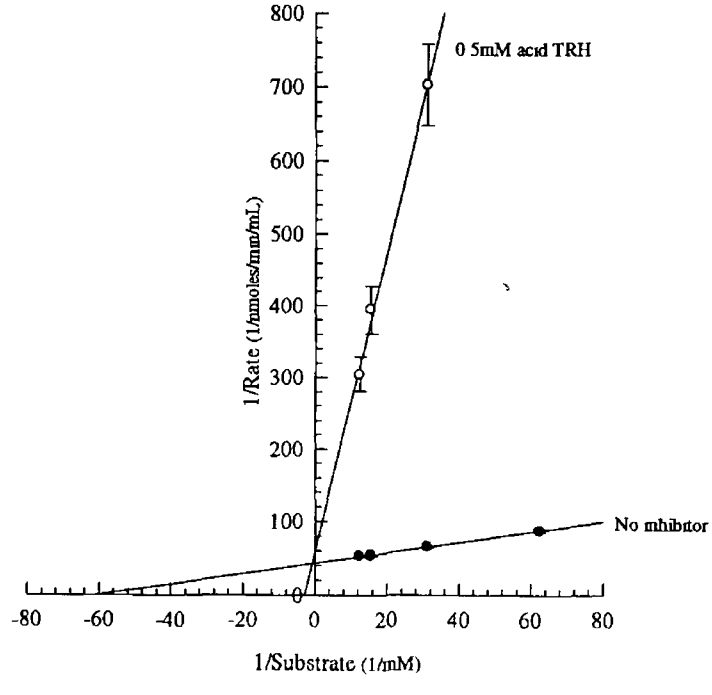
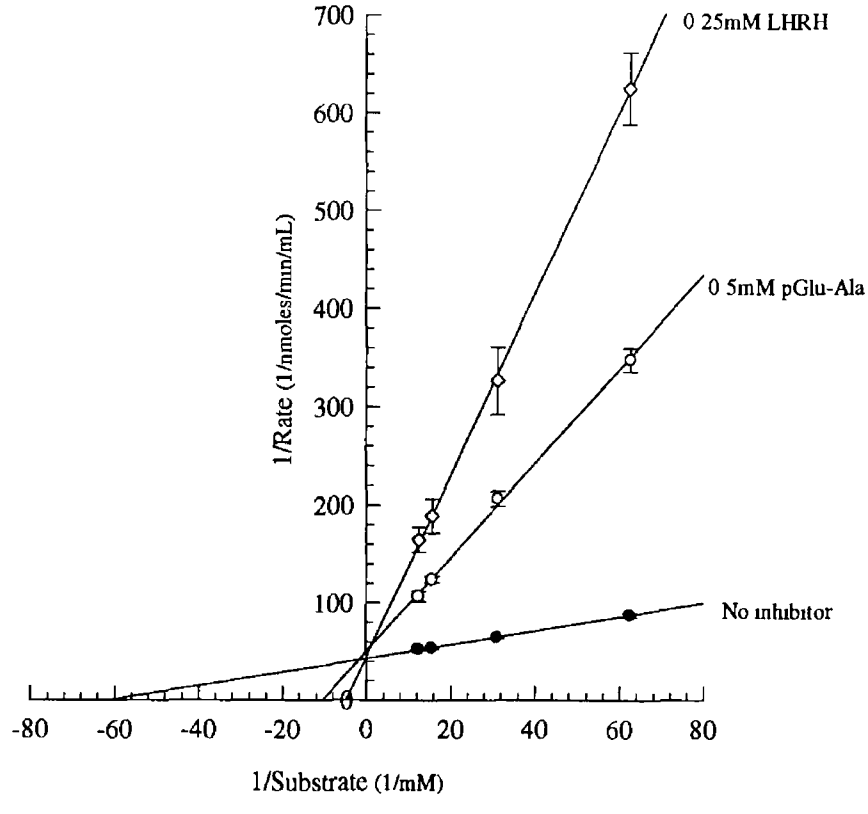


Fig 3 42 Determination of cytosolic PAP K_i for pGlu-Ala and LH-RH



3 7.10 The substrate specificity of cytosolic PAP as examined by HPLC

Section 2 7 10 1 describes the incubation of post anion-exchange PAP with a range of synthetic pyroglutamyl substrates Enzyme/substrate incubations were allowed to proceed for up to 40 hours in parallel with negative control incubations in which enzyme was excluded until after the 40 hour incubation period The peptidase inhibitors, bestatin and Z-pro-prolinal, were also included in all incubations Samples and negative controls were subsequently analysed using a C-8 (octyl) reversed phase HPLC column as described in section 2 7 10 2

Scanning spectrophotometry using a Beckman photo-diode array detector (Diode Array Detector Module 168) revealed a single absorbance maxima at approximately 207nm for pyroglutamic acid Due to its relatively hydrophilic properties, pyroglutamic acid elutes quite rapidly (6 3 minutes) from the reversed phase C-8 column, well away from virtually all of the other substrates and metabolites under investigation in this study

3 7 10 1 The degradation of TRH and acid TRH by cytosolic PAP

Following incubation of post anion-exchange PAP with TRH (pGlu-His-Pro-NH_2), subsequent HPLC analysis of the enzyme digest revealed two main cleavage products, namely, pyroglutamic acid and cyclo(His-Pro) (see Figs 3 43a/b) Similarly, incubation of cytosolic PAP with acid TRH (pGlu-His-Pro), a primary TRH metabolite, generated pyroglutamic acid and His-Pro as the main cleavage products (see Figs 3 44a/b) Both substrates were hydrolysed almost completely over the 40 hour incubation Both chromatograms are overlayed with the corresponding negative control chromatogram From the control chromatograms, one can observe the presence of small amounts of the cleavage products pyroglutamic acid, His-Pro and cyclo(His-Pro)

Fig. 3.43a *Degradation of TRH by cytosolic PAP (HPLC analysis)*

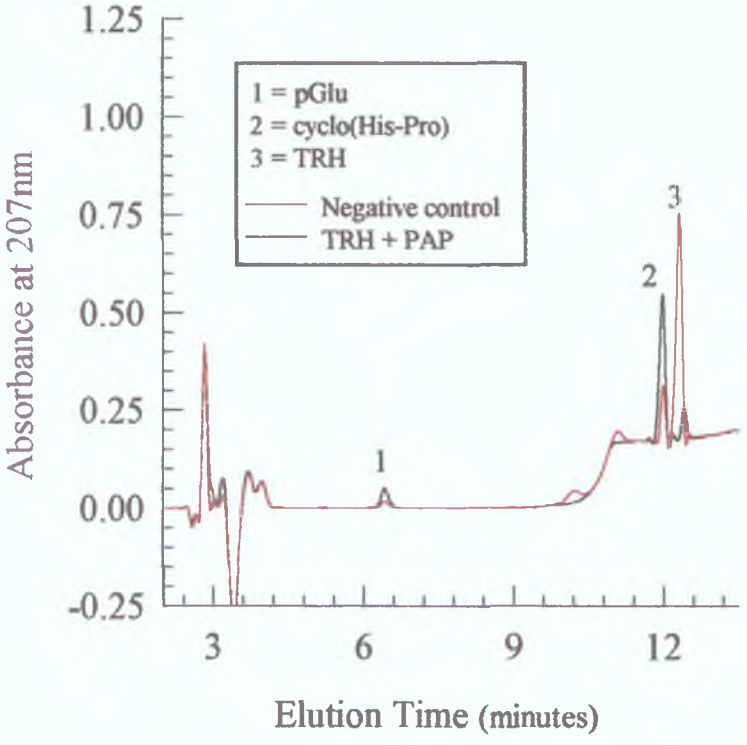


Fig. 3.43b *Magnification of the pGlu peak (1)*

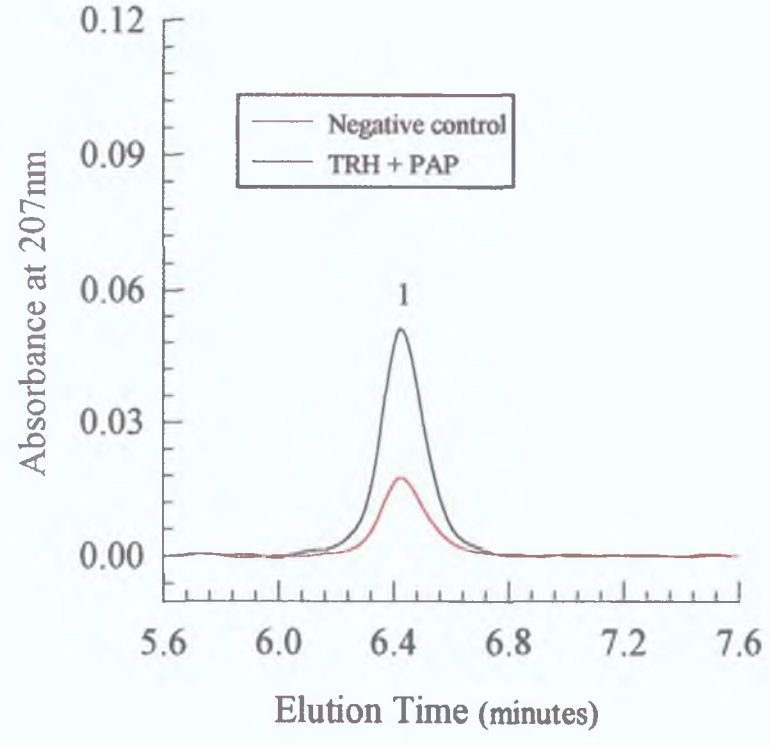


Fig. 3.44a *Degradation of acid TRH by cytosolic PAP (HPLC analysis)*

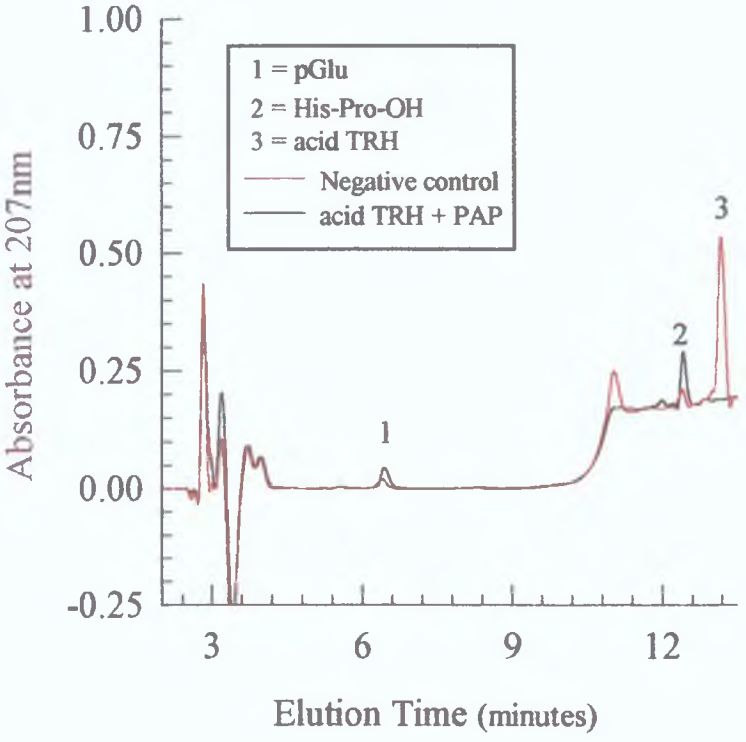
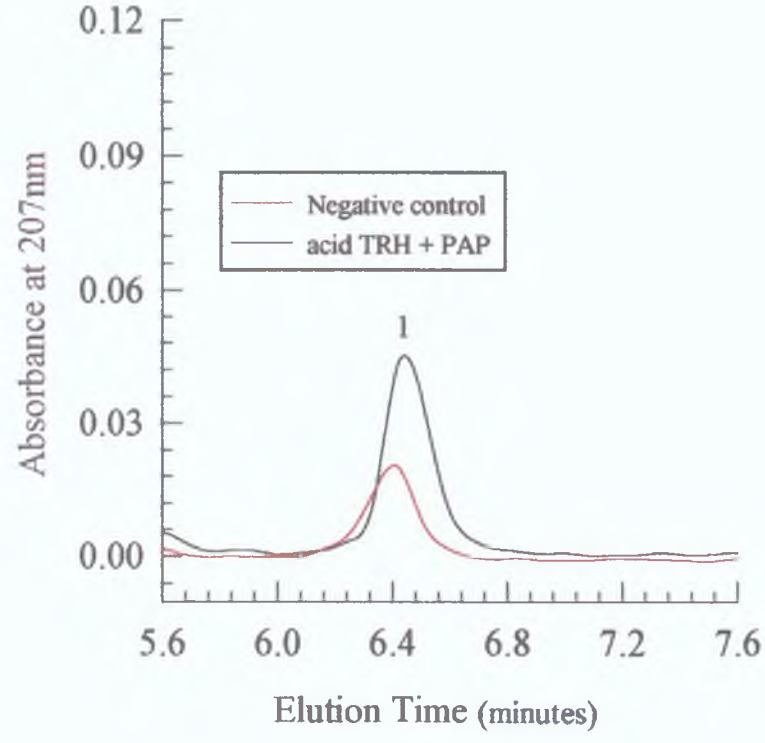


Fig. 3.44b *Magnification of the pGlu peak (1)*



3 7 10 2 Ability of cytosolic PAP to degrade various pyroglutamyl substrates

Table 3 8 highlights the complete range of synthetic pyroglutamyl substrates which were used to examine the substrate specificity of cytosolic PAP, as outlined in section 2 7 10 All of the substrates investigated, with the exception of LHRH (pGlu-His-Trp-) and eledoisin (pGlu-Pro-Ser-) were hydrolysed at this bond by post anion-exchange PAP

Table 3 8 Hydrolysis of pyroglutamyl peptides by purified PAP

Substrate	Position of cleavage	Substrate concentration during assay (mM)	Hydrolysis of substrate
1 TRH	pGlu-His	0 55	+
2 Acid TRH	pGlu-His	0 55	+
3 pGlu-His-Gly	pGlu-His	0 55	+
4 pGlu-His-Gly-NH_2	pGlu-His	0 55	+
5 pGlu-Ala	pGlu-Ala	0 55	+
6 pGlu-His	pGlu-His	0 55	+
7 pGlu-Val	pGlu-Val	0 55	+
8 LH-RH	-	0 28	-
9 Neurotensin	pGlu-Leu	0 16	+
10 Bombesin	pGlu-Gln	0 1	+
11 Eledoisin	-	0 13	-

+ Indicates hydrolysis - Indicates no hydrolysis

4. DISCUSSION

PREFACE

Cytosolic pyroglutamyl aminopeptidase (EC 3 4 19 3) can be classified as an omega peptidase (McDonald and Barrett, 1986) which hydrolytically removes the pyroglutamyl residue from the amino terminus of pGlu-peptides and proteins (Fig 1 1) This enzyme (referred to as PAP-I in animals) has been shown to display a specificity for L-pGlu-L-amino acid optical isomers (Uliana and Doolittle, 1969) Several studies have previously described the purification and characterisation of soluble PAP activities from different mammalian tissues including guinea-pig brain (Browne and O'Cuinn, 1983a) and human cerebral cortex, kidney and skeletal muscle (Lauffart *et al* 1989, Mantle *et al*, 1990, 1991) However, despite earlier work by Wilk *et al* (1985) and Mudge and Fellows (1973) detailing the partial purification of a soluble PAP activity from bovine whole brain and pituitary respectively, with a brief examination of some of the enzymes main properties, the full purification and characterisation of this soluble enzyme activity from bovine brain has never been reported This research project subsequently describes the complete purification of a PAP activity from the soluble fraction of bovine whole brain. A detailed examination of the biochemical properties of the purified enzyme is also presented.

The ensuing sections will endeavour to examine some of the more interesting aspects of this work in closer detail and will include a comparison of the current findings with those previously reported for this enzyme by other workers

4.1 MCA standard curve preparation: The effect of "quenching"

As outlined in section 2 2 1, the specific fluorimetric substrate, pGlu-MCA, was used to assay for cytosolic PAP activity in bovine brain, the enzymatic release of MCA subsequently being monitored fluorimetrically at excitation and emission wavelengths of 370 and 440nm respectively MCA release could then be quantitated by using a standard curve prepared with the latter compound under corresponding assay conditions and read at the corresponding slit widths For quantitative purposes, one set of MCA standard curves was prepared in the absence of enzyme, whilst one set incorporated crude enzyme samples (i e a standard curve incorporating crude homogenate and a standard curve incorporating crude cytosol) in order to correct for the fluorimetric "quenching" effect observed with crude samples (section 3 1) Quenching, unless corrected for, can result in falsely low measurements of enzyme activity in crude enzyme samples, and results from the absorbance of electromagnetic radiation, at both the excitation and emission wavelengths, by proteins and other contaminants present in crude samples

Fig 3 3 compares an MCA standard curve prepared normally (i e in the absence of crude sample) to MCA standard curves prepared under identical conditions in the presence of crude homogenate and crude cytosol respectively From this plot it can be seen that in the presence of crude homogenate the MCA standard curve is quenched by up to 17 2%, whilst in the presence of crude cytosol, this figure is as high as 27 5% The apparently lower degree of quenching observed with crude homogenate (17 2%) can be explained thus, Prior to reading the fluorescence of any given MCA standard (or sample) prepared with crude homogenate, this standard must be spun down at 10,000rpm for 10 minutes in a microfuge to remove particulate material present in the homogenate The fluorescence of the supernatant is then determined as previously described in sections 2 2 1 and 3 1 2 If one can assume that MCA is excluded from the pellet, then this compound will be present at a slightly higher concentration in the supernatant than it would be in a corresponding MCA standard simply prepared with crude cytosol Consequently, an MCA standard prepared with crude homogenate will give a slightly higher fluorescence reading than the same MCA standard prepared with crude cytosol, and subsequently, an apparently lower degree of quenching

Overall therefore, these results would appear to justify the decision to prepare separate MCA standard curves for crude samples derived from this tissue when determining enzyme activity within these samples This quenching phenomenon was not observed with any of the partially purified (post column) enzyme samples

4.2 Subcellular localisation studies

High speed centrifugation and salt washing procedures, as outlined in section 2 4 were employed in order to identify the subcellular location of the pGlu-MCA hydrolysing PAP activity observed in

bovine brain homogenates From Fig 3 6 it can be seen that over 85% of the pGlu-MCA hydrolysing PAP activity was found to be located in the soluble fraction of bovine brain, a significantly smaller proportion of this enzyme activity (13%) was found to be associated with the particulate fraction, whilst 1 9% of the total PAP activity initially observed in the crude homogenate remained unaccounted for following the centrifugation process (Fig 3 6) The possibility that weak, non-specific interactions between the enzyme molecule and membrane components might be responsible for the observation of this enzyme activity within the particulate fraction was also investigated, as outlined in section 2 4 2 Salt washing of the particulate fraction with up to 3M NaCl had no effect on the release of pGlu-MCA hydrolysing PAP activity from the membranes Based on previous findings that the high molecular mass, membrane-bound PAP activity (PAP-II) observed in mammalian brain membrane preparations displays an extremely high degree of substrate specificity restricted to TRH or closely related peptides (O'Connor and O'Cuinn, 1984, O'Leary and O'Connor, 1995a), it was concluded that the pGlu-MCA hydrolysing PAP activity observed in the particulate fraction was, most likely, soluble PAP activity which had become "occluded" within vesicles that are formed under the vigorous homogenisation conditions Van Amsterdam *et al* (1983) have previously claimed that proteins loosely adsorbed to membrane structures can be mistaken for membrane-bound activities, due to insufficient washing of the membranes These researchers have also suggested the use of an osmotic shock technique which entails washing the membranes in distilled water in order to lyse entrapped vesicles formed during the homogenisation procedure which may be harbouring occluded soluble enzyme activity This technique was not performed in this instance

These results are consistent with previous findings that the pGlu-MCA hydrolysing PAP activity observed in different mammalian tissues typically has a cytosolic location (Browne and O'Cuinn, 1983a, Mantle *et al*, 1990, 1991, Lauffart *et al*, 1989, O'Connor and O'Cuinn, 1984) Indeed, Mudge and Fellows (1973) have previously demonstrated that a pGlu-Ala hydrolysing PAP activity observed in bovine pituitary homogenates could be completely localised to the soluble fraction following an ultracentrifugation procedure (100,000g) Noteworthy also is the finding that bacterial PAP activities, with the exception of the *K cloacae* PAP (Kwiatkowska *et al*, 1974), have all been shown to be soluble proteins located in the cell cytosol (Awade *et al*, 1992a, 1992b, Tsuru *et al*, 1978, Cleuziat *et al*, 1992a) More recently, the characterisation of a number of bacterial PAP genes has revealed that these enzymes lack a post-translational processed signal sequence, a finding consistent with a cytosolic location (Gonzales *et al*, 1992, Awade *et al*, 1992b)

4.3 Purification of PAP activity from bovine brain cytosol

4 3 1 Column chromatography

Anion-exchange chromatography using DEAE Sepharose Fast Flow proved to be an ideal first step in the purification of PAP from bovine brain cytosol Ease of use, high loading capacity, relatively good separation of cytosolic PAP activity away from the bulk of contaminating proteins (Fig 3 7) and

excellent active recovery (85 8% of total applied activity) characterised the use of this technique The majority of cytosolic components did not bind to the exchanger matrix under running buffer conditions, described in section 2 5 2, and simply ran through the column

Gel-filtration chromatography of post anion-exchange PAP activity on a Sephacryl S-200 HR column enabled excellent separation of PAP from other cytosolic components to be achieved (Fig 3 8) Unfortunately, the recovery of active enzyme from this column was quite poor (17 7% of total applied activity) In an earlier attempt to partially purify cytosolic PAP activity from bovine pituitary using a Sephadex G-200 gel-filtration column, Mudge and Fellows (1973) have reported a similarly low active recovery (21 8% of total applied activity), whilst Wilk *et al* (1985) also report such a finding following the partial purification of cytosolic PAP activity, by gel-filtration, from bovine whole brain The exact cause(s) of such poor active recovery have yet to be definitively identified, although proteolytic hydrolysis of the enzyme, the instability of the enzyme in dilute solution and/or the separation of the enzyme from low molecular mass modulatory peptides (stabilising or inhibiting) remain distinct possibilities Initial attempts to partially purify soluble PAP activities from pigeon and rabbit liver (Szewczuk and Kwiatkowska, 1970), rat liver (Armentrout, 1969) and bacteria (Doolittle and Armentrout, 1968, Armentrout and Doolittle, 1969) clearly highlight the extreme instability of this enzyme in dilute solution The recent findings of Ohmori *et al* (1994) however, lend credence to the latter possibility These researchers have demonstrated the existence of endogenous low molecular mass peptides in bovine brain cytosol which display specific inhibitory activity towards prolyl endopeptidase isolated from this source and from bacteria

Affinity chromatography of post gel-filtration PAP activity exploited the sulphydryl nature of this enzyme and subsequently proved to be an efficient final "clean-up" step Using an Activated Thiol Sepharose 4B column, PAP activity could be specifically bound to the column through the formation of disulphide linkages between the matrix functional groups and intact thiol groups located within the active site of the enzyme (Figs 3 9 and 3 10) The recovery of cytosolic PAP activity from this column was moderately good (54% of total applied activity)

The overall recovery of cytosolic PAP activity was approximately 6 6% This result can be attributed to the very poor recovery of active enzyme from the gel-filtration step As a consequence of this, a purification factor of 91 01 (lower than initially expected) was obtained

4 3 2 SDS PAGE analysis

The photographic evidence presented in Figs 3 13 and 3 14a/b demonstrate that the gel-filtration and affinity chromatography steps in particular, have a very significant effect on the separation of soluble PAP activity away from other cytosolic components Noteworthy also is the difference in band staining efficiency observed when using both Coomassie Brilliant Blue G and silver stain For example, the post gel-filtration PAP in lanes 4 and 5 of Fig 3 13, as visualised by Coomassie staining appears to be

present as a single band Subsequent staining of either of these lanes with silver stain however reveals the presence of several minor bands (Fig 3 14a, Lane 2)

The silver stained protein band corresponding to purified cytosolic PAP activity is displayed in Lane 1 of Fig 3 14a Towards the top of this lane, two additional bands can also be seen In order to demonstrate the "artefactual" nature of these two bands, a 3 6 fold concentrate of the purified enzyme (prepared by reverse osmosis using PEG 6000) was re-analysed by SDS PAGE to yield the result shown in Fig 3 14b (Lane 1) One clearly notes the absence of these bands Indeed, Bauer (1994) has suggested that artefactual bands can arise during silver staining when 2-mercaptoethanol is used in the sample buffer (as in this case) From Fig 3 14b it would also appear that the enzyme has been purified to homogeneity However, at least two additional faint minor bands were observed, in the vicinity of the PAP band, which subsequently faded following reduction of the gel to remove background staining (Table 2 3) Consequently, one can only conclude that the enzyme has apparently been purified to near homogeneity using this purification scheme

From these gels, the purified (and partially purified) cytosolic PAP can be seen to migrate slightly further than the carbonic anhydrase marker (29,000 daltons) to a position representing a relative molecular mass of approximately 22,450 daltons (non-native, denaturing conditions)

4 3 3 Monitoring of other TRH-inactivating enzyme activities throughout the PAP purification scheme

In addition to PAP, two other soluble enzyme activities known to participate in TRH inactivation, *in vitro*, were observed in bovine brain cytosol Using specific fluorimetric substrates (see section 2 2) dipeptidyl aminopeptidase (DAP) and prolyl endopeptidase (PE) were both detected in bovine brain cytosol (the reader is directed to section 1 3 5 for a more detailed review of these enzyme activities in regard to TRH catabolism) Sephacryl S-200 HR gel-filtration chromatography was sufficient to resolve soluble PAP activity from both of these enzymes (Fig 3 12)

DAP activity, detected using the dipeptidyl substrates Lys-Ala-MCA and Gly-Pro-MCA, appears to correspond to a single, high molecular mass enzyme (Fig 3 11 - 190,600 daltons) displaying a sensitivity to puromycin In addition, this enzyme was seen to hydrolyse the Gly-Pro-MCA substrate approximately 2 4 times more rapidly than the Lys-Ala-MCA substrate, indicative of a preference for N-terminal dipeptide moieties containing a proline residue The puromycin-sensitivity of this enzyme suggests that it could possibly be classified as a dipeptidyl aminopeptidase II (EC 3 4 14 2) (McDonald and Barrett, 1986), an activity normally associated with lysosomes McDonald *et al* (1968a, 1968b) however, have previously described the purification and characterisation of a puromycin-sensitive lysosomal DAP activity from bovine pituitary (dipeptidyl arylamidase II) which preferentially hydrolyses Lys-Ala-ßNA at pH 5 5 (approximately 23 times more rapidly than Gly-Pro-ßNA) and which exhibits a molecular mass of 130,000 daltons The reported properties of this enzyme therefore appear to be quite different from those of the DAP activity observed here Moreover, the DAP activity

described in this study is very similar to post proline dipeptidyl aminopeptidase (PPDA) described by Browne and O'Cuinn (1983a) in guinea-pig brain Guinea-pig brain PPDA, like bovine brain DAP, exhibits a cytosolic location, high molecular mass (200,000 daltons) and sensitivity to puromycin as well as an ability to hydrolyse Gly-Pro-MCA at neutral pH These observations suggest therefore, that the cytosolic DAP activity of this study should probably be classified as a PPDA which displays properties distinct from those one would normally expect for a classical DAP-II

PE, detected using the substrate Z-Gly-Pro-MCA, was shown to exhibit a molecular mass of 74,500 daltons which is consistent with previous estimates of 75,000 (Hersh, 1981), and 76,000 daltons (Yoshimoto *et al*, 1983, Knisatschek and Bauer, 1979) observed for this enzyme from the soluble fraction of bovine brain tissue

4.4 Characterisation studies

4 4 1 Molecular mass determination

The relative molecular mass of cytosolic PAP as determined by gel-filtration chromatography under native, non-denaturing conditions (section 2 7 1 1) was found to be approximately 23,700 daltons This molecular mass estimate compares well with the value of 22,450 daltons estimated for this enzyme via SDS PAGE under non-native, denaturing conditions (section 2 7 1 2) On the basis of the relatively small size of the native enzyme and the similarity of these estimates under both native and non-native conditions, this enzyme would appear to exist as a monomer Several workers have previously reported that PAP activity observed in the soluble fraction of other mammalian tissues exhibits a similar native molecular mass Mantle *et al* (1990, 1991) have reported a native molecular mass of 22,000 daltons for soluble PAP activity isolated from human kidney and skeletal muscle Estimates of 23,000 and 24,000 daltons for the native enzyme from human brain (Lauffart *et al*, 1989) and guinea-pig brain (Browne and O'Cuinn, 1983a) respectively have also been reported

4 4 2 Assay linearity with respect to assay time and enzyme concentration

The importance of assay linearity was addressed in this study (see section 2 7 2) For an enzyme assay to be used in a reproducible and quantitative manner, the rate of substrate hydrolysis should be linear with respect to both assay time and enzyme concentration Only under such circumstances can one conclude that an enzyme assay proceeds in an optimal steady-state fashion, free from the influence of limiting factors such as substrate depletion, enzyme denaturation and product inhibition, all of which can affect the assay outcome The results of this study, as outlined in section 3 7 2, clearly demonstrate that during the assay of soluble PAP activity in crude cytosol and in purified PAP preparations containing 0 5%w/v BSA, using the substrate pGlu-MCA as outlined in section 2 2 1, substrate hydrolysis proceeds linearly with respect to both assay time and enzyme concentration (Figs 3 16 - 3 19) Interestingly, when purified PAP was assayed under standard assay conditions in the absence of 0 5%w/v BSA, the assay was seen to proceed linearly for up to 10 minutes after which the enzyme

behaved in a completely non-linear fashion (Fig 3 17) From this latter result it would appear that BSA has a stabilising effect on purified cytosolic PAP activity in dilute solution Moreover, further experimentation revealed that a minimum protein concentration of 1mg/ml (0 1%w/v BSA) was necessary to maintain the stability of the purified enzyme during the standard assay (Fig 3 20)

The use of BSA to stabilise dilute enzyme solutions during assay and storage (see below) is widely practised, though not well understood BSA is a very hydrophobic protein (Bigelow, 1967), therefore hydrophobic interactions with exposed hydrophobic groups may possibly lead to increased stability Browne and O'Cuinn (1983a) have previously used BSA (0 5%w/v) to store soluble PAP purified from guinea-pig brain, whilst Bauer (1994) reports that during the radiochemical assay of PAP-II isolated from rat and pig brain, 2%w/v BSA is incorporated into the assay to minimise adsorption of the purified enzyme to glass Noteworthy also is a recent report by Kita *et al* (1989) which indicates that following the isolation of *Fok* I restriction endonuclease overproduced in an *E coli* system, the quantitative and reproducible assay of the purified enzyme depended on the inclusion of 0 01%w/v BSA, a requirement which apparently pertains to the purified soluble PAP activity of this study

4 4 3 Storage stability of purified cytosolic PAP

As described in section 3 7 3, the inclusion of 0 5%w/v BSA in purified PAP preparations significantly improves the storage stability of this enzyme, with 65% and 81% of the original activity still present after 4 weeks storage at 4 C and room temperature respectively In the absence of BSA, the enzyme activity at these temperatures decreased to 4% and 31% of the original activity respectively after only 1 week of storage, *indicative of enzyme instability in dilute solution* It is noted that earlier attempts to partially purify soluble PAP activities from mammalian and avian liver (Armentrout, 1969, Szewczuk and Kwiatkowska, 1970) and bacteria (Doolittle and Armentrout, 1968, Armentrout and Doolittle, 1969) have focused on the extreme instability of this enzyme in dilute solution Mudge and Fellows (1973) have also reported the destabilisation of partially purified PAP from bovine pituitary cytosol, stored for more than 24 hours at 4 C in potassium phosphate buffer (pH 7 3) containing 1mM EDTA and 30mM 2-mercaptoethanol, with less than 20% of the original activity remaining after 6 days storage Interestingly, many of these researchers have demonstrated the ability of 2-pyrrolidone, a reversible non-competitive inhibitor of soluble PAP activity, to stabilise purified preparations of this enzyme during storage at 4 C

Freezing of purified PAP activity at -80 C in either the absence or presence of BSA was also seen to prolong the storage life of the enzyme In addition, it was noted that purified enzyme activity stored at -80°C in the absence of BSA appeared to increase over the four week storage period This trend however, can probably be attributed to the lower reproducibility (between consecutive assays) observed when assaying purified samples which contain no BSA (i e [protein] < 10µg/mL)

4 4 4 Effect of DTT and EDTA

As reported in section 3 7 4 1, the expression of soluble PAP activity purified from bovine brain was found to be dependent on the presence of a disulphide bond-reducing agent such as DTT, suggesting the participation of active site thiol groups in enzyme activity (Fig 3 23) Indeed, numerous reports have cited the sulphydryl-dependent nature of cytosolic PAP from various sources including bovine pituitary (Mudge and Fellows, 1973), guinea-pig brain (Browne and O'Cuinn, 1983a), human brain (Lauffart *et al*, 1989) and rat adenohypophysis (Bauer and Kleinkauf, 1980)

EDTA was included in all purification and assay buffers at a concentration of 2mM The reasons for this were threefold (1) The inhibition of metalloproteases which could potentially reduce the active recovery of cytosolic PAP during purification, (2) to chelate free metal cations which can speed up the oxidative breakdown of DTT by facilitating the formation of free radicals and (3) to inhibit PAP-II, a particulate form of pyroglutamyl aminopeptidase which is reportedly inhibited by metal chelating agents (Browne *et al*, 1981, O'Connor and O'Cuinn, 1984) Consequently, the effect of different EDTA concentrations on the expression of soluble PAP activity was examined, as outlined in section 2 7 4 2 DTT was also included in this study at a concentration of 2mM since its removal would simply abolish enzyme activity As can be seen from Fig 3 24, 0-10mM EDTA has a relatively negligible effect on purified PAP activity

4 4 5 pH and thermostability profiles

Using a selection of 5 different buffers, soluble PAP activity was assessed over the pH range 4 5-10 5 The purified enzyme was subsequently found to display an optimum activity between pH 8 5-9 0 (Fig 3 25) In contrast, Mudge and Fellows (1973) have previously reported a pH optimum of 7 3 for soluble PAP activity partially purified from bovine pituitary One can suggest a number of reasons to account for this difference Unlike whole brain, the pituitary is a site of hormonal biosynthesis, release and catabolism, a highly efficient and well balanced process which may possibly exhibit a neutral pH dependency Enzymes present in the soluble fraction of this tissue would therefore be expected to function optimally at such a pH The use of a different substrate (pGlu-Ala) and assay temperature (40 C) might also account for the observed difference in pH optimum

The pH optimum obtained for the enzyme in this study does however compare well with pH optima previously reported for soluble PAP activities examined in other mammalian tissues pH optimum values ranging from pH 8 0-8 5 have been reported for this enzyme from rat liver (Busby *et al*, 1982) as well as human whole brain , kidney and skeletal muscle (Lauffart *et al*, 1989 Mantle *et al*, 1990, 1991) Soluble PAP activity from human skeletal muscle was also reported to possess up to 50% of optimum activity at pH 9 5 (Mantle *et al*, 1990), a finding reasonably consistent with the bovine brain activity in this study (75% of optimal activity at pH 9 5)

The thermal stability of purified PAP was investigated, as outlined in section 2 7 6 1 Incubating the enzyme at 37 C for up to 45 minutes had no apparent effect on enzyme activity By simply increasing

the incubation temperature a mere 3 C however, PAP activity decreases by up to 17% after a similar incubation period, indicative of a narrow thermal sensitivity range Incubation of the enzyme at higher temperatures had significantly greater destabilising effects on enzyme activity after shorter incubation periods (Fig 3 26) Unlike the soluble bovine brain PAP activity of this study however, soluble PAP activities isolated from pigeon liver (Szewczuk and Kwiatkowska, 1970) and chicken liver (Tsuru *et al* , 1982) have been shown to be quite stable at 50 C for up to 15-30 minutes Such an apparent difference in thermal stability may possibly derive from the functionally dissimilar nature of whole brain and liver tissue Finally, it should be noted that these thermostability studies were initially performed with a view to developing techniques aimed at stabilising soluble bovine brain PAP activity at high temperatures (in conjunction with another research group at Dublin City University) Unfortunately, this particular study was not pursued

4 4 6 Effect of metal ions

Table 3 3 describes the effects of a range of metal ions, at a concentration of 1mM, on soluble PAP activity Whilst none of the metal ions tested were found to stimulate PAP activity above control levels, only the transition metal ions Hg^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} were found to be significantly inhibitory (over 80% inhibition) In parallel assays for PAP activity in the presence of Hg^{2+}, Zn^{2+} and Cu^{2+} ions, the inclusion of 2mM EDTA was found to completely reverse the inhibitory effect of Zn^{2+} ions The inhibitory effects of Hg^{2+} and Cu^{2+} ions were unaffected by the presence of EDTA however, suggesting that either these ions inhibit the enzyme in an irreversible manner or that they are not chelated in significant quantities by EDTA These findings compare well with those reported for soluble PAP activities in other species Strong inhibition by metal ions such as Hg^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Cd^{2+} have been reported for this enzyme in pigeon and rabbit liver (Szewczuk and Kwiatkowska, 1970), chicken liver (Tsuru *et al* , 1982), hamster hypothalamus (Prasad and Peterkofsky, 1976) and human skeletal muscle (Mantle *et al* , 1991) The inhibitory effects of these metal ions have also been reported for soluble PAP activities observed in *S pyogenes* (Awade *et al* , 1992a), *B subtilis* (Szewczuk and Mulczyk, 1969) and *B amyloliquifaciens* (Tsuru *et al* , 1978) It is also noted that , in contrast to the findings of this study, the latter research group (Tsuru *et al* 1978) were able to partially reverse the inhibitory effects of Hg^{2+} and Cu^{2+} ions on PAP activity by the inclusion of 5mM EDTA in the enzyme assay

4 4 7 Effect of various functional reagents

Tables 3 4 and 3 5 detail the effects of a range of functional reagents on purified PAP activity In Table 3 4, one notes the strong inhibitory influence of sulphydryl-blocking agents such as 2-iodoacetamide, iodoacetate, N-ethylmaleimide and PCMB on this activity at concentrations ranging from 0 1mM to 1mM E-64, a novel thiol protease inhibitor originally isolated from a culture extract of *Aspergillus japonicus* TPR-64 (Hanada *et al* , 1978) had a significantly milder inhibitory effect than expected, although this could be attributed to the low concentrations of inhibitor used In contrast, the serine protease inhibitors PMSF and benzamidine, at a concentration of 1mM, had a negligible effect on the

activity of this enzyme These findings are consistent with the thiol-dependent nature of this bovine brain enzyme reported earlier (see sections 3 7 4 1 and 4 4 4) The inhibitory effects of sulphydryl-blocking agents on soluble PAP activities have also been reported for many other animal tissues including guinea-pig brain (Browne and O'Cuinn, 1983a), hamster hypothalamus (Prasad and Peterkofsky, 1976), chicken liver (Tsuru *et al*, 1982) and rat adenohypophysis (Bauer and Kleinkauf, 1980) in addition to numerous microbial sources of the enzyme (Tsuru *et al*, 1978, Szewczuk and Mulczyk, 1969, Awade *et al*, 1992a)

Metal chelating agents, with the exception of 1,10-phenanthroline (28% inhibition at 1mM) had no inhibitory effect on soluble bovine whole brain PAP activity (Table 3 5), suggesting the lack of a metal requirement for the expression of enzyme activity Of the other inhibitors tested, only 2-pyrrolidone displayed any significant inhibitory activity towards this enzyme (95% inhibition at 0 1M), consistent with previous observations that this compound specifically inhibits soluble PAP activity, in a reversible and non-competitive manner, from both microbial (Armentrout and Doolittle, 1969) and mammalian sources including bovine pituitary (Mudge and Fellows, 1973, Armentrout, 1969) Indeed, the reversibility of 2-pyrrolidone inhibition on the bovine brain activity was subsequently demonstrated, as outlined in section 3 7 8 2 (Fig 3 28)

The speed with which certain inhibitors act on purified cytosolic PAP was also investigated using a continuous real-time fluorimetric assay, as outlined in section 2 7 8 3 This assay enabled one to continuously monitor the enzymatic hydrolysis of the substrate pGlu-MCA, by purified PAP in the presence or absence of an inhibitory compound Figs 3 29 And 3 30 clearly demonstrate that when the purified enzyme is assayed continuously in the presence or absence of either 2-pyrrolidone (10mM and 0 5mM) or 1,10-phenanthroline (1mM and 0 2mM), pGlu-MCA hydrolysis proceeds linearly with respect to assay time Based on the assumption that a "slow acting" inhibitor would cause the enzyme to behave in a non-linear fashion over this period of time, one might therefore conclude that both 2-pyrrolidone and 1,10-phenanthroline act on cytosolic PAP in an extremely rapid, if not instantaneous manner, with complete inhibition achieved within a matter of seconds or less Consistent with these observations are the previous findings outlined in section 3 7 2 1 of this report in which the linear rate of pGlu-MCA hydrolysis with respect to assay time was demonstrated with purified cytosolic PAP using a discontinuous approach which involved assaying for PAP activity at discrete time intervals

4 4 8 Kinetic analyses

Using an extensive range of pyroglutamyl substrates, the catalytic properties of purified bovine brain PAP were examined, as outlined in section 2 7 9 Studies revealed that hydrolysis of the synthetic substrates pGlu-MCA, pGlu-ßNA and pGlu-His-Pro-MCA (a TRH analog) by the purified enzyme was found to obey Michaelis-Menten steady-state kinetics (Michaelis and Menten, 1913) Using a number of different kinetic models (see section 3 7 9 2), all of which are based upon manipulations of the Michaelis-Menten equation, the Michaelis-Menten constant or K_m of the purified enzyme for each of the above substrates was evaluated Using the same kinetic models, the inhibition constant or K_I value

of the purified enzyme was ascertained for substrates which could not be assayed for directly, based on their ability to inhibit the hydrolysis of pGlu-MCA, as described in section 2 7 9 3 Analyses subsequently revealed that the K_m or K_I obtained for any given substrate from each of the kinetic models was very similar (see sections 3 7 9 2 and 3 7 9 3)

It should be noted that the use of a number of different kinetic models to analyse the data obtained for each substrate enabled one to minimise the influence of inherent inaccuracies present in any single kinetic model Henderson (1992) for example, is highly critical of the Lineweaver-Burk model as a method for the determination of kinetic constants because it relies upon a double reciprocal transformation of the Michaelis-Menten equation Consequently, this plot groups data points close to the origin and ignores the increased margin of error associated with using lower substrate concentrations (the reader is directed to Figs 3 33 and 3 37 - lowering substrate concentration clearly has the effect of increasing the size of error bars) This author also suggests that the Hanes-Woolf and Eadie-Hofstee models are more accurate because they only rely upon a single reciprocal transformation of the Michaelis Menten equation The Direct-Linear plot (Eisenthal and Cornish-Bowden, 1974) is widely regarded as the most accurate model for the evaluation of kinetic constants With this model, observations are plotted as lines in parameter space, instead of points in observation space Kinetic constants can subsequently be read directly off the plot without the necessity for calculations

Table 3 6 displays the K_m values obtained for the aforementioned substrates pGlu-MCA, pGlu-ßNA and pGlu-His-Pro-MCA using the Direct-Linear plot method The K_m values obtained for these substrates are significantly lower than those previously obtained (Table 3 6) with values observed within the lower micromolar range, suggestive of strong enzyme/substrate affinity Hydrolysis of pGlu-pNA, a chromogenic substrate, by the purified enzyme was also clearly demonstrated However, an accurate K_m for this substrate could not be obtained with-pure enzyme due to a combination of low V_{max} and poor instrument sensitivity (the spectrophotometer, a Shimadzu UV 160-A, was observed to be several orders of magnitude less sensitive than the fluorimeter, a Perkin-Elmer LS-50) The K_I values obtained for a range of pyroglutamyl substrates (LHRH, acid TRH, TRH, pGlu-His-Gly, bombesin, pGlu-Ala, pGlu-His, pGlu-Val and pGlu-Pro-NH_2) are displayed in Table 3 7 All of the substrates tested were found to be competitive inhibitors Indeed, LHRH, acid TRH and TRH were found to be particularly effective in this regard Unfortunately, K_I values for either eledoisin or neurotensin could not be ascertained due to insufficient quantities of substrate

A comparison with published values, of the kinetic constants (K_m or K_I) evaluated for purified cytosolic PAP with different substrates reveals a number of interesting observations The bovine brain enzyme has been shown to exhibit a K_m of 15 36µM and 20 76µM for the fluorimetric substrates pGlu-MCA and pGlu-ßNA respectively These would appear to be among the lowest K_m values ever determined for a soluble PAP activity with either of these substrates as seen from Table 4 1 Equally low was the K_m value of 13 64µM obtained with the TRH analog, pGlu-His-Pro-MCA This value is very similar to the K_I value of 24 9µM obtained with synthetic TRH (pGlu-His-Pro-NH_2), suggesting

that replacement of the amide group of TRH with MCA has a relatively small effect on enzyme-substrate binding A similar observation with PAP-II isolated from a rabbit brain membrane preparation has also previously been reported by Wilk and Wilk (1989) K_m values of 44μM and 70μM were obtained for this enzyme with pGlu-His-Pro-ßNA and TRH respectively, indicating that the substitution of ßNA for NH_2 in TRH has very little effect on PAP-II activity

The extremely low K_i values obtained for the bovine brain enzyme with the neuropeptide substrates TRH, acid TRH and LHRH are also noteworthy Workers have previously reported K_m values of 420μM and 727μM with TRH as substrate for soluble PAP activities isolated from human (Lauffart *et al*, 1989) and guinea-pig (Browne and O'Cuinn, 1983a) brain respectively The bovine brain enzyme of this study however, has demonstrated a significantly lower K_i value of 24 9μM for this substrate, a value which actually compares well with K_m values of 40μM, *50μM and 25μM which have previously been obtained with TRH for the TRH-specific PAP-II activities isolated from guinea-pig (O'Connor and O'Cuinn, 1985), bovine (*K_i value - O'Leary and O'Connor, 1995) and rat/porcine (Bauer, 1994) brain membrane preparations respectively The observation of similarly low K_i values for the bovine brain enzyme with LHRH (20 6μM) and acid TRH (23 1μM) may possibly signify an important role for this enzyme *in vivo*, in the intracellular catabolism of these three neuropeptides, although the existence of such a role has yet to be verified Only the findings of Busby *et al* (1982) have demonstrated consistency with current observations in this regard As with the purified bovine brain PAP activity, these researchers have reported very low K_m/K_i values for a soluble PAP activity isolated from rat brain with TRH (K_m = 40μM) and LHRH (K_i = 22 2μM)

Table 4 1 *Reported K_m values for soluble PAP activities as determined with the substrates pGlu-MCA and pGlu-ßNA*

Substrate	K_m (μM)	Enzyme source	Reference
pGlu-MCA	80	Human skeletal muscle	Mantle *et al* (1991)
	80	Human brain	Lauffart *et al* (1989)
	210	Human kidney	Mantle *et al* (1990)
	150	Guinea-pig brain	Browne and O'Cuinn (1983a)
	40	Chicken liver	Tsuru *et al* (1982)
	330	*Bacillus amyloliquifaciens*	Fujiwara and Tsuru (1978)
pGlu-ßNA	700	Chicken liver	Tsuru *et al* (1982)
	130	*Bacillus amyloliquifaciens*	Tsuru *et al* (1978)
	1700	*Bacillus subtilis*	Szewczuk and Mulczyk (1969)
	1790	*Streptococcus pyogenes*	Awade *et al* (1992a)
	220	Pigeon kidney	Albert and Szewczuk (1972)
	500	Pigeon liver	Albert and Szewczuk (1972)
	77	pigeon intestine	Albert and Szewczuk (1972)

A closer examination of the K_I values obtained for the purified enzyme with different pyroglutamyl substrates (Table 3 7) also enables one to make a number of comparisons One notes that, of the substrates tested, dipeptides (including pGlu-His) gave the highest K_I values, whilst substrates consisting of three peptides or more gave significantly lower K_I values for this enzyme This would serve to indicate that the affinity of the enzyme for a pGlu-X bond may depend on the length of the X moiety Also of interest were the K_I values obtained with the tripeptides pGlu-His-Pro (acid TRH) and pGlu-His-Gly (anorexigenic peptide) The latter tripeptide gave a K_I (58 8μM) over twice the magnitude of the K_I (23 1μM) obtained with the former tripeptide, indicating that the moiety immediately following the N-terminal pGlu dipeptide would appear to have an affect on the affinity of the enzyme for the pGlu-X bond

4 4 9 Substrate specificity studies

As described in section 2 7 10, bovine brain cytosolic PAP was incubated with a range of synthetic pyroglutamyl substrates for a period of up to 40 hours at 37 C The resultant digests and corresponding negative controls prepared under identical assay conditions were subsequently analysed by HPLC using a C-8 reversed phase column Digestion products were detected by scanning at 207nm, the absorbance maxima determined for pyroglutamic acid Initial studies using a pyroglutamic acid standard revealed that, even at millimolar concentrations, this compound absorbs very poorly at 207nm A consequence of this was the inability to detect the extremely low concentrations of pyroglutamic acid generated by the action of the purified bovine brain enzyme on the pyroglutamyl substrates However, the use of partially purified cytosolic PAP (post anion-exchange) consisting of a much more active and concentrated PAP component enabled one to circumvent this problem, although it necessitated the inclusion of Z-Pro-Prolinal (PE inhibitor) and bestatin (aminopeptidase inhibitor) in the enzyme/substrate incubation mixture in order to minimise unwanted substrate hydrolysis by other cytosolic peptidases also present in the test sample

Table 3 8 displays the findings of this study With the exception of eledoisin and LHRH, all of the substrates tested were found to be cleaved by the bovine brain activity at the pGlu-X bond (where X = 1 or more amino acids) This would serve to indicate a broad pyroglutamyl substrate specificity for this enzyme Particularly noteworthy is the ability of this enzyme to cleave TRH to yield pGlu and cyclo(His-Pro) which is in agreement with the reported ability of soluble PAP activities from other sources, including bovine pituitary, to cleave TRH at the pGlu-His bond *in vitro* (Table 4 2) From Figs 3 43a/b and 3 44a/b, one also notes the presence of small quantities of the cleavage products pGlu, cyclo(His-Pro) and His-Pro in the control chromatograms This would seem to suggest the spontaneous release of N-terminal pyroglutamic acid from these substrates (i e TRH and acid TRH) in aqueous solution at 37°C over the 40 hour reaction period In the case of TRH, this process would yield the TRH metabolite His-Pro-NH_2, which would spontaneously cyclise under assay conditions (Bauer and Nowak, 1979, Bauer and Kleinkauf, 1980) to yield cyclo(His-Pro) Consistent with this hypothesis

was the observed spontaneous release of MCA from the substrates pGlu-MCA and pGlu-His-Pro-MCA when stored in aqueous solution at 4 C for an extended period of time

The inability of bovine brain PAP to cleave eledoisin (pGlu-Pro-X) is also consistent with published findings Numerous studies have reported the inability of this enzyme activity to cleave the pGlu-Pro bond of natural and synthetic substrates in various mammalian (Browne and O'Cuinn 1983a, Tsuru *et al* , 1982) and microbial (Fujiwara *et al* , 1979, Uliana and Doolittle, 1969) sources It is worth noting however, that this bond is hydrolysed by a particulate pyroglutamyl aminopeptidase activity isolated from *Klebsiella cloacae* (Kwiatkowska, 1974)

The inability to detect the release of any pyroglutamic acid following incubation of partially purified enzyme with LHRH is an exceptionally surprising result as numerous studies have reported on the ability of cytosolic PAP activities from various sources to cleave this neuropeptide at the $pGlu^1$-His^2 bond (the reader is directed to Table 4 2) This observation is also in contrast with the low competitive K_I value determined for the bovine brain enzyme with this substrate (see Table 3 7) which is indicative of a strong enzyme-substrate interaction One can simply speculate therefore, as to the reasons which may account for this apparently anomalous result Very low levels of peptide hydrolysis coupled to insufficient instrument sensitivity may represent one possibility

Table 4 2 *Hydrolysis of TRH and LHRH at the $pGlu^1$-His^2 bond by soluble PAP activities from different sources*

Substrate	Enzyme source	Reference
TRH	Human skeletal muscle	Mantle *et al* (1991)
	Human brain	Lauffart *et al* (1989)
	Human kidney	Mantle *et al* (1990)
	Guinea-pig brain	Browne and O'Cuinn (1983a)
	Chicken liver	Tsuru *et al* (1982)
	Rat adenohypophysis	Bauer and Kleinkauf (1980)
	Rat brain	Busby *et al* (1982)
	Bovine pituitary	Mudge and Fellows (1973)
	Hamster hypothalamus	Prasad and Peterkofsky (1976)
	Bacillus amyloliquifaciens	Fujiwara *et al* (1979)
LHRH	Human brain	Lauffart *et al* (1989)
	Human kidney	Mantle *et al* (1990)
	Human skeletal muscle	Mantle *et al* (1991)
	Chicken liver	Tsuru *et al* (1982)
	Guinea-pig brain	Browne and O'Cuinn (1983a)
	Bacillus amyloliquifaciens	Fujiwara *et al* (1979)

Conversely, the inability to detect cleavage of the pGlu^1-His^2 bond of LHRH may derive from the use of partially purified PAP during the enzyme/substrate incubation O'Cuinn *et al* (1990) in a recent review on the degradation of TRH and LHRH by enzymes of brain tissue reports that, in addition to PAP and PE, at least two other soluble enzyme activities have been isolated which are capable of hydrolysing LHRH These are neutral endopeptidases, both of which have the ability to cleave LHRH at the Tyr^5-Gly^6 bond but which can be differentiated by their abilities to introduce secondary cleavages between His^2-Trp^3 (Camargo *et al*, 1973, Horsthemke and Bauer, 1980) and Trp^3-Ser^4 (Wilk and Orlowski, 1980, Dresdner *et al*, 1982) respectively One might expect therefore that the action of either of these enzymes on LHRH, if present in the partially purified PAP sample, would tend to limit the availability of this substrate to other enzymes including PAP Consistent with this hypothesis is the finding that much of the LHRH originally incubated with partially purified PAP was found to be degraded to a number of peptide fragments, although the size and amino acid composition of these fragments remains undetermined In addition, soluble PAP activity may exhibit poor binding characteristics for pGlu-containing peptide fragments generated from the metabolism of LHRH by neutral endopeptidases

In view of the inconsistent nature of this result with respect to previously published findings, the noted insensitivity of the pGlu detection method used in this instance (i e absorbance at 207nm) and the use of a partially purified source of PAP, one is prompted to view this result with some degree of caution until more definitive confirmation is available

4.5 Summary

Since the initial isolation of pyroglutamyl aminopeptidase (PAP) from a strain of *Pseudomonas fluorescens* by Doolittle and Armentrout in 1968, similar enzyme activities have been isolated and characterised from a multitude of prokaryotic and eukaryotic sources Studies on eukaryotic PAPs have been done mainly in mammals, typically with a view to elucidating the potential role of this class of enzymes in the catabolism of various pGlu-terminating peptides, including neuropeptides, *in vivo* The central aim of this study was to undertake the complete purification and characterisation of a PAP activity observed within the soluble or cytosolic fraction of bovine whole brain Several workers have previously described the purification and characterisation of soluble PAP activities from different mammalian tissues including guinea-pig brain (Browne and O'Cuinn, 1983a) and human bram (Lauffart *et al*, 1989) However, other than some minor details furnished by the earlier studies of Wilk *et al* (1985) and Mudge and Fellows (1973), little was known of the bovine brain activity

From a practical viewpoint, the relatively large size (approx 300g wet weight) and easy availability (local abattoir) of this tissue source greatly facilitated this study A combination of different chromatographic methodologies subsequently generated a soluble PAP activity with a total active yield of 6 6% which had been purified to near homogeneity, as judged by SDS PAGE and silver staining techniques The unstable nature of the purified enzyme in dilute solution was very apparent, prompting

the usage of 0 5%w/v BSA to stabilise PAP activity during both assay and storage Characterisation of this enzyme activity subsequently revealed a number of interesting results, many of which compared well with findings previously reported for soluble PAP activities examined in other sources In addition to a predominantly cytosolic subcellular location, this enzyme was found to exhibit a low relative molecular mass Gel-filtration chromatography revealed a native molecular mass of approximately 23,700 daltons, a value which compares well with that obtained for the enzyme under denaturing conditions via SDS PAGE (22,450 daltons), supporting the likelihood that the soluble bovine brain PAP exists as a monomer A pH optimum of 8 5-9 0, as determined with pGlu-MCA at 37 C, was also demonstrated for this enzyme, whilst the expression of PAP activity exhibited an absolute requirement for the presence of a disulphide bond-reducing agent such as DTT, suggesting the participation of active site thiol groups in enzyme activity (i e a thiol protease) Strong inhibition of purified PAP activity was observed with a number of different agents which included the transition metal ions Hg^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+}, the sulphydryl-blocking agents iodoacetate, 2-iodoacetamide, N-ethylmaleimide and PCMB and the reversible inhibitor 2-pyrrolidone Serine protease inhibitors and metal chelating agents (with the exception of 1,10-phenanthroline) as well as the compounds bacitracin, puromycin and bestatin had no effect on enzyme activity

The cleavage of the N-terminal pGlu residue from a wide range of pyroglutamyl substrates including TRH, acid TRH, pGlu-His-Pro-MCA (a TRH analog), bombesin and neurotensin was clearly demonstrated for the soluble bovine brain PAP activity N-terminal pGlu cleavage of eledoisin and LHRH could not be detected however Whereas this was expected for eledoisin, a substrate which commences with the sequence pGlu-Pro, such a finding for LHRH was quite unexpected Subsequent kinetic analysis also revealed that the purified PAP activity displays K_m and K_I values within the lower micromolar range for a number of these substrates (TRH, acid TRH, LHRH, pGlu-MCA, pGlu-ßNA and pGlu-His-Pro-MCA) indicative of a strong enzyme-substrate interaction–In-addition, all of the pGlu-peptides for which K_I values were estimated proved to be competitive inhibitors

Based on a comparison of these findings with those reported for soluble PAP activities in other mammalian tissues, the soluble PAP enzyme activity observed in bovine whole brain can tentatively be classified as a pyroglutamyl aminopeptidase type-1 or PAP-I (EC 3.4 19.3)

A definitive physiological role for PAP-I has yet to be identified The relatively ubiquitous distribution of PAP-I in such functionally dissimilar mammalian tissues as brain, liver and kidney (Mantle *et al*, 1990, 1991, Lauffart *et al*, 1989) would appear to support a role for this enzyme in the intracellular catabolism of peptides to free amino acids Thus PAP-I may, at least in part, be involved in the regulation of the cellular pool of free pyroglutamic acid Indeed, workers have hypothesised that cytosolic enzymes may represent a mechanism for returning neuropeptides released from damaged or ageing vesicles to the cellular amino acid pool (O Cuinn *et al*, 1990), or, in cases where secretion from neuropeptide-synthesising cells is suppressed, cytosolic degradation of neuropeptides might conceivably represent a security device system to ensure the degradation of neuropeptides which are

produced in excess (Bauer, 1987b) In this regard, the potential role of PAP-I in the intracellular metabolism of physiologically important neuropeptides such as TRH has received much attention However, despite the ability of this enzyme from different mammalian tissues, including the aforementioned bovine brain PAP activity, to degrade putative neuropeptide substrates such as TRH and acid TRH *in vitro*, several studies have cast doubt on a similar role for PAP-I *in vivo* (Bauer, 1987b, Charli *et al*, 1987, Torres *et al*, 1986, Salers *et al*, 1991, 1992, Mendez *et al*, 1990) Conversely, some researchers have provided evidence in support of the notion that cytosolic enzymes may have some role, direct or otherwise, in regulating intracellular levels of TRH Faivre-Bauman *et al* (1986) for example, have reported that addition of specific inhibitors of PAP-I and PE to TRH-synthesising hypothalamic cells in primary culture results in a significant increase in their TRH content and especially a pronounced increase in the amount of TRH being released from these cells under basal or potassium-stimulated conditions In view of these findings, and by virtue of its unique catalytic properties, it would therefore be of great interest to identify mechanisms which may possibly exist to bring neuropeptides into contact with PAP-I, and indeed other cytosolic peptidases, as neuropeptides do not normally have access to the cytosolic compartment

On a final note therefore, it is hoped that the work outlined in this report will ultimately serve to compliment other studies currently in progress in this laboratory, which concern the purification and characterisation of various neuropeptide-degrading enzyme activities in different bovine tissues

5. REFERENCES

Akita, S , Tanaka, K and Kinoshita, S (1959), Enzymic dehydration of L-glutamic acid, *Biochemical and Biophysical Research Communications*, **1**, 179-181

Albert, Z and Szewczuk, A (1972), Pyrrolidonyl peptidase in some avian and rodent tissues Histochemical localization and biochemical studies, *Acta Histochemica*, **44**, 98-105

Aoyagi, T , Hatsu, M , Kojima, F , Hayashi, C , Hamada, M and Takeuchi, T (1992a) Benarthin A new inhibitor of pyroglutamyl peptidase I Taxonomy, fermentation, isolation and biological activities, *Journal of Antibiotics*, **45**(7), 1079-1083

Aoyagi, T , Hatsu, M , Imada, C , Naganawa, H , Okami, Y and Takeuchi, T (1992b), Pyrizinostatin A new inhibitor of pyroglutamyl peptidase, *Journal of Antibiotics*, **45**(11), 1795-1796

Aratan-Spire, S , Moilanen, K and Czernichow, P (1983), Postnatal developmental pattern of thyrotropin releasing hormone-degrading activity in rat plasma, hypothalamus and liver Role of tri-iodothyronine, *Journal of Endocrinology*, **97**, 409-418

Aratan-Spire, S , Wolf, B and Czernichow, P (1986), Enzymatic degradation of thyrotropin releasing hormone by pancreatic homogenates Failure to detect His-Pro-diketopiperazine as TRH metabolite, *Neuroendocrinology*, **42**, 399-406

Armentrout, R W and Doolittle, R F (1969), Pyrrolidonecarboxylyl peptidase Stabilization and purification, *Archives of Biochemistry and Biophysics*, **132**, 80-90

Armentrout, R W (1969), Pyrrolidonecarboxylyl peptidase from rat liver, *Biochemica et Biophysica Acta*, **191**, 756-759

Ashworth, R , Yu, R , Nelson, E J , Dermer, S , Gershengorn, M C and Hinkle, P M (1995), Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling, *Proceedings of the National Academy of Science U S A* , **92**, 512-516

Attali, J R , Valensi, P , Darnis, D and Sebaoun, J (1984) Thyrotrophin releasing hormone (TRH) stimulating effect on thyroxine secretion by perfused rat thyroid fragments, *Comptes Rendus de L Academie des Sciences Serie III - Sciences de la Vie*, **298**, 93-96

Awade, A , Gonzales, Th , Cleuziat, Ph and Robert-Baudouy, J (1992a), One step purification and characterisation of the pyrrolidone carboxyl peptidase of *Streptococcous pyogenes* over-expressed in *Escherichia coli*, *FEBS Letters*, **308**(1), 70-74

Awade, A , Cleuziat, Ph , Gonzales, Th and Robert-Baudouy, J (1992b), Characterisation of the pcp gene encoding the pyrrolidone carboxyl peptidase of *Bacillus subtilis*, *FEBS Letters*, **305**(1), 67-73

Awade, A , Cleuziat, Ph , Gonzales, Th and Robert-Baudouy, J (1994), Pyrrolidone carboxyl peptidase (Pcp) An enzyme that removes pyroglutamic acid (pGlu) from pGlu-peptides and pGlu-proteins, *Proteins Structure, Function, and Genetics*, **20**, 34-51

Bauer, K , Sy, J and Lipmann, F (1973), *Federal Proceedings*, **32**, 482

Bauer, K (1976), Regulation of degradation of thyrotropin releasing hormone by thyroid hormones, *Nature*, 259, 591-593

Bauer, K and Lipmann, F (1976), Attempts towards biosynthesis of the thyrotropin-releasing hormone and studies on its breakdown in hypothalamic tissue preparations, *Endocrinology*, **99**(1), 230-242

Bauer, K and Nowak, P (1979), Characterisation of a thyroliberin-degrading serum enzyme catalyzing the hydrolysis of thyroliberin at the pyroglutamyl-histidine bond, *European Journal of Biochemistry*, **99**, 239-246

Bauer, K , Nowak, P and Kleinkauf, H (1979), The degradation of thyroliberin (TRF) by serum enzymes is initiated by an apparently TRF-specific "pyroglutamate aminopeptidase", *Hoppe-Seyler's Z. Physiological Chemistry* , **360**(3), 229-230

Bauer, K and Kleinkauf, H (1980), Catabolism of thyroliberin by rat adenohypophyseal tissue extract, *European Journal of Biochemistry*, **106**, 107-117

Bauer, K , Nowak, P and Kleinkauf, H (1981), Specificity of a serum peptidase hydrolysing thyroliberin at the pyroglutamyl-histidine bond, *European Journal of Biochemistry*, **118**, 173-176

Bauer, K (1987a), Adenohypophyseal degradation of thyrotropin releasing hormone regulated by thyroid hormones, *Nature*, **330**, 375-377

Bauer, K (1987b), Degradation and biological inactivation of thyrotropin-releasing hormone and other neuropeptides, *Integrative Neuroendocrinology Molecular, Cellular and Clinical Aspects*, McCann and Weiner (eds), Karger, Basel, 102-114

Bauer, K (1988), Degradation and biological inactivation of thyrotropin releasing hormone (TRH) Regulation of the membrane-bound TRH-degrading enzyme from rat anterior pituitary by estrogens and thyroid hormones, *Biochimie*, **70**, 69-74

Bauer, K , Carmeliet, P , Schulz, M , Baes, M and Denef, C (1990), Regulation and cellular localization of the membrane-bound thyrotropin-releasing hormone-degrading enzyme in primary cultures of neuronal, glial and adenohypophyseal cells, *Endocrinology*, **127**(3), 1224-1233

Bauer, K (1994), Purification and characterization of the thyrotropin-releasing-hormone-degrading ectoenzyme, *European Journal of Biochemistry*, **224**, 387-396

Bieber, A L , Becker, R R , McParland, R , Hunt, D F , Shabanowitz, J , Yates, J R III, Martino, P A and Johnson, G R (1990), The complete sequence of the acidic subunit from Mojave toxin determined by Edman degradation and mass spectrometry, *Biochimic et Biophysica Acta*, **1037**, 413-421

Bigelow, C C (1967), *Journal of Theoretical Biology*, **16**, 187-211

Bloom, F E (1979), Contrasting principles of synaptic physiology Peptidergic and non-peptidergic neurons, In *Central Regulation of the Endocrine System*, Fuxe, K , Hokfelt, T and Luft, R (eds), Nobel Foundation Symposium 42, Plenum, New York, 173-187

Boler, J , Enzmann, F , Folkers, K , Bowers, C Y and Schally, A V (1969), The identity of chemical and hormonal properties of the thyrotropin releasing hormone and pyroglutamyl-histidyl-proline-amide, *Biochemical and Biophysical Research Communications*, **37**(4), 705-711

Boschi, G , Launay, N and Rips, R (1980), Induction of wet-dog shakes by intracerebral "acid" TRH in rats, *Neuroscience Letters*, **16**, 209-212

Borden, M , Holm, J , Laeslie, J , Sweetman, L , Nyhan, W L , Nadler, H , Lewis, D and Scott, C R (1992), Hawkinsinuria in two families, *American Journal of Medical Genetics*, **44**, 52-56

Bowers, C Y , Redding, T W and Hawley, W D (1966), *Program of the 48th Meeting of the Endocrine Society*, Chicago, p 43

Bowman, W C and Rand, M J (1980), Textbook of Pharmacology - 2nd Edition, Blackwell Scientific Publications, Oxford

Brabant, G , Wickings, E J and Nieschlag, E (1981), The TRH metabolite histidyl-proline-diketopiperazine (DKP) inhibits prolactin secretion in male Rhesus monkeys, *Acta Endocrinologica*, **98**, 189-194

Bradbury, A , Finme, M and Smyth D (1982), Mechanism of C-terminal amide formation by pituitary enzymes, *Nature*, **298**, 686-688

Bradford, M (1976), A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry*, **72**, 248-254

Brocklehurst, K , Carlsson, J , Kierstan, M P J and Crook E M (1974), Covalent chromatography by thiol-disulfide interchange, In *Methods in Enzymology*, Jacoby, W B and Wilchek, M (eds), Academic Press, New York, **34**, 531-544

Browne P , Phelan, J and O'Cuinn, G (1981), TRH pyroglutamate aminopeptidase activity in two different subcellular fractions of guinea-pig brain, *Irish Journal of Medical Sciences*, **150**, 348-349

Browne, P and O'Cuinn, G (1983a), An evaluation of the role of a pyroglutamyl peptidase, a post-proline cleaving enzyme and a post-proline dipeptidyl amino peptidase, each purified from the the soluble fraction of guinea-pig brain, in the degradation of thyroliberin *in vitro*, *European Journal of Biochemistry*, **137**, 75-87

Browne, P and O'Cuinn, G (1983b), The purification and characterisation of a proline dipeptidase from guinea-pig brain, *Journal of Biological Chemistry*, **258**, 6147-6154

Bruhn, T O , Taplin, J H and Jackson, I M D (1991), Hypothyroidism reduces content and increases *in vitro* release of pro-thyrotropin-releasing hormone peptides from the median eminence *Neuroendocrinology*, **53**, 511-515

Bulant, M , Delfour, A , Vaudry, H and Nicolas, P (1988), Processing of thyrotropin-releasing hormone prohormone (Pro-TRH) generates pro-TRH-connecting peptides, *Journal of Biological Chemistry*, **263**, 17189-17196

Bulant, M , Roussel, J P , Astier, H , Nicolas, P and Vaudry, H (1990), Processing of thyrotropin-releasing hormone prohormone (pro-TRH) generates a biologically active peptide, prepro-TRH-(160-169), which regulates TRH-induced thyrotropin secretion, *Proceedings of the National Academy of Science U S A* , **87**, 4439-4443

Bulant, M , Richter, K , Kuchler, K and Kreil, G (1992a), A cDNA from brain of *Xenopus laevis* coding for a new precursor of thyrotropin-releasing hormone, *FEBS Letters*, **296**, 292-296

Bulant, M , Ladram, A , Montagne, J J , Delfour, A , Nicolas, P (1992b), Isolation and amino acid sequence of the TRH-potentiating peptide from bovine hypothalamus, *Biochemical and Biophysical Research Communications*, **189**, 1110-1118

Bundgaard H and Moss, J (1989), Prodrugs of peptides IV Bioreversible derivatization of the pyroglutamyl group by N-acylation and N-aminomethylation to effect protection against pyroglutamyl aminopeptidase, *Journal of Pharmaceutical Sciences*, **78**(2), 122-126

Burgus, R , Dunn, T F , Desiderio, D , Vale, W and Guillemin, R (1969), Derives polypeptidiques de syntheses doues d'activite hypophysiotropic TRF, *Comptes Rendus de L Academie des Sciences (Paris)*, **226**, 321-325

Burgus, R , Dunn, T F , Desiderio, D , Ward, D N , Vale, W and Guillemin, R (1970), Characterisation of ovine hypothalamic hypophysiotrophic TSH-releasing factor, *Nature*, **226**, 321-325

Busby Jr , W H , Youngblood, W W and Kizer, J S (1982), Studies of substrate requirements, kinetic properties, and competitive inhibitors of the enzymes catabolizing TRH in rat brain, *Brain Research*, **242**, 261-270

Busby Jr , W H , Quakenbush, G E , Humm, J , Youngblood, W W and Kizer, J S (1987), An enzyme(s) that converts glutaminyl-peptides into pyroglutamyl-peptides Presence in pituitary, brain, adrenal medulla, and lymphocytes, *Journal of Biological Chemistry*, **262**(18), 8532-8536

Camargo, A C M , Shapanka, R and Greene, L J (1973), Preparation, assay and partial characterisation of a neutral endopeptidase from rabbit brain, *Biochemistry*, **12**, 1838-1844

Capecchi J T and Loudon, G M (1985), Substrate specificity of pyroglutamylaminopeptidase, *Journal of Medicinal Chemistry*, **28**, 140-143

Caraty, A , Locatelli, A , Moenter, S M and Karsch, F J (1994), Sampling of hypophyseal portal blood of conscious sheep for direct monitoring of hypothalamic neurosecretory substances, In *Methods in Neuroscences*, Conn, M (ed), Academic Press San Diego, **9** 162-183

Carr, F E , Fein, H G , Fisher, C U , Wessendorf, M W and Smallridge, R C (1992), A cryptic peptide (160-169) of thyrotropin-releasing hormone prohormone demonstrates biological activity *in vitro* and *in vivo*, *Endocrinology*, **131**, 2653-2658

Charli, J -L , Mendez, M , Joseph-Bravo, P and Wilk, S (1987), Specific inhibitors of pyroglutamyl peptidase I and prolyl endopeptidase do not change the *in vitro* release of TRH or its content in rodent brain, *Neuropeptides*, **9**, 373-378

Charli, J -L , Cruz, C , Vargas, M-A and Joseph-Bravo, P (1988), The narrow specificity pyroglutamate aminopeptidase degrading TRH in rat brain is an ectoenzyme, *Neurochemistry International*, **13**(2), 237-242

Charli, J -L , Mendez, M , Vargas, M-A , Cisneros, M , Assai, M , Joseph-Bravo, P and Wilk, S (1989), Pyroglutamyl peptidase II inhibition specifically increases recovery of TRH released from rat brain slices, *Neuropeptides*, **14**, 191-196

Cleuziat, P , Awade, A and Robert-Baudouy, J (1992a), Molecular characterisation of pcp, the structural gene encoding the pyrrolidone carboxylyl peptidase from *Streptococcous pyogenes*, *Molecular Microbiology*, **6**(15), 2051-2063

Cleuziat, P , Gayral, J -P , Allibert, P , Cros, P and Mandrand, B (1992b), Automated detection of group A *streptococci* by DNA probe, *Proceedings of the Conference on Taxonomy and Automated Identification of Bacteria (Praga)*, 70-73

Cockle, S M and Smyth, D G (1986), Processing of thyrotropin releasing hormone (TRH) precursor in *Xenopus* skin and bovine hypothalamus Evidence for the existence of extended forms of TRH, *Regulatory Peptides*, **14**, 217-227

Cockle, S M and Smyth, D G (1987), Specific processing of the thyrotropin-releasing prohormone in rat brain and spinal cord, *European Journal of Biochemistry*, **165**, 693-698

Crawley, J N (1990), Coexistence of neuropeptides and "classical" neurotransmitters Functional interactions between galanin and acetylcholine, *Annals of the New York Academy of Science*, **579**, 233

Cruz, C , Charli, J -L , Vargas, M -A , Joseph-Bravo, P (1991), Neuronal localisation of pyroglutamate aminopeptidase II in primary cultures of fetal mouse brain, *Journal of Neurochemistry*, **56**(5), 1594-1601

Dahl, G E , Evans, N P , Thrun, L A and Karsch, F J (1994), A central negative feedback action of thyroid hormones on thyrotropin-releasing hormone secretion, *Endocrinology*, **135**(6), 2392-2397

Dealler, S F , Campbell, L , Kerr, K G , McGoldrick, J , Flannigan, K A and Hawkey, P M (1989), Reliable five-minute test strip method for identification of *Streptococcus pyogenes*, *European Journal of Clinical Microbiology and Infectious Disease*, **8**, 308-310

de Gandarias, J M , Casis, O , Echevarria, E , Irazusta, J and Casis, L (1992), Pyroglutamil-peptidase I activity in the cortex of the cat brain during development, *International Journal of Developmental Biology*, **36**, 335-337

de la Pena, P , Delgado, L M , Del Camino, D and Barros, F (1992a), Cloning and expression of the TRH-receptor from GH_3 rat anterior pituitary cells, *Biochemical Journal*, **284**, 891-899

de la Pena, P , Delgado, L M , Del Camino, D and Barros, F (1992b), Two isoforms of the TRH receptor generated by alternative splicing have indistinguishable functional properties, *Journal of Biological Chemistry*, **267**, 25703-25708

de la Pena, P , del Camino, D , Pardo, L A , Dominguez, P and Barros F (1995), Gs couples thyrotropin-releasing hormone receptors expressed in *Xenopus oocytes* to phospholipase C, *Journal of Biological Chemistry*, **270**(8), 3554-3559

Docherty, K and Steiner, D F (1982), Post-translational proteolysis in polypeptide hormone biosynthesis, *Annual Review of Physiology*, **44**, 625-638

Doolittle, R F and Armentrout, R W (1968), Pyrrolidonyl peptidase An enzyme for selective removal of pyrrolidonecarboxylic acid residues from polypeptides, *Biochemistry*, 7(2), 516-521

Dresdner, K , Barker, L A , Orlowski, M and Wilk, S (1982), Subcellular distribution of prolyl endopeptidase and cation sensitive neutral endopeptidase in rabbit brain, *Journal of Neurochemistry*, **38**, 1151-1154

Drummond, A H (1986), Inositol lipid metabolism and signal transduction in clonal pituitary cells, *Journal of Experimental Biology*, **124**, 337-358

Dupont, A , Labrie, F , Levasseur, L , Dussault, J-H and Schally, A (1976), Effect of thyroxine on the inactivation of thyrotrophin-releasing hormone by rat and human plasma, *Clinical Endocrinolgy*, **5**, 323-330

Duthie, S M , Taylor, P L , Anderson, L , Cook, J and Eidne, K A (1993), Cloning and functional characterisation of the human TRH receptor, *Molecular and Cellular Endocrinology*, **95**, R11-R15

Dyess, E M , Segerson, T P , Liposits, Z , Paull, W , Kaplan, M M , Wu, P and Jackson, I M D (1988), Triiodothyronine exerts direct cell-specific regulation of thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus, *Endocrinology*, **123**, 2291-2297

Edman, P (1950), Method for the determination of the amino sequence in peptides *Acta Chemica Scandinavica*, **4**, 283-293

Edwardson, J A and Benett, G W (1977), In *Peptides - Proceedings of the Fifth American Peptide Symposium*, Goodman, M and Meienhofer, J (eds), John Wiley, New York, 183-185

Elmore, M A , Griffiths, E C , O'Connor, B and O'Cuinn, G (1990), Further characterisation of the substrate specificity of TRH hydrolysing pyroglutamate aminopeptidase from guinea-pig brain, *Neuropeptides*, **15**, 31-36

Eipper, B A and Mains, R E (1988), Peptide alpha-amidation, *Annual Review of Physiology*, **50**, 333-344

Eisenthal, R and Cornish-Bowden, A (1974), The direct linear plot A new graphical procedure for estimating enzyme kinetic parameters, *Biochemical Journal*, **139**, 715-720

Emerson, C H and Wu, C F (1987), Thyroid status influences rat serum but not brain TRH pyroglutamyl aminopeptidase activities, *Endocrinology*, **120**(3), 1215-1217

Engler, D , Scanlon M F and Jackson, I M D , (1981), Thyrotropin-releasing hormone in the systemic circulation of the neonatal rat is derived from the pancreas and other extraneural tissues, *Journal of Clinical Investigation*, **67**, 800-808

Faivre-Bauman, A , Knisatchek, H , Tixier-Vidal, A and Bauer, K (1981), Ontogenesis of neuropeptide degrading enzymes in the mouse brain, *Journal of Neurosci ence Research*, **6**, 63-74

Faivre-Bauman, A , Loudes, C , Barret, A , Tixier-Vidal, A and Bauer, K (1986), Possible role of neuropeptide degrading enzymes on thyroliberin secretion in fetal hypothalamic cultures in serum free medium *Neuropeptides*, **7**, 125-138

Fellows, R E and Mudge, A (1971a), Isolation and characterisation of *B subtilis* pyrrolidonecarboxylyl peptidase as an adjunct for the investigation of peptide structure, *Federal Proceedings*, **30**, 1078(151)

Fellows, R E and Mudge, A (1971b), Degradation of thyrotropin releasing factor by pituitary pyrrolidonecarboxylyl peptidase, *Federal Proceedings*, **30**(2), A534(1899)

Fischer, W H and Speiss, J (1987), Identification of a mammalian glutaminyl cyclase converting glutaminyl into pyroglutamyl peptides, *Proceedings of the National Academy of Sciences USA*, **84**, 3628-3632

Folkers, K , Enzmann, F , Boler, J (1969), Discovery of modification of the synthetic tripeptide-sequence of the thyrotropin releasing hormone having activity, *Biochemical and Biophysical Research Communications*, **37**(1), 123-126

Friedman, T C , Orlowski, M and Wilk, S (1984), Prolyl endopeptidase Inhibition *in vivo* by *N*-benzyloxycarbonyl-prolyl-prolinal, *Journal of Neurochemistry*, **42**(1), 237-241

Friedman, T C , Kline, T B and Wilk, S (1985), 5-oxoprolinal Transition-state aldehyde inhibitor of pyroglutamyl-peptide hydrolase, *Biochemistry*, **24**, 3907-3913

Friedman, T C and Wilk, S (1986), Delineation of a particulate thyrotropin-releasing hormone-degrading enzyme in rat brain by the use of specific inhibitors of prolyl endopeptidase and pyroglutamyl peptide hydrolase, *Journal of Neurochemistry*, **46**(4), 1231-1239

Fujiwara, K and Tsuru, D (1978), New chromogenic and fluorogenic substrates for pyrrolidonyl peptidase, *Journal of Biochemistry*, **83**, 1145-1149

Fujiwara, K , Kobayashi, R and Tsuru, D (1979), The substrate specificity of pyrrolidone carboxylyl peptidase from Bacillus amyloliquifaciens, *Biochimica et Biophysica Acta*, **570**, 140-148

Fujiwara, K , Kitagawa, T and Tsuru, D (1981a), Inactivation of pyroglutamyl ammopeptidase by L-pyroglutamyl chloromethyl ketone, *Biochimica et Biophysica Acta*, **655**, 10-16

Fujiwara, K , Matsumoto, E , Kitagawa, T and Tsuru, D (1981b), Inactivation of pyroglutamyl aminopeptidase by N^{alpha}-carbenzoxy-L-pyroglutamyl chloromethyl ketone, *Journal of Biochemistry* **90** 433-437

Fujiwara, K , Matsumoto, E , Kitagawa, T and Tsuru, D (1982), N-alpha-carbenzoxy pyroglutamyl diazomethyl ketone as active-site-directed inhibitor for pyroglutamyl peptidase, *Biochimica et Biophysica Acta*, **702**, 149-154

Furukawa, K , Nomoto, T and Tonoue, T (1980), Effects of thyrotropin-releasing hormone (TRH) in the isolated small intestine and taenia coli of the guinea pig, *European Journal of Pharmacology*, **64**, 279-287

Fuse, Y , Polk, D H , Lam, R W , Reviczky, A L and Fisher, D A (1990), Distribution and ontogeny of thyrotropin-releasing hormone degrading enzymes in rats, *American Journal of Physiology*, **259**(6), E787-E791

Gainer, H , Russell, J T and Loh, Y P (1985), The enzymology and intracellular organization of peptide precursor processing - the secretory vesicle hypothesis, *Neuroendocrinology*, **40**, 176-184

Garat, B , Miranda, J , Charli, J -L and Joseph-Bravo, P (1985), Presence of a membrane bound pyroglutamyl amino peptidase degrading thyrotropin releasing hormone in rat brain, *Neuropeptides*, **6**, 27-40

Gautvik, K M , Tashjian Jr , A H , Kourides, I A , Weintraub, B D , Graeber, C T , Maloof, F , Suzuki, K and Zuckerman, J E (1974), Thyrotropin-releasing hormone is not the sole physiologic mediator of prolactin release during suckling, *New England Journal of Medicine*, **290**, 1162-1165

Gershengorn, M C (1978), Bi-hormonal regulation of thyrotropin-releasing-hormone receptor in mouse pituitary thyrotropic tumor-cells in culture, *Journal of Clinical Investigation*, **62**(5), 937-943

Gershengorn, M C (1986), Mechanism of thyrotropin releasing hormone stimulation of pituitary hormone secretion, *Annual Review of Physiology*, **48**, 515-526

Gkonos, P J , Tavianini, M A , Liu, C C and Roos, B A (1989), Thyrotropin-releasing hormone gene expression in normal thyroid parafollicular cells, *Molecular Endocrinology*, **3** 2101-2109

Gonzales, T , Awade, A , Besson, C and Robert-Baudouy, J (1992), Purification and characterisation of recombinant pyrrolidone carboxyl peptidase of *Bacillus subtilis*, *Journal of Chromatography*, **584**, 101-107

Gonzales, T and Robert-Baudouy, J (1994), Characterization of the pcp gene of *Pseudomonas fluorescens* and its product, pyrrolidone carboxyl peptidase (pcp), *Journal of Bacteriology*, **176**(9), 2569-2576

Greaney, A , Phelan, J and O'Cuinn, G (1980), Localisation of thyroliberin pyroglutamyl peptidase on synaptosomal-membrane preparations of guinea-pig brain tissue, *Biochemical Society Transactions*, **8**(4), 423

Griffiths, E C , Kelly, J A , White, N and Jeffcoate, S L (1979), Hypotalamic inactivation of thyroliberin (thyrotropin-releasing hormone), *Biochemical Society Transactions*, **7**, 74-75

Griffiths, E C , Kelly, J A , White, N and Jeffcoate, S L (1980), Further studies on the inactivation of thyrotrophin releasing hormone (TRH) by enzymes in the rat hypothalamus, *Acta Endocrinologica*, **93**, 385-391

Griffiths, E C , Widdowson, P S and Slater, P (1982), Antagonism of TRH-induced wet-dog shaking in rats by neurotensin and a neurotensin fragment, *Neuroscience Letters*, **31**, 171-174

Griffiths, E C , Slater, P and Widdowson, P S (1983a), Effects on limbic tone of neurotensin, TRH and ß-endorphin injected into the periaqueductal grey region of rat brain, *British Journal of Pharmacology*, **80**, 657

Griffiths, E C , Millar, R P and McKelvy, J F (1983b), In *Thyrotropin Releasing Hormone*, Griffiths, E C and Bennett, G W (eds), Raven Press, New York, 45-49

Griffiths, E C and Bennett, G W (1983), In *Thyrotropin Releasing Hormone*, Griffiths, E C and Bennett, G W (eds), Raven Press, New York

Griffiths, E C and McDermott, J R (1984), Biotransformation of neuropeptides, *Neuroendocrinology*, **39**, 573-581

Griffiths, E C (1985), Thyrotrophin releasing hormone Endocrine and central effects, *Psychoneuroendocrinology*, **10**(3), 225-235

Grioli, S , Lomeo, C , Quattropani, M C , Spignoli, G and Vilardita, C (1990), Pyroglutamic acid improves the age-associated memory impairment, *Fundamental Clinical Pharmacology*, **4**, 169-173

Grimm-Jorgensen, Y and Reichlin, S (1973), Thyrotropin-releasing hormone (TRH) Neurotransmitter regulation of secretion by mouse hypothalamic tissue *in vitro*, *Endocrinology*, **93**, 626-631

Guerineau, N C , Lledo, P M , Verrier, D and Israel, J M (1995), Evidence that TRH controls prolactin release from rat lactotrophs by stimulating a calcium influx, *Cell Biology and Toxicology*, **10**(5-6), 311-316

Guillemin, R , Yamakazi, E , Jutisz, M and Sakiz, E (1962), *Comptes Rendus de L Academie des Sciences (Paris)*, **255**, 1018

Guillemin, R (1967), *Annual Review of Physiology*, **29**, 313

Guillemin, R (1971), *In vitro* synthesis of thyrotropin releasmg factor, *Program of the 1st Annual Meeting of the Society of Neuroscience*, Washington D C , p70

Guillemin, R and Burgus, R (1972), Hormones of hypothalamus, *Scientific American*, **227**(5), 24

Haitinger, L (1882), Vorlauge mittheilung uber glutaminsaure und pyrrol, *Monatsh Chem* , **3**, 228-229

Hall, T R (1984), Control of prolactin secretion in vertebrates - a comparative study, *General Pharmacology*, **15**, 189-195

Hanada, K , Tamai, M , Yamagishi, M , Ohmura, S , Sawada, J and Tanaka, I (1978), Isolation and characterisation of E-64, a new thiol protease inhibitor, *Agricultural and Biological Chemistry*, **42**, 523

Hanes, C S (1932), *Biochemical Journal*, **26**, 1406

Harris, A C , Christianson, D , Smith, M S , Fang, S -L , Braverman, L E and Vagenakis, A G (1978), The physiological role of thyrotropin-releasing hormone in the regulation of thyroid-stimulating hormone and prolactin secretion in the rat, *Journal of Clinical Investigation*, **61**, 441-448

Hatsu, M , Naganawa, H , Aoyagi, T and Takeuchi T (1992a), Benarthin A new inhibitor of pyroglutamyl peptidase II Physico-chemical properties and structure determination, *Journal of Antibiotics*, **45**(7), 1084-1087

Hatsu, M , Naganawa, H , Aoyagi, T and Takeuchi, T (1992b), The structure of pyrizinostatin, *Journal of Antibiotics*, **45**(12), 1961-1962

Hayes, D J , Phelan, J J and O'Cuinn, G (1979), The metabolism of thyrotropin-releasing hormone by guinea-pig brain, *Biochemical Society Transactions*, **7**, 59-62

Heal, D J , Pycock, C J , Youdim, M B H *et al* (1983), Actions of TRH and its analogues on the mesolimbic dopamine system, In *Thyrotropin-Releasing Hormone*, Griffiths, E C and Bennett, G W (eds), Raven Press, New York, 271-282

Hedner, J , Hedner, T , Wessberg, P , Lundberg, D and Jonason, J (1983), Effects of TRH and TRH analogues on the central regulation of breathing in rat, *Acta Physiologica Scandinavica*, **117**, 427-437

Henderson, P J F (1992), Statistical analysis of enzyme kinetic data, In *Enzyme Assays*, Eisenthal, R and Danson, M J (eds), IRL Press, Oxford, 277-316

Herkenham, M and McLean, S (1986), Mismatches between receptor and transmitter localisations in the brain, In *Quantitative Receptor Autoradiography*, Boast, C , Snowhill, E W and Altar, A (eds), Liss, New York, 137-171

Hersh, L B (1981), Immunological, physical, and chemical evidence for the identity of brain and kidney post-proline cleaving enzyme, *Journal of Neurochemistry*, **37**(1), 172-178

Hershman, J M and Pekary, A E (1985), In *The Pituitary Gland*, Imura, H (ed), Raven Press, New York, 149-188

Heukshoven, J and Dernick, R (1985), Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining, *Electrophoresis*, **6**(3), 103-112

Hinkle, P M and Tashjian Jr , A H (1973), Receptors for thyrotropin-releasing hormone in prolactin-producing rat pituitary cells in culture, *Journal of Biological Chemistry*, **248**(17), 6180-6186

Hinkle, P M , Perrone, M H and Schonbrunn, A (1981), Mechanism of thyroid hormone inhibition of thyrotropin releasing hormone action, *Endocrinology*, **108**, 199-205

Hirooka, Y , Hollander, C S , Suzuki, S , Ferdinand, P , and Juan, S -I (1978), Somatostatin inhibits release of thyrotropin releasing factor from organ cultures of rat hypothalamus, *Proceedings of the National Academy of Science U S A* , **75**, 4509-4513

Hofstee, B H J , Dixon, M and Webb E C (1959), Non-inverted versus inverted plots in enzyme kinetics, *Nature (London)*, **184**, 1296-1294

Hokfelt, T , Lundberg, J M , Schultzerg M , Johansson, O , Ljungdahl, A and Rehfeld, J (1980), Co-existence of peptides and putative neurotransmitters in neurons, In *Neural Peptides and Neuronal Communication*, Costa, E and Trabucchi, M (eds), Raven Press, New York, 1-23

Hokfelt, T , Everitt, B , Holets, V R , Meister, B , Melander, T *et al* (1986), Coexistence of peptides and other active molecules in neurons Diversity of chemical signalling potential, In *Fast and Slow Chemical Signalling in the Nervous System*, Iverson, L L , Maidment, J and Goodman, E C (eds), Oxford University Press, Oxford, 205-231

Hollenberg A N , Monden T , Flynn, T R , Boers, M E , Cohen, O and Wondisford, F E (1995), The human thyrotropin-releasing hormone gene is regulated by thyroid hormone through two distinct classes of negative thyroid hormone response elements, *Molecular Endocrinology*, **9**(5), 540-550

Horsthemke, B and Bauer, K (1980), Characterisation of a non-chymotrypsin-like endopeptidase from anterior pituitary that hydrolyses luteinising hormone-releasing hormone at the tyrosyl-glycine and histidyltryptophan bonds, *Biochemistry*, **19**, 2867-2873

Hu, L M and Lawson, D (1995), Prolactin release from subpopulations of rat lactotrophs in response to thyrotropin-releasing hormone, *Endocrine*, **2**(8), 703-708

Huang, S W , Tsai, S C , Tung, Y F and Wang, P S (1995), Role of progesterone in regulating the effect of estradiol on the secretion of thyrotropin-releasing hormone and dopamine into hypophyseal portal blood in ovariectomized rats, *Neuroendocrinology*, **61**(5), 536-541

Iverson, L L (1984), Amino acids and peptides Fast and slow chemical signals in the nervous system, *Proceedings of the Royal Society of London Series B - Biological Sciences*, **221**, 245-260

Jackson, I M D and Reichlin, S (1979) Distribution and biosynthesis of TRH in the nervous system, In *Central nervous system effects of hypothalamic hormones and other peptides*, Collu, R , Barbeau, A , Ducharme, J R , Rochefort, J G *et al* (eds), Raven Press, New York, 3-54

Jackson, I M D , Papapetrou, P D and Reichlin, S (1979), Metabolic clearance of thyrotropin-releasing hormone in the rat in hyperthyroid states Comparison with serum degradation *in vitro*, *Endocrinology*, **104**, 1292-1298

Jackson, I M D (1982), Thyrotropin-releasing hormone, *New England Journal of Medicine*, **306**, 145-155

Jackson, I M D , Lechan, R M and Lee S L (1990), TRH prohormone Biosynthesis, anatomic distribution and processing, *Frontiers in Neuroendocrinology*, **11**, 267-312

Johansson, O and Hokfelt, T (1980), Thyrotropin releasing hormone, somatostatin and enkephalin Distribution studies using immunohistochemical techniques, *Journal of Histochemistry and Cytochemistry*, **28**, 364-366

Joseph-Bravo, P , Charli, J -L , Palacios, J M and Kordon, C (1979), Effect of neurotransmitters on the *in vitro* release of immunoreactive thyrotropin releasing hormone from rat mediobasal hypothalamus, *Endocrinology*, **104**, 801-806

Kakucska, I , Rand, W and Lechan, R M (1992), Thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is dependant upon feedback regulation by both triiodothyronine and thyroxine, *Endocrinology*, **130**, 2845-2850

Kalivas, P W and Horita, A (1983), Involvement of the septohippocampal system in TRH antagonism of pentobarbital narcosis, In *Thyrotropin Releasing Hormone*, Griffiths, E C and Bennett, G W (eds), Raven Press, New York, 283-290

Kanda, Y , Koike, K , Ohmichi, M , Sawada, T , Hirota, K and Miyake, A (1994), A possible involvement of tyrosine kinase in TRH-induced prolactin secretion in GH_3 cells, *Biochemical and Biophysical Research Communications*, **199**(3), 1447-1452

Kato, T , Nagatsu, T , Kimura, T and Sakakibara, S (1978), Fluorescence assay of X-prolyl dipeptidyl-aminopeptidase activity with a new fluorigenic substrate, *Biochemical Medicine*, **19**, 351-359

Keller, H H , Bartholini, G and Pletscher, A (1974), Enhancement of cerebral noradrenalin turnover by thyrotropin-releasing hormone, *Nature*, **248**, 528-529

Kerdlehue, B , Palkovits, M , Karteszi, M and Reinberg, A (1981), Circadian variation in substance P, luliberin (LH-RH) and thyroliberin (TRH) contents in hypothalamic and extrahypothalamic brain nuclei of adult male rats, *Brain Research*, **206**, 405-412

Khandke, K M , Fairwel, T , Chait, B T and Manjula, B N (1989), Influence of ions on cyclization of the amino terminal glutamine residues of tryptic peptides of streptococcal PepM49 protein

Resolution of cyclized peptides by HPLC and characterization by mass spectrometry, *International Journal of Peptide and Protein Research*, **34**, 118-123

Kikawa, U , Kishimoto, A and Nishizuka, Y (1989), The protein kinase-C family - heterogeneity and its implications, *Annual Review of Biochemistry*, **58**, 31-44

Kita, K , Kotani, H , Hiraoka, N , Nakamura, T and Yonaha, K (1989), Overproduction and crystallization of *Fok* I restriction endonuclease, *Nucleic Acids Research*, **17**(21), 8741-8753

Knisatschek, H and Bauer, K (1979), Characterization of "thyroliberin-deamidating enzyme" as a post-proline-cleaving enzyme Partial purification and enzyme-chemical analysis of the enzyme from anterior pituitary tissue, *Journal of Biological Chemistry*, **254**(21), 10936-10943

Koch, Y and Okon, E (1979), Localisation of releasing hormones in the human brain, *International Review of Experimental Pathology*, **19**, 45-62

Koida, M and Walter, R (1976), Post-proline Cleaving Enzyme Purification of this endopeptidase by affinity chromatography, *Journal of Biological Chemistry*, **251**(23), 7593-7599

Koivusalo, F (1980), Degradation of TRF immunoreactivity in rat hypothalamus, extrahypothalamic brain and pancreas, *Acta Physiologica Scandinavica*, **110**, 303-306

Kow, L -M and Pfaff, D W (1987), Neuropeptides TRH and cyclo(His-Pro) share neuromoulatory, but not stimulatory,-action on hypothalamic neurons *in vitro* Implication on the regulation of feeding, *Experimental Brain Research*, **67**, 93-99

Kow, L -M , and Pfaff, D W (1988), Neuromodulatory actions of peptides, *Annual Review of Pharmacology and Toxicology*, **28**, 163-188

Krieger, D T (1983), Brain peptides What, where, and why?, *Science*, **22**, 975-985

Kreil, G (1981), Transfer of proteins acrosss membranes, *Annual Review of Biochemistry*, **50**, 317-348

Kuchler, K , Richter, K , Trnovsky, J , Egger R and Kreil, G (1990), Two precursors of thyrotropin-releasing hormone from skin of *Xenopus laevis*, *Journal of Biological Chemistry*, **265**, 11731-11733

Kwiatkowska, J , Toram, B and Glenner, G G (1974), Pyrrolidone carboxylate peptidase from particulate fraction of *Klebsiella cloacae* - purification of stable enzyme and its use in releasing NH_2 terminus from pyrrolidone carboxylate peptides and proteins, *Journal of Biological Chemistry*, **249**(24), 7729-7736

Kyte, J and Doolittle, R F (1982), A simple method for displaying the hydropathic character of a protein, *Journal of Molecular Biology*, **157**, 105-132

Ladram, A , Bulant, M , Delfour, A , Montogne, J J , Vaudry, H and Nicolas, P (1994), Modulation of the biological activity of thyrotropin-releasing hormone by alternate processing of pro-TRH, *Biochimie*, **76**, 320-328

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

Lauffart, B and Mantle, D (1988), Rationalization of aminopeptidase activities in human skeletal muscle soluble extract, *Biochimica et Biophysica Acta*, **956**, 300-306

Lauffart, B , McDermott, J R , Biggins, J A , Gibson, A M and Mantle, D (1989), Purification and characterization of pyroglutamyl aminopeptidase from human cerebral cortex, *Biochemical Society Transactions*, **17**(1), 207-208

Lazdunski, A M (1989), Peptidases and proteases of *E coli* and *Salmonella typhimurium*, *FEMS Microbiology Reviews*, **63**, 265-276

Lechan, R M , Wu, P , Jackson, I M D , Wolf, H , Cooperman, S , Mandel, G and Goodman, R H (1986), Thyrotropin releasing hormone precursor Characterisation in the rat brain, *Science*, **231**, 159-161

Le Roith, D , Shiloach, J , Roth, J and Lesniak, M A (1980), Evolutionary origins of vertebrate hormones - substances similar to mammalian insulins are native to unicellular eukaryotes, *Proceedings of the National Academy of Science U S A* , **77**(10), 6184-6188

Le Roith, D , Shiloach, J , Roth, J and Lesniak, M A (1981), Insulin or a closely related molecule is native to *Escherichia coli* *Proceedings of the National Academy of Science U S A* , **256**(13), 6533-6536

Le Roith, D , Liotta, A S , Roth, J , Shiloach, J , Lewis, M E , Pert, C B and Krieger, D T (1982), Corticotropin and beta-endorphin-like materials are native to unicellular animals, *Proceedings of the National Academy of Science U S A* , **79**(6), 2086-2090

Liao, N , Bulant, M , Nicolas, P , Vaudry, H and Pelletier, G (1989), Thyroid hormone regulation of neurons staining for a pro-TRH derived cryptic peptide sequence in the rat hypothalamic paraventricular nucleus, *Neuroendocrinology*, **50**, 217-221

Lineweaver, H and Burk D (1934), Determination of enzyme disassociation constants, *Journal of the American Chemical Society*, **56**, 658

Loudes, C , Faivre-Bauman, A , Patte, C and Tixier-Vidal, A (1988), Involvement of DHP voltage-sensitive calcium channels and protein kinase C in thyroliberin (TRH) release by developing hypothalamic neurons in culture, *Brain Research*, **456**, 324-332

Lu, H S , Clogston, C L , Wypych, J , Fausset, P R , Lauren, S , Mendiaz, E A , Zsebo, K M and Langley, K E (1991), Amino acid sequence and post translational modification of stem cell factor isolated from buffalo rat liver cell-conditioned medium, *Journal of Cell Biology*, **266**, 8102-8107

Lundberg, J M and Tatemoto, K (1982), Pancreatic polypeptide family (APP, BPP, NPY and PYY) in relation to sympathetic vasoconstriction resistant to alpha-adrenoreceptor blockade, *Acta Physiologica Scandinavica*, **116**, 393-402

Lundberg, J and Hokfelt, T (1983), Coexistence of peptides and classical neurotransmiters, *Trends in Neuroscience*, **6**, 325-333

Maeda, K and Frohman, L A (1980), Release of somatostatin and thyrotropin-releasing hormone from rat hypothalamic fragments *in vitro*, *Endocrinology*, **106**, 1837-1842

Mains, R E , Dickerson, I M , May, V *et al* (1990), Cellular and molecular aspects of peptide hormone biosynthesis, In *Frontiers in Neuroendocrinology*, Ganong, W F and Martini, I (eds), Raven, New York, **11**, 52-89

Mannisto, P T (1983), Central regulation of thyrotropin secretion in rats Methodological aspects, problems and some progress, *Medical Biology*, **61**, 92-100

Mantle, D , Lauffart, B , McDermott, J and Gibson, A (1990), Characterization of aminopeptidases in human kidney soluble fraction, *Clinica Chimica Acta*, **187**, 105-114

Mantle, D , Lauffart, B and Gibson, A (1991), Purification and characterization of leucyl aminopeptidase and pyroglutamyl aminopeptidase from human skeletal muscle, *Clinica Chimica Acta*, **197**, 35-46

Martino, E , Seo, H , Lernmark, A and Refetoff, S (1980) Ontogenetic patterns of thyrotropin-releasing hormone-like material in rat hypothalamus, pancreas and retina Selective effect of light deprivation, *Proceedings of National Academy of Science U S A* , **77**, 4345-4348

Maruo, T , Cohen, H , Segal, S J and Koide, S S (1979), *Proceedings of the National Academy of Science U S A* , **76**, 6622

Matre, V , Karlsen, H E , Wright, M S , Lundell, I , Fjeldheim, A K , Gabrielsen, O S , Larhammar, D and Gautvik, K M (1993), Molecular cloning of a functional human thyrotropin-releasing hormone receptor, *Biochemical and Biophysical Research Communications*, **195**, 179-185

Matsui, T , Prasad, C and Peterkofsky, A (1979), Metabolism of thyrotropin releasing hormone in brain extracts Isolation and characterisation of an imidopeptidase for histidylprolineamide, *Journal of Biological Chemistry*, **254**(7), 2439-2445

McDonald, J K , Reilly, T J , Zeitman, B B and Ellis, S (1968a), Dipeptidyl arylamidase II of the pituitary Properties of Lysylalanyl-ß-naphthylamide hydrolysis, Inhibition by cations, distribution in tissues, subcellular localization, *Journal of Biological Chemistry*, **243**(8), 2028-2037

McDonald, J K , Leibach, F H , Grindeland, R E and Ellis, S (1968b), Purification of dipeptidyl aminopeptidase II (dipeptidyl arylamidase II) of the anterior pituitary gland Peptidase and dipeptide esterase activities, *Journal of Biological Chemistry*, **243**(15), 4143-4150

McDonald, J K and Barrett, A J (1986), *Mammalian Proteases A Glossary and Bibliography Vol 2 Exopeptidases*, Academic Press, London

McKelvy, J F , Sheridan, M , Joseph, S , Phelps, C H and Perrie, S (1975), Biosynthesis of thyrotropin releasing hormone in organ cultures of the guinea-pig median eminence, *Endocrinology*, **97**, 908-918

McKelvy, J F (1977), Biosynthesis of hypothalamic peptides, *Advances in Experimental Medicine and Biology*, **87**, 77-98

Meister, A , Bukenberger, M W and Strassburger, M (1963), The optically-specific enzymatic cyclization of D-glutamate, Biochem Z , **338**, 217-229

Meister, A (1968), The specificity of glutamine synthetase and its relationship to substrate conformation at the active site, *Advances in Enzymology*, **31**, 183

Melmed, S , Carlson, H E and Hershman, J M (1982), Histidyl-proline diketopiperazine suppresses prolactin secretion in human pituitary tumor cell culture, *Clinical Endocrinology*, **16**, 97-100

Mendez, M , Cruz, C , Joseph-Bravo, P , Wilk, S and Charli, J -L (1990), Evaluation of the role of prolyl endopeptidase and pyroglutamyl peptidase I in the metabolism of LH-RH and TRH in brain, *Neuropeptides*, **17**(2), 55-62

Metcalf, G (1974), TRH A possible mediator of thermoregulation, *Nature*, **252**, 310-311

Michaelis, L and Menten, M L (1913), *Biochem Z* , **49**, 333-369

Mitnick, M A and Reichlin, S (1971), Biosynthesis of TRH by rat hypothalamic tissue *in vitro*, *Science*, **172**, 1241-1243

Mitnick, M A and Reichlin, S (1972), Enzymatic synthesis of thyrotropin releasing hormone, *Endocrinology*, **91**, 1145

Miyatake, N , Kamo, M , Satake, K , Uchiyama, Y and Tsugita, A (1993), Removal of N-terminal formyl groups and deblocking of pyrrolidone carboxylic acid of proteins with anhydrous hydrazine vapor, *European Journal of Biochemistry*, **212**, 785-789

Morley, J E (1979), Extrahypothalamic thyrotropin releasing hormone (TRH) - its distribution and its functions, *Life Sciences*, **25**, 1539-1550

Morley J E , Bayshore R A , Reed, A Carlson H E and Hershman, J M (1979), Thyrotropin-releasing-hormone and thyroid-hormones in amniotic fluid, *American Journal of Obstetrics and Gynecology*, **134**, 581-584

Morley, J E (1981), Neuroendocrine control of thyrotropin secretion, *Endocrine Reviews*, **2** 396-436

Morrison, N , Duthie, S M , Boyd, E , Eidne, K A and Connor J M (1994), Assignment of the gene encoding the human thyrotropin releasing hormone receptor to 8q23 by fluorescence *in situ* hybridisation, *Human Genetics*, **93**, 716-718

Moss, J and Bundgaard, H (1989), Prodrugs of peptides 5 Protection of the pyroglutamyl residue against pyroglutamyl aminopeptidase by bioreversible derivitization with glyoxylic acid derivatives, *International Journal of Pharmaceutics*, **52**(3), 255-263

Moss, J and Bundgaard, H (1992) Prodrugs of peptides 19 Protection of the pyroglutamyl residue against pyroglutamyl aminopeptidase by N-acycloxymethylation and other means, *Acta Pharmaceutica Nordica*, **4**(4), 301-308

Mudge, A W and Fellows, R E (1973), Bovine pituitary pyrrolidonecarboxylyl peptidase, *Endocrinology*, **93**, 1428-1434

Mulczyk, M and Szewczuk, A (1970), Pyrrolidonyl peptidase in bacteria A new colorimetric test for differentiation of *Enterobacteriaceae*, *Journal of General Microbiology*, **61**, 9-13

Mulczyk, M and Szewczuk, A (1972), Pyrrolidonyl peptidase activity A simple test for differentiating *staphlococci*, *Journal of General Microbiology*, **70**, 383-384

Neary, J T , Kieffer, J D , Federico, P , Maloof, H M F and Soodak, M (1976), Thyrotropin releasing hormone Development of inactivation system during maturation of the rat, *Science*, **193**, 403-405

Nemeroff, C B , Kalivas, P W , Golden, R N and Prange, A J (1984), Behavioural effects of hypothalamic hypophysiotropic hormones, neurotensin, substance P and other neuropeptides, *Pharmacol ogy and Therapeutics*, **24**, 1-56

Nikodemova, M and Strbak, V (1995), Different regulation of thyrotropin releasing hormone content and release in paraventricular nucleus (PVN) and median eminence (ME) of rat hypothalamus during *in vitro* incubation, *Life Sciences*, **56**(18), 1511-1521

O'Connor B and O'Cuinn, G (1984), Localization of a narrow-specificity thyroliberin-hydrolysing pyroglutamate ammopeptidase in synaptosomal membranes of guinea-pig brain, *European Journal of Biochemistry*, **144**, 271-278

O'Connor, B and O'Cuinn, G (1985), Purification and kinetic studies on a narrow specificity synaptosomal membrane pyroglutamate ammopeptidase from guinea-pig brain, *European Journal of Biochemistry* **150** 47-52

O'Connor, B and O'Cuinn, G (1986), Post-proline dipeptidyl aminopeptidase from synaptosomal membranes of guinea-pig brain A possible role for this activity in the hydrolysis of His-Pro-

NH_2, arising from the action of synaptosomal membrane pyroglutamate aminopeptidase on thyroliberin, *European Journal of Biochemistry*, 154, 329-335

O'Cuinn, G , O'Connor, B and Elmore, E (1990), Short Review Degradation of thyrotropin-releasing hormone and luteinising hormone-releasing hormone by enzymes of brain tissue, *Journal of Neurochemistry*, **54**(1), 1-13

Ohmori, T , Nakagami, T , Tanaka, H and Maruyama, S (1994), Isolation of prolylendopeptidase-inhibiting peptides from bovine brain, *Biochemical and Biophysical Research Communications*, **202**, 809-815

O'Leary, R and O'Connor, B (1995a), A study of a synaptosomal thyrotrophin releasing hormone-inactivating pyroglutamyl aminopeptidase from bovine brain, *International Journal of Biochemistry and Cell Biology*, In press

O'Leary, R and O'Connor, B (1995b), Identification and localisation of a synaptosomal membrane prolyl endopeptidase from bovine brain, *European Journal of Biochemistry*, **227**, 277-283

Oliveira, E B , Martins, A P and Camargo, C M (1976), Isolation of brain peptidases Influence of size and sequence of substrates structurally related to bradykinin, *Biochemistry*, **15**, 1967-1974

Oliver, C , Charvet, J P , Codaccioni, J L , Vague, J and Porter, J C (1974a), TRH in human CSF, *Lancet*, **1**, 873

Oliver, C , Charvet, J P , Codaccioni, J L and Vague, J (1974b), Radioimmunoassay of thyrotropin-releasing hormone in human plasma and urine, *Journal of Clinical Endocrinology and Metabolism*, **39**, 406-409

Oliver, C , Tuarog, A and Porter, J C (1974c), Physiology of TRH secretion, *Nouvelle Presse Medicale*, **3**, 1941-1944

Orlowski, M and Meister, A (1971), Enzymology of pyrrolidone carboxylic acid, *The Enzymes*, **4**, 123-151

Orlowski, M , Wilk, E , Pearce, S and Wilk, S (1979), Purification and properties of a prolyl endopeptidase from rabbit brain, *Journal of Neurochemistry* **33**(2) 461-469

Oron, Y , Gillo, B , Straub, R E and Gershengorn, M C (1987a), Membrane electrical response to thyrotropin-releasing-hormone in *Xenopus* oocytes injected with GH_3 pituitary cell messenger ribonucleic acid, *Molecular Enocrinology*, **1**(12), 918-925

Oron, Y , Straub, R E , Traktman, P and Gershengorn, M C (1987b), Decreased TRH receptor messenger-RNA activity precedes homologous down-regulation - assay in oocytes, *Science*, **238**, 1406-1408

Page, I H and Bumpus, F M (1961), *Physiological Review*, **41**, 331

Pan, J -T , Kow, L -M and Pfaff, D W (1988), Modulatory actions of luteinizing-hormone-releasing hormone on electrical activity of preoptic neurons in brain slices, *Neuroscience*, **27**(2), 623-628

Panosian, K J and Edberg, S C (1989), Rapid identification of *streptococcus bovis* by using a combination of constitutive enzyme substrate hydrolyses, *Journal of Clinical Microbiology*, **27**, 1719-1722

Patchett, A A , Harris, E , Tristam, E W , Wyvratt, M J , Wu, M T , Taub, D , Peterson, E R , Ikeler, T J , Ten Broeke, J , Payne, L G , Ondeyka, D L , Thorsett, E D , Grenniee, W J , Lohr, N S , Hoffsommer, R D , Joshua, A , Ruyle, W V , Rothrock, J W , Aster, S D , Maycock, A L , Robinson, F M and Hirschmann, C R (1980), A new class of angiotensin-converting enzyme inhibitors, *Nature*, **288**, 280-283

Perlman, J H , Thaw, C N , Laakkonen, L , Bowers, C Y , Osman, R and Gershengorn, M (1994), Hydrogen bonding interaction of thyrotropin releasing hormone (TRH) with transmembrane tyrosine 106 of the TRH receptor, *Journal of Biological Chemistry*, **269**(3), 1610-1613

Peterkofsky, A , Battaini, F , Koch, Y , Takahara, Y and Dannies, P (1982), Histidyl-proline diketopiperazine Its biological role as a regulatory peptide, *Molecular and Cellular Biochemistry*, **42**, 45-62

Petrou, C P and Tashjian Jr , A H (1995), Evidence that the thyrotropin-releasing hormone receptor and its ligand are recycled dissociated from each other, *Biochemical Journal*, **306**, 107-113

Pierro, M and Orsatti, M (1970), Intestinal hydrolysis of pyrrolidonyl peptides, *Bollettino Della Societa Italiana Di Biologia Sperimentale*, **45**, 1630

Podell, D N and Abraham, G N (1978), A technique for the removal of pyroglutamic acid from the amino terminus of proteins using calf liver pyroglutamate amino peptidase, *Biochemical and Biophysical Research Communications*, **81**(1), 176-185

Prasad, C and Peterkofsky, A (1976), Demonstration of pyroglutamylpeptidase and amidase activities toward thyrotropin-releasing hormone in hamster hypothalamus extracts, *Journal of Biological Chemistry*, **251**(11), 3229-3234

Prasad, C , Jacobs, J J and Wilber, J F (1980), Thermoregulation A new, non-endocrine, role for endogenous thyrotropin-releasing hormone (TRH), *Clinical Research*, **28**(2), 481A

Prasad, C , Wilber, J F and Amborski, R L (1982a), Thyrotropin-releasing hormone Its distribution and metabolism during development in bullfrog (*Rana catesbeiana*), *Developmental Neuroscience*, **5**(4), 293-297

Prasad, C , Mori, M , Wilber, J F , Pierson, W , Pegues, J and Jayaraman, A (1982b), Distribution and metabolism of cyclo(His-Pro) A new member of the neuropeptide family, *Peptides*, **3**, 591-598

Prasad, C , Mori, M , Pierson, W , Wilber, J F and Ewards, R M (1983), Developmental changes in the distribution of rat brain pyroglutamate aminopeptidase, a possible determinant of endogenous cyclo(His-Pro) concentrations, *Neurochemical Research*, **8**(3), 389-399

Prasad, C and Jayarman, A (1986), Metabolism of thyrotropin-releasing hormone in human cerebrospinal fluid Isolation and characterization of pyroglutamate aminopeptidase activity, *Brain Research*, **364**, 331-337

Prasad, C (1987), Activation/inactivation of rat tissue pyroglutamate aminopeptidase by disulfide bond-reducing agents, *Neuropeptides*, **9**(3), 211-215

Ramirez, M , Sanchez, B , Arechaga, G , Garcia, S , Lardelli, P , Venzon, D and de Gandarias, J M (1991), Diurnal variation and left-right distribution of pyroglutamyl peptidase I activity in rat brain and retina, *Acta Endocrinologica*, **125**(9), 570-573

Redding, T W and Schally, A V (1969), Studies on the inactivation of thyrotropin-releasing hormone (TRH), *Proceedings of the Society of Experimental Biology and Medicine*, **131** 415-420

Reichlin, S , Martin, J B and Jackson, I M D (1978), Regulation of thyroid-stimulating hormone (TSH) secretion, In *The Endocrine Hypothalamus*, Jeffcoate, S L and Hutchinson, J S M (eds), Academic Pres, New York, 229-269

Richter, K , Kawashima, E , Egger, R and Kreil, G (1984), Biosynthesis of thyrotropin releasing hormone in the skin of *Xenopus laevis* Partial sequen ce of the precursor deduced from cloned cDNA, *EMBO Journal*, **3**, 617-621

Roussel, J P , Hollande, F , Bulant, M and Astier, H (1991), A prepro-TRH connecting peptide (prepro-TRH 160-169) potentiates TRH-induced TSH release from rat perifused pituitaries by stimulating dihydropyridine- and omega-connotoxin-sensitive Ca^{2+} channels, *Neuroendocrinology*, **54**, 559-565

Rupnow, J H , Hinkle, P M and Dixon, J E (1979a), Macromolecule which gives rise to thyrotropin releasing-hormone, *Biochemical and Biophysical Research Communications*, **89**(2), 721-728

Rupnow, J H , Taylor, W L and Dixon, J E (1979b), Purification and characterisation of a thyrotropin-releasing hormone deamidase from rat brain, *Biochemistry*, **18**, 1206-1212

Sachs, H (1969), *Advances in Enzymology*, **32**, 327

Salers, P , Ouafik, L , Giraud, P , Dutour, A , Maltese, J-Y and Oliver, C (1991), Evidence for pyroglutamyl peptidase I and prolyl endopeptidase activities in the rat insulinoma cell line RINm 5F Lack of relationship with TRH metabolism, *Molecular and Cellular Biochemistry*, **106**(1), 15-24

Salers, P , Ouafik, L , Giraud, P , Maltese, J-Y , Dutour, A and Oliver, C (1992), Ontogeny of prolyl endopeptidase, pyroglutamyl peptidase I, TRH and its metabolites in rat pancreas *American Journal of Physiology*, **262**, E845-E850

Sanger, F and Thompson, E O P (1953), The amino-acid sequence in the glycyl chain of insulin 2 The investigation of peptides from enzymic hydrolysates, *Biochemical Journal*, **53**, 366

Sanger, F , Thompson, E O P and Kitai, R (1955), The amide groups of insulin, *Biochemical Journal*, **59**, 509-518

Satoh, T , Yamada, M , Monden, T , Lizuka, M and Mori, M (1992), Cloning of the mouse hypothalamic preprothyrotropin-releasing hormone cDNA and tissue distribution of its mRNA, *Molecular Brain Research* **14** 131-135

Schaffer, J M , Brownstein, M J and Axelrod, J (1977), Thyrotropin-releasing hormone-like material in the rat retina Changes due to environmental lighting, *Proceedings of the National Academy of Science U S A* , **74**, 3579-3581

Schally, A V , Bowers, C Y , Redding, T W and Barrett, J F (1966), *Biochemical and Biophysical Research Communications*, **25**, 165

Schally, A V , Arimura, A , Bowers, C Y , Kastin, A J , Sawano, S and Redding, T W (1968), *Recent Progress in Hormone Research*, **24**, 497

Schally, A V , Redding, T W , Bowers, C Y ad Barrett, J F (1969), *Journal of Biological Chemistry*, **244**, 4077

Scharfmann, R and Aratan-Spire, S (1991), Ontogeny of two topologically distinct TRH-degrading pyroglutamate aminopeptidase activities in the rat liver, *Regulatory Peptides*, **32**(2), 75-83

Schauder, B , Schomburg, L , Kohrle, J and Bauer, K (1994), Cloning of a cDNA encoding an ectoenzyme that degrades thyrotropin-releasing hormone, *Proceedings of the National Academy of Science USA*, **91**, 9534-9538

Schwabe, C , Le Roith, D , Thompson, R P , Shiloach, J and Roth, J (1983), Relaxin extracted from protozoa (*Tetrahymena pyriformis*) - molecular and immunological properties, *Journal of Biological Chemistry*, **258**(5), 2778-2781

Seeburg, P H and Adelman, J P (1984), Characterization of cDNA for precursor of human luteinizing hormone releasing hormone, **Nature**, *311*, 666-668

Segerson, T P , Kauer, J , Wolfe, H C , Mobtaker, H , Wu P , Jackson I M D and Lechan, R M (1987), Thyroid hormone regulates TRH biosynthesis in the paraventricular nucleus of the rat hypothalamus, *Science*, **238**, 78-80

Shambaugh III, G , Kubek, M and Wilber, J F (1979), Thyrotropin-releasing hormone activity in the human placenta, *Journal of Clinical Endocrinology and Metabolism*, **48**, 483-486

Shupnik, M A , Chin, W W , Ross, D S , Downing, M F , Habener, J F and Ridgway, E C (1983), Regulation by thyroxine of the messenger-RNA encoding the alpha-subunit of mouse thyrotropin *Journal of Biological Chemistry*, **253**, 5120-5124

Shupnik, M A , Chin, W W , Habener, J F and Ridgway, E C (1985), Transcriptional regulation of thyrotropin beta-subunit genes by thyroid hormone, *Journal of Biological Chemistry*, **260**, 2900-2903

Shupnik, M A , Ardisson, L J , Meskell, J F , Bornstein, J and Ridgway, E C (1986), Triiodothyronine (T3) regulation of thyrotropin subunit gene-transcription is proportional to T3 nuclear receptor occupancy, *Endocrinology (Baltimore)*, **118**(1), 367-371

Smith, P K , Krohn, R I , Hermanson, G T , Mallia, A K , Gartner, F H , Provenzano, M D , Fujimoto, E K , Goeke, N M , Olsen, B J and Klenk, D C (1985), Measurement of protein using bicinchoninic acid, *Analytical Biochemistry*, **150**, 76-85

Smyth, D G , Stein W H and Moore, S (1962), On the sequence of residues 11 to 8 in bovine pancreatic ribonuclease, *Journal of Biological Chemistry*, **237**, 1845-1850

Smyth, M and O'Cuinn, G (1994a), Dipeptidyl aminopeptidase activities of guinea-pig brain, *International Journal of Biochemistry*, **26**(7), 913-921

Smyth, M and O'Cuinn, G (1994b), Alanine aminopeptidase of guinea-pig brain A broad specificity cytoplasmic enzyme capable of hydrolysing short and intermediate length peptides, *International Journal of Biochemistry*, **26**(10/11), 1287-1297

Spignoli, G , Magnani, M , Giovannini, M and Pepeu, G (1987), Effect of pyroglutamic acid stereoisomers on ECS and scopolamine-induced memory disruption and brain acetylcholine levels in rat, *Pharmacological Research Communications*, **19**, 901-912

Steiner, D F , Clark, J L , Nolan, C , Rubenstein, A H , Aten, B and Oyer, P E (1969), *Recent Progress in Hormone Research*, **25**, 207

Straub, R E , Frech, G C , Joho, R H and Gershengorn, M C (1990), Expression cloning of a cDNA encoding the mouse pituitary thyrotropin-releasing hormone receptor, *Proceedings of the National Academy of Sciences U S A* , **87**, 9514-9518

Suen, C -S and Wilk, S (1989), Regulation of thyrotropin releasing hormone degrading enzymes in rat brain and pituitary by L-3,5,3'-triiodothyronine, *Journal of Neurochemistry*, **52**(3), 884-888

Sullivan, J J and Jago, G R (1970), A model for bitter peptide formation and degradation in cultured dairy products, *Australian Journal of Dairy Technology*, **25**, 111

Sullivan, J J , Muchmicky, E E , Davidson, B E and Jago, G R (1977), Purification and properties of the pyrrolidonecarboxylate peptidase of *Streptococcus faecium*, *Australian Journal of Biological Sciences*, **30**, 543-552

Svoboda, K S and Currie, B L (1992), Evaluation of some substrates and potential inhibitors of tissue pyroglutamyl aminopeptidase I, *Neuropeptides*, **21**(2), 85-92

Szewczuk, A and Mulczyk, M (1969), Pyrrolidonyl peptidase in bacteria The enzyme from *Bacillus subtilis*, *European Journal of Biochemistry*, **8**, 63-67

Szewczuk, A and Kwiatkowska, J (1970), Pyrrolidonyl peptidase in animal, plant and human tissues Occurrence and some properties of the enzyme, *European Journal of Biochemistry*, **15**, 92-96

Tate, S S (1981), Purification and properties of a bovine brain thyrotropin-releasing-factor deamidase A post-proline cleaving enzyme of limited specificity, *European Journal of Biochemistry*, **118**, 17-23

Tatsuta, K and Kitagawa, M (1994), One step synthesis of a pyroglutamyl peptidase inhibitor, pyrizinostatin, from an antibiotic, 2-methylfervenulone, *Journal of Antibiotics*, **47**(3), 389-390

Taylor, W L and Dixon, J E (1978), Characterisation of a pyroglutamate aminopeptidase from rat serum that degrades thyrotropin-releasing hormone, *Journal of Biological Chemistry*, **253**(19), 6934-6940

Taylor, W L and Dixon, J E (1980), Catabolism of neuropeptides by a brain proline endopeptidase, *Biochemica et Biophysica Acta*, **94**, 9-15

Thomas, G B , Cummins, J T , Yao, B , Gordon, K and Clarke, I (1988), Release of prolactin is independent of the secretion of thyrotropin-releasing hormone into pituitary portal blood of sheep, *Journal of Endocrinology*, **117**, 115-122

Thompson, R C (1973), Use of peptide aldehydes to generate transition-state analogs of elastase, *Biochemistry*, **12**, 47

Tixier-Vidal, A and Fairvre-Bauman, A (1992), Ontogeny of thyrotropin-releasing hormone biosynthesis and release in hypothalamic neurons, *Trends in Endocrinology and Metabolism*, **3**(2), 59-64

Torres, H , Charli, J -L , Gonzalez-Noriega, A , Vargas, M A and Joseph-Bravo, P (1986), Subcellular distribution of the enzymes degrading thyrotropin releasing hormone and metabolites in rat brain, *Neurochemistry International*, **9**(1), 103-110

Tsuru D , Fujiwara, K and Kado, K , (1978), Purification and characterisation of L-pyrrolidonecarboxylate peptidase from *Bacillus amyloliquefaciens*, *Journal of Biochemistry*, **84**, 467-476

Tsuru, D , Sakabe, K , Yoshimoto, T and Fujiwara, K (1982), Pyroglutamyl peptidase from chicken liver Purification and some properties, *Journal of Pharmacobio-Dynamics*, **5**(11), 859-868

Tsuru, D , Nakamura, K , Yoshimoto, T and Fujiwara, K (1984), Pyroglutamyl peptidase from *Bacillus amyloliquefaciens* An improved purification method and some properties of the enzyme, *Biochimica et Biophysica Acta*, **791**, 117-122

Uliana, J A and Doolittle, R F (1969), Pyrrolidonecarboxylyl peptidase Studies on the specificity of the enzyme, *Archives of Biochemistry and Biophysics*, **131**, 561-565

Van Amsterdam, J G C , Van Buuren, K J H and Soudijn, W (1983), Purification and characterisation of enkephalin-degrading enzymes from calf-brain striatum, *Biochemical and Biophysical Research Communications*, **115**, 632-641

Van Der Werf, P , Griffith, O W and Meister, A (1975), 5-oxo-L-prolinase (L-pyroglutamate hydrolase) Purification and catalytic properties, *Journal of Biological Chemistry*, **250**, 6686-6692

Vanhaasteren, G A C , Linkels, E , Klootwijk, W , Vantoor, H , Rondeel, J M M , Themmen, A P N , Dejong, F H , Valentijn, K , Vaudry, H , Bauer, K , Visser, T J and Degreef, W J (1995), Starvation-induced changes in the hypothalamic content of prothyrotropin-releasing hormone (proTRH) mRNA and the hypothalamic release of proTRH-derived peptides Role of the adrenal gland, *Journal of Endocrinology*, **145**(1), 143-153

Vargas, M , Mendez, M Cisneros, M , Joseph-Bravo, P and Charli, J -L (1987), Regional distribution of the membrane-bound pyroglutamate amino peptidase-degrading thyrotropin-releasing hormone in rat brain, *Neuroscience Letters*, **79**, 311-314

Vargas, M A , Cisneros, M , Herrera, J , Joseph-Bravo, P and Charli, J -L (1992a) Regional distribution of pyroglutamyl peptidase II in rabbit brain, spinal cord, and organs *Peptides*, **13**, 255-260

Vargas, M A , Herrera, J , Uribe, R M , Charli, J -L and Joseph-Bravo, P (1992b), Ontogenesis of pyroglutamyl peptidase II activity in rat brain, adenohypophysis and pancreas, *Developmental Brain Research*, **66**, 251-256

Wang, P S , Huang, S W , Tung, Y F , Pu, H F , Tsai, S C , Lau, C P , Chien, E J and Chien, E C (1994), Interrelationship between thyroxine and estradiol on the secretion of thyrotropin-releasing hormone and dopamine into hypophysial portal blood in ovariectomized-thyroidectomized rats, *Neuroendocrinology*, **59**(3), 202-207

Walter, R , Shlank, H , Glass, D J , Schwartz, I L and Kerenyi, T D (1971), Leucylglycinamide released from oxytocin by a human uterine enzyme, *Science*, **173**, 827-829

Wellstood, S A (1987), Rapid, cost-effective identification of group A *streptococci* and *enterococci* by pyrrolidonyl-ß-naphthylamide hydrolysis, *Journal of Clinical Microbiology*, **25**, 1805-1806

Westerik, J O and Wolfenden, R (1972), Aldehydes as inhibitors of papain, *Journal of Biological Chemistry*, **247**(24), 8195

White, N , Jeffcoate, S L , Griffiths, E C and Hooper, K C (1976), Effect of thyroid status on the thyrotropin-releasing hormone-degrading activity of rat serum, *Journal of Endocrinology*, **71**, 13-19

Wilk, S and Orlowski, M (1980), Cation-sensitive neutral endopeptidase Isolation and specificity of the bovine pituitary enzyme, *Journal of Neurochemistry*, **35**, 1172-1182

Wilk, S and Orlowski, M (1982), Determination of specificity of endopeptidases by combined high-performance liquid chromatography and amino acid analysis, *Journal of Chromatography*, **249**, 121-129

Wilk, S (1983), Prolyl endopeptidase (minireview), *Life Science*, **33**, 2149-2157

Wilk, S and Orlowski, M (1983), Inhibition of rabbit brain prolyl endopeptidase by *N*-benzyloxycarbonyl-prolyl prolinal, a transition state aldeyde inhibitor, *Journal of Neurochemistry*, **41**, 69-75

Wilk, S , Friedman, T C and Kline, T B (1985), Pyroglutamyl diazomethyl ketone Potent inhibitor of mammalian pyroglutamyl peptide hydrolase, *Biochemical and Biophysical Research Communications*, **130**(2), 662-668

Wilk, S (1986), Neuropeptide-specific peptidases Does brain contain a specific TRH-degrading enzyme (minireview), *Life Science*, **39**, 1487-1492

Wilk, S (1989), Inhibitors of TRH-degrading enzymes *Annals of the New York Academy of Science*, **553**, 252-264

Wilk, S and Wilk, E K (1989), Pyroglutamyl peptidase II, a thyrotropin releasing hormone degrading enzyme Purification and specificity studies of the rabbit brain enzyme, *Neurochemistry International*, **15**(1), 81-89

Winstead, J A and Wold, F (1962), Studies on rabbit muscle enolase Chemical evidence for two polypeptide chains in the active enzyme, *Biochemistry*, **3**, 791-795

Winters, A J , Eskay, R L and Porter, J C (1974), Concentration and distribution of TRH and LRH in the human fetal brain, *Journal of Clinical Endocrinology and Metabolism*, **39**, 960-963

Wu, P , Lechan, R M and Jackson, I M D (1987), Identification and characterisation of thyrotropin-releasing hormone peptides in rat brain, *Endocrinology*, **121**, 108-115

Wu, P and Jackson, I M D (1988), Post-translational processing of thyrotropin-releasing hormone precursor in rat brain Identification of 3 novel peptides derived from pro-TRH, *Brain Research*, **456**, 22-28

Wurtman, R J (1971), Brain monoamines and endocrine function, *Neuroscience-Research Progress Bulletin*, **9**, 172-197

Yalow, R S and Berson, S A (1971), Further studies on the nature of immunoreactive gastrin in human plasma, *Gastroenterology*, **60**(2), 203

Yamada, M , Radovick, S , Wondisford, F E , Nakayama, Y , Weintraub, B D and Wilber, J F (1990), Cloning and structure of human genomic DNA and hypothalamic cDNA encoding human preprothyrotropin-releasing hormone, *Molecular Endocrinology*, **4**, 551-556

Yarbrough, G G (1976), TRH potentiates excitatory actions of acetylcholine on cerebral cortical neurones, *Nature*, **263**, 523-524

Yoshimoto, T , Orlowski, R C and Walter, R (1977), Postproline cleaving enzyme Identification as serine protease using active site specific inhibitors, *Biochemistry*, **16**(13), 2942-2948

Yoshimoto, T , Ogita, K , Walter, R , Koida, M and Tsuru, D (1979), Post proline cleaving enzyme Synthesis of a new fluorogemc substrate and distribution of the endopeptidase in rat tissues and body fluids of man, *Biochimica et Biophysica Acta*, **569**, 184-192

Yoshimoto, T , Simmons, W H , Kita, T and Tsuru, D (1981), Post proline cleaving enzyme from lamb brain, *Journal of Biochemistry*, **90**, 325-334

Yoshimoto, T , Nishimura, T , Kita, T and Tsuru, D (1983), Post-proline cleaving enzyme (prolyl endopeptidase) from bovine brain, *Journal of Biochemistry*, **94**, 1179-1190

Yoshimoto, T , Shimoda, T , Kitazano, A , Kabashima, T , Ito, K and Tsuru, D (1993), Pyroglutamyl peptidase gene from *Bacillus amyloliquifaciens* Cloning, sequencing, expression and crystallization of the expressed enzyme, *Journal of Biochemistry*, **113**, 67-73

APPENDICES

A1. Kinetic analysis

Key

[S] = Substrate concentration

[I] = Inhibitor concentration

K_I = Rate constant (inhibition)

V_O = Initial velocity

V_{max} = Maximal velocity

K_m = Rate constant (Michaelis-Menten)

1 Michaelis-Menten equation

This is the rate equation for a one-substrate enzyme-catalysed reaction

$$V_O = \frac{V_{max}[S]}{K_m + [S]}$$

2 Lineweaver-Burk equation

This involves a double reciprocal transformation of the Michaelis-Menten model (i.e. [S] and V_O)

$$\frac{1}{V_O} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Plotting $1/V_O$ vs $1/[S]$ yields a straight line

$$\text{Slope} = \frac{K_m}{V_{max}} \qquad \text{y-axis intercept} = \frac{1}{V_{max}} \qquad \text{x-axis intercept} = \frac{-1}{K_m}$$

In the presence of a competitive inhibitor, the x-axis intercept becomes

$$\frac{-1}{K_m\left(1 + \frac{[I]}{K_I}\right)}$$

3. Hanes-Woolf equation

This equation is derived from the Michaelis-Menten model and only involves plotting one reciprocal value (i e V_0)

$$\frac{[S]}{V_0} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}}$$

Plotting $[S]/V_0$ vs [S] yields a straight line

$$\text{Slope} = \frac{1}{V_{max}} \qquad \text{y-axis intercept} = \frac{K_m}{V_{max}} \qquad \text{x-axis intercept} = -K_m$$

In the presence of a competitive inhibitor, the x-axis intercept becomes

$$-K_m\left(1 + \frac{[I]}{K_I}\right)$$

4. Eadie-Hofstee equation

This equation is derived from the Michaelis-Menten model and only involves plotting one reciprocal value (i e [S])

$$\frac{V_0}{[S]} = \frac{V_{max}}{K_m} - \frac{V_0}{K_m}$$

Plotting $V_0/[S]$ vs V_0 yields a straight line

$$\text{Slope} = \frac{-1}{K_m} \qquad \text{y-axis intercept} = \frac{V_{max}}{K_m} \qquad \text{x-axis intercept} = V_{max}$$

In the presence of a competitive inhibitor, the slope becomes

$$\frac{-1}{K_m\left(1 + \frac{[I]}{K_I}\right)}$$

A2. Statistical analysis

Key

S D = Standard deviation
a,b,c = Individual determinations or replicates

For statistical purposes, most results (where relevant) are expressed as the mean of three individual determinations +/- S D

$$\text{Mean} = \frac{a + b + c}{3}$$

$$\text{Variance} = \frac{a^2 + b^2 + c^2}{3} - (\text{Mean})^2$$

$$\text{S D} = \sqrt{\text{Variance}}$$