A Study of the Primary Inactivating Enzymes of Thyroliberin in the Synaptosomal Membranes of Bovine Brain

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I declare that all the work reported in this thesis was performed by Rhona O'Leary, unless otherwise stated.

Rhona O'Leary

Rhona O'Leary

This work is dedicated to my parents, Michael and Nannette, for their endless support, encouragement and love.....long may it continue.

He Wishes For The Cloths Of Heaven

Had I the heavens' embroidered cloths,
Enwrought with golden and silver light,
The blue and the dim and the dark cloths
Of night and light and the half-light,
I would spread the cloths under your feet:
But I, being poor, have only my dreams;
I have spread my dreams under your feet;
Tread softly because you tread on my dreams.

c. W.B.Yeats

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to Louise, whose laugh is so infectious

to Ina, who fed, spoilt and pampered me - now it is your turn!

to the McCabes, my home away from home

to Ger, who was always there to listen

and finally to all my family, for everything that you have given to me, no words of thanks can ever express how I feel - Thank you.

Abstract

Over the past twenty years, there has been an accumulation of evidence indicating that certain peptides that exert biological effects outside the CNS, may also possess neurotransmitter or neuromodulator functions in brain. Thyroliberin (Thyrotropin-Releasing Hormone) was the first of these such peptides displaying a dual role as a hormone and as a neurotransmitter. Its ubiquitous distribution in the hypothalamus and in the extra-hypothalamic regions, and its diverse pharmacological and physiological effects are all features of its dual functions. The study of brain peptidases that hydrolyse thyroliberin is of importance in securing an understanding of the possible mechanisms for termination of its neurotransmitter or neuromodulator actions and the possible enzymic biotransformation of the the original peptide to cleavage products that themselves may exhibit biological activity.

The brain peptidases involved in the primary degradation of thyroliberin studied to date are three forms of pyroglutamate aminopeptidase (PAP), a cytosolic, particulate and serum form, and a cytosolic prolyl endopeptidase (PE) activity.

This work studied the primary degradation of thyroliberin by the enzymes found in the particulate fraction of bovine brain. It began by investigating the methods available for the quantitation of the particulate PAP activity, and the development of two new assays for its detection, the first a coupled enzyme fluorimetric assay, and the second, a spontaneous cyclisation assay. Using these assays, PAP activity from the synaptosomal membranes of bovine brain was purified and characterised for the first time. This enzyme proved to have many of the characteristics of the previously studied particulate PAP activities, with some notable differences. The particulate PAP activity was shown to be 230kDa in size, had a narrow substrate specificity, cleaving only thyroliberin or very closely related peptides, and displayed a high affinity for thyroliberin. It was inhibited by thiol protease inhibitors, had a requirement for DTT and was unaffected by EDTA. By combining these features, the particulate PAP from bovine brain appears to be a 'hybrid' form of the cytosolic and the particulate PAP activities previously studied.

Prolyl endopeptidase is widely distributed, has a broad substrate specificty and has been previously characterised in the cytosolic fractions of many species. It is involved in the primary degradation of thyroliberin, producing the bioactive peptide, acid thyroliberin. Prior to this study, PE had only been characterised as a cytosolic activity, and despite references to the possibility of there being a particulate activity, it was never identified. PE activity was located on the synaptosomal membranes of bovine brain, and following vigorous salt-washing, osmotic shock and solubilisation with detergents, has been identified conclusively as a membrane-associated activity. It was purified from the synaptosomal membranes, and characterised as having a broad substrate specificity and a high affinity for thyroliberin (in fact a higher affinity than the particulate PAP). It was inhibited by some of the thiol protease inhibitors and some of the metal chelators, suggesting that it may be a thimet protease. It also is inhibited by the specific prolyl endopeptidase inhibitor, Z-Pro-prolinal.

List of Abbreviations

ACE: Angiotensin Converting Enzyme
ACTH: Adrenocorticotrophin
AMP: Adenosine Monophosphate
CCK: Cholecystokinin
cDNA: Complementary DNA
cGMP: 3', 5'-Cyclic Guanosine Monophosphate
CNS: Central Nervous System
CoA: Coenzyme A
CRS: Cold Restraint Stress
CSF: CerebroSpinal Fluid
Cyclo(His-Pro): His-Pro Diketopiperazine
DAP IV: Dipeptidyl Aminopeptidase IV
DFP: Diisopropyl Fluorophosphate
DMSO: Dimethylsulphoxide
DTT: Dithiothreitol
EDTA: Ethylenediaminetetra Acetic Acid
GABA: Gamma Aminobutyric Acid
GH: Growth Hormone
Gly-ProMCA: Glycylproline-7-amino-4-methyl Coumarin
Z-Gly-ProMCA: N-Benzyloxycarbonyl-glycylproline-7-amino-4-methyl Coumarin
<Glu: Pyroglutamic Acid (5-pyrrolidone-2-carboxylic acid)
<Glu-His: Pyroglutamyl Histidine
<Glu-His-Pro: Acid Thyroliberin
<Glu-His-ProNH_2: Thyroliberin
<Glu-His-ProOH: Acid Thyroliberin
<Glu-MCA: Pyroglutamate-7-amino-4-methyl Coumarin
<Glu-(Me)His-ProNH_2: Pyroglutamyl methyl-histidine proline 7-amino-4-methyl Coumarin (a Thyroliberin Analogue)
GPCR: G-Protein Coupled Receptors
<Glu-ProNH_2: Pyroglutamyl Proline Amide (a Thyroliberin Analogue)
5-HT: Seretonin
Ki: Inhibitor Constant
Km: Michaelis Menten Constant
LDH: Lactate Dehydrogenase
LHRH: Luteinising Hormone Releasing Hormone
MCA: 7-Amino-4-Methyl Coumarin
ME: Median Eminence
mRNA: Messenger Ribonucleic Acid
MSH: Melanocyte-Stimulating Hormone
NA: Naphthyl Amide

PAP: Pyroglutamate Aminopeptidase

PCMB: p-Chloromercuribenzoate

PDMK: Pyroglutamyl Diazomethyl Ketone

PE: Prolyl Endopeptidase

PIT: Pituitary

PKC: Protein Kinase C

PMSF: Phenylmethane Sulphonyl Fluoride

PPCE: Post Proline Cleaving Enzyme

PRL: Prolactin

P_S4: Preprothyroliberin

PTU: Propylthiouracil

SM: Sulphamethoxazole

T_3: Triiodothyronine

T_4: Tetraiodothyronine (thyroxine)

TEMED: $N_1N_1N',N',$-Tetramethyl Sulphonic Acid

TPA:12-o-Tetra-Decanyl-Phorbal-13-Acetate

TRH: Thyrotropin Releasing Hormone

TSH: Thyrotropin

VIP: Vasoactive Intestinal Peptide

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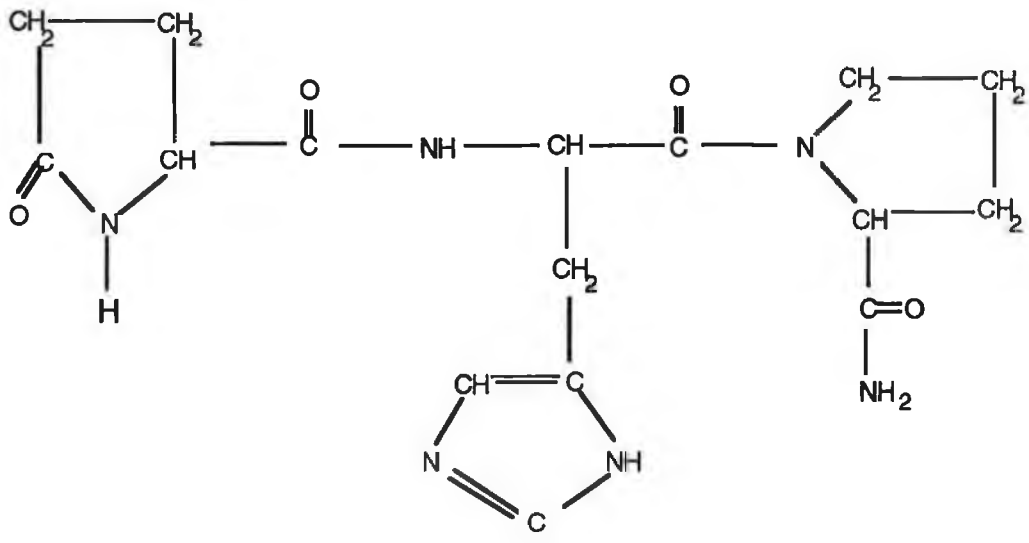
1. Introduction

1. Thyrotropin Releasing Hormone

The hypothalamic control of the anterior pituitary function through endogenous chemical substances was postulated several years ago, but it was only in the mid 1950s that the search began for the hypothalamic release-modulating substances (Harris,1955). Thyrotropin releasing hormone (TRH) from porcine hypothalamus was the first hypothalamic hypophysiotropic hormone to be purified and characterised in the laboratories of Schally et al. (1969) and Guillemin (1970). Working independently, they reported that TRH was a weakly basic tripeptide with the amino acid sequence pyroglutamyl-histidyl-proline amide (<Glu-His-Pro-NH_2) (Fig 1.1). It has strict conformational requirements for biological activity and almost any departure from the structure of native thyroliberin results in substantial if not complete loss of biological activity (Guillemin and Burgus,1972).

Fig. 1.1 Structure of thyroliberin



The availability of TRH in pure form led to the development of specific radioimmunoassays for thyroliberin and the realisation that thyroliberin, identical to that found in the hypothalamus, also occurs in extrahypothalamic brain regions as well as outside the CNS altogether (Jackson and Reichlin, 1979). At this point, it was rapidly realised that in both its amino acid sequence and in its biological activity, TRH exhibited no species specificity and that <Glu-His-ProNH_2 was readily active

in all mammalian species including humans (Fleischer and Guillemin, 1976). It was also observed that while its functions as a hypothalamic regulatory hormone adequately identified this tripeptide as a hormone, (stimulating the release of thyrotropin, and perhaps other trophic hormones from the anterior pituitary), its widespread extrahypothalamic distribution and its functions which are independent of its endocrine activity in the hypothalamus-pituitary-thyroid axis suggested that TRH had a much broader functional significance within the body.

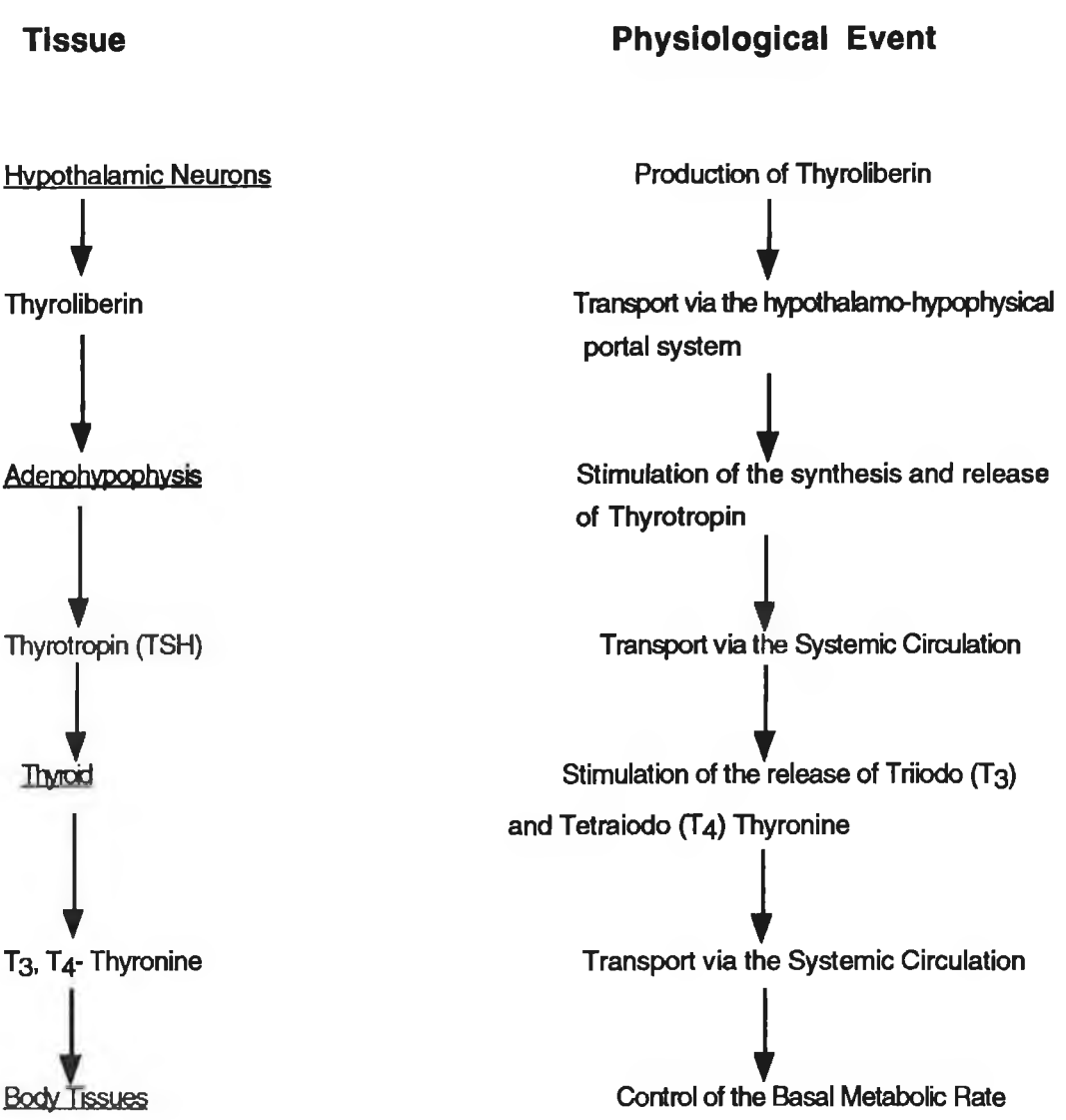
In view of the increasing neurological role of thyrotropin releasing hormone within the CNS, as well as its neurocrine function, the IUPAC-IUB Commission on Biochemical Nomenclature assigned the term 'Thyroliberin' to thyrotropin releasing hormone to reflect its dual roles.

1.1 Physiological actions of thyroliberin at the level of the adenohypophysis

The most obvious effect of thyroliberin, from which it derives its name, is the ability to stimulate the release of thyrotropin (TSH) from the anterior pituitary through binding high affinity receptors and subsequent activation of adenyl cyclase (Jackson,1982). Thyroliberin receptors in brain are ubiquitously distributed, but are found in highest densities in limbic structures, especially the amygdala and hypothalamus, and in the lowest densities in brain stem and cerebellum (Parker and Capdevila,1984). The receptor is membrane-bound, digestible by trypsin and phospholipase and inactivated by various thiol reagents and metal salts (Sharif and Burt, 1984).

The effect of thyroliberin on the thyrotroph (cell types of the anterior pituitary), resulting in TSH release, is balanced by the powerful negative feedback control over TSH release exerted by thyroid hormones. Besides this feedback, other factors such as noradrenaline, dopamine and somatostatin (both of hypothalamic origin) may well be involved in modifying thyroliberin-stimulated TSH release (Jackson, 1982, Mannisto,1983). Other neurotransmitters such as serotonin, gamma-aminobutyric acid (GABA) and histamine may influence the hypothalamic release of thyroliberin (Mannisto,1983). The catecholamines and somatostatin could provide short term inhibitory influences, while the thyroid hormones themselves have a much longer-lasting effect. Modulation of the TSH response to thyroliberin also may be brought about through changes in the circulating levels of sex steroids, cortisol and growth hormone (GH) (Jackson,1982). At the level of the thyroid gland itself, it has been shown that thyroliberin can directly stimulate the release of thyroxine (T_4) from perfused rat thyroid gland fragments *in vitro* (Attali et al.,1985), adding another complexity to the control of thyroid hormone secretion.

Fig. 1.2 Thyroliberin actions in the adenohypophysis



In considering the physiological role of thyroliberin in the release of prolactin, it has been shown that thyroliberin is at least equipotent in releasing prolactin and thyrotropin from the pituitary and that prolactin precedes the release of TSH (Jacobs et al.,1971). However it is unlikely that thyroliberin is the only peptide prolactin-releasing factor, since vasoactive intestinal peptide (VIP), substance P and bombesin can all stimulate prolactin release.

A further effect of thyroliberin on the anterior pituitary may be the release of GH. Although thyroliberin does not stimulate GH release normally in man, it does so in several endocrine, neuropsychiatric and metabolic disorders (Jackson, 1982; Scanlon et al.,1983), which may be due to a change in balance of other factors regulating GH release.

The effects of thyroliberin on other endocrine tissues include the stimulation of arginine vasopressin and oxytocin release from posterior pituitary, enhancement of arginine-induced glucagon release from the rat pancreas, inhibition of gastrin-stimulated acid secretion in dog and human, and attenuation of pancreatic polypeptide release during insulin-induced hypoglycemia (Morley et al.,1977).

Thyroliberin-related changes have been implicated in the pathophysiologies of various disease states. These include Huntington's chorea (Spindel et al., 1980), schizophrenia (Nemeroff et al., 1984), epilepsy (Kubek et al., 1993), Alzheimers disease (Albert et al., 1993), primary thyroid diseases, pituitary adenomas, neuropsychiatric disorders (anorexia nervosa) and other endocrine or metabolic disorders such as chronic renal failure and starvation (Duthie et al., 1993).

1.2 Distribution of thyroliberin

Thyroliberin-like immunoreactivity or biological activity is widely distributed throughout the nervous system of vertebrate species including human, rat, guinea-pig, mouse, frog, chicken and salmon (Nemeroff, 1978). While hypothalamic thyroliberin, transported to the anterior pituitary via the portal vessel circulation, acts on thyrotrophs (cell types of the anterior pituitary) in a classical fashion to cause the release of TSH, there is also much evidence to suggest that the thyroliberin found in extra-pituitary locations has a role in neurotransmission (neurocrine function) and in cell-to-cell regulation (paracrine function) (Jackson, 1983). Over 70% of the total thyroliberin in the CNS is found outside the hypothalamus, although the concentrations are lower than those within the hypothalamus or posterior pituitary (Jackson and Reichlin, 1979).

Table 1.1 The distribution of thyroliberin in tissues and body fluids of various species (Prasad, 1987)

Species	Tissue/ Body Fluid	Conc (fmol/mg Protein)
Human	Cerebellum	15.0
	Hypothalamus	1.1
	Placenta	54.6
	Pineal Gland	71.1
	Peripheral Blood	78 pg/ml
Rat	Cerebellum	31.5
	Hypothalamus	2208.5
	Caecum	91.0
	Rectum	27.6
	Pancreas	93.8
	Retina	828.0
	Peripheral Blood	77.0
	Hypophyseal Portal Plasma	801.0
Frog	Brain	6.9
	Skin	2.6
	Blood	227.0
Monkey	Cerebellum, anterior	2.3
	Hypothalamus, anterior	150.8
Chicken	Hypothalamus	938-1352
Snake	Hypothalamus	10884-20171
Salmon	Hypothalamus	5188-7284

Thyroliberin is distributed throughout the extra-hypothalamic CNS (Winokur and Utiger,1974) and spinal cord (Hokfelt et al.,1975), in the retina, (Schaeffer et al.,1977), in the pancreas and gastrointestinal tract (Morley et al.,1977), in the placenta (Shambaugh et al.,1978), in amniotic fluid (Morley,1979) and in breast milk (Baram et al.,1977).

Distribution of thyroliberin is uneven throughout the gastrointestinal tract, with the highest concentration in the pancreas and caecum, and the lowest concentrations in rectum, jejunum and duodenum, (Morley,1979; Leppäluoto et al.,1978). Within the pancreas, thyroliberin has been localised in the islets of Langerhans. Ontogenic studies have shown the pancreas to contain much higher levels of thyroliberin than the hypothalamus during the early neonatal period (Engler et al.,1981), but the concentrations in the pancreas and the gastrointestinal tract subsequently decline with age, while hypothalamic and brain levels gradually increase.

Amniotic fluid thyroliberin appears to be immunologically similar to synthetic thyroliberin, and its activity increases with gestational age (Morley et al.,1977). Thyroliberin-like material has been shown to be present in both cisternal fluid and in lumbar cerebrospinal fluid (Oliver et al.,1974a) and its levels are increased in depressed patients (Oliver et al.,1974b). There are considerable difficulties in measuring thyroliberin in the systemic circulation because of rapid degradation by proteolytic enzymes and its presence in human peripheral blood is controversial.

Table 1.2 Distribution of thyroliberin in the brain and spinal cord (Leppäluoto et al.,1978)

Tissue	Tissue Wet Weight (mg)	Thyroliberin Concentration (pg/mg)	Thyroliberin Total Amount (ng)
Hypothalamic-Pituitary Complex			
Whole Hypothalamus	33.00	410.00	13.53
Posterior Pituitary (Neurohypophysis)	0.80	380.00	0.30
Anterior Pituitary (Adenohypophysis)	8.00	18.00	0.14
Extrahypothalamic Brain and Spinal Cord			
Pons and medulla	275.00	49.00	13.48
Cerebrum	1170.00	2.90	3.39
Cerebellum	300.00	1.30	0.39
Cervical Spinal Cord	67.00	49.00	3.28
Thoracal Spinal Cord	60.00	84.00	5.04
Lumbar Spinal Cord	65.00	115.00	7.48

Within the hypothalamus, a study by Jackson and Reichlin in 1974 showed that in rat, the highest concentration of thyroliberin lies in the median eminence (ME) region with levels in excess of 3.5ng/mg tissue.

Using immunohistochemistry, it was shown that the most dense concentration of thyroliberin is

found in the external zone of the ME juxtaposed to portal capillaries, consistent with its hypophysiotropic role. Thyroliberin is also found in the posterior pituitary of the rat, its concentration there being exceeded in the CNS only by that of the hypothalamus .
As can be seen from the Table1.2, the highest total amount of thyroliberin found in the extrahypothalamic brain was found to be in the pons and medulla (13.48ng).

The distribution of thyroliberin, its rapid degradation in blood and tissue, its concentration in synaptosomes and its demonstrated actions on isolated neurons, have led to the concept that thyroliberin is an ubiquitous neurotransmitter that has been co-opted by the pituitary as a releasing factor.

1.3 Thyroliberin as a possible neuroregulator

Information processing in the brain largely involves chemical communication among neurons through substances called neuroregulators. These substances may be subdivided into those that convey information between adjacent nerve cells(neurotransmitters) and those that amplify or dampen neuronal activity (neuromodulators). The criteria by which a compound is classified as a neurotransmitter or neuromodulator have been extensively reviewed and debated. These include:

1. Localisation within presynaptic nerve terminals
2. Release upon nerve stimulation
3. Attachment to specific post-synaptic receptors
4. Induction of biological effects identical to that achieved by direct nerve stimulation ('synaptic mimicry')
5. Termination of effects by a specific inactivatiing or reuptake mechanism
6. The capacity of the nerve cell to synthesize the neurotransmitter

In considering the central effects of thyroliberin, it is worth noting that this peptide was originally classified as a hormone through its effects on the pituitary, but it also qualifies as a neurotransmitter. Its extrahypothalamic distribution in the brain, 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 presence of brain peptidases capable of inactivating the tripeptide provide a formidable list of criteria consistent with such a neuronal function (Jackson, 1982; Griffiths and Bennett, 1983).

Both hypothalamic and extrahypothalamic synaptosomes are richly endowed with immunoreactive thyroliberin (Winokur et al., 1977). Immunocytochemically, thyroliberin has been shown to be present in nerve endings (Johansson and Hokfelt, 1980). In the hypothalamus, immunoreactive

thyroliberin is found in two subcellular populations, consisting of large and small particles, with sedimentation characteristics similar to those of synaptosomes containing noradrenaline and dopamine (Barnea et al.,1976). Following hypo-osmotic shock or exposure to solubilising agents, most of the thyroliberin appears near the top of the gradient. The remaining fraction of thyroliberin was associated with small particles with a sedimentation coefficient similar to that of synaptic vesicles containing acetylcholine. These properties suggest that both thyroliberin-containing particles are of synaptosomal origin.

1.3.1 Action of thyroliberin on neurons

Thyroliberin has been shown to inhibit a significant portion of hypothalamic, cerebral cortical and cerebellar neurons (Renaud,1978). Winokur and Beckman, (1978) have shown thyroliberin to cause a selective reduction in neuronal excitation evoked by L-glutamate but not by acetylcholine in rat cerebral cortex. In the cat spinal cord, thyroliberin increases the frequency of spontaneous ventral root action potentials and increases the amplitude of the monosynaptic reflex and the amplitude and onset of polysynaptic potentials, leading to a general activation of muscle tonus (Cooper and Boyer, 1978) .

1.3.2 Release and reuptake of thyroliberin at synaptic terminals

The termination of neurotransmitter action at the synapse may be regulated by removal of the neurotransmitter from the synaptic cleft. Reuptake of neurotransmitters is one mechanism by which neurotransmitters are removed. Such an uptake process may take place either at the pre- or postsynaptic neuronal elements or by glial cells that form the synaptic capsule. Studies of the *in vitro* uptake of exogenous thyroliberin by rat cerebellar slices in the presence of bacitracin (a protease inhibitor) suggested that thyroliberin is taken up via a process involving many of the properties of a high-affinity transport system including (1) saturation kinetics, (2) high-affinity kinetic constants, (3) temperature dependence, (4) sodium dependence and (5) a high tissue/medium ratio. After 1 hour of incubation at 27°C, the tissue-medium ratio was 5:1 and 70% of the total radioactivity was discovered as thyroliberin (Pacheco, 1981).

1.3.3. Behavioural effects of thyroliberin

Plotnikoff and his co-workers (1974), were the first to demonstrate the action of thyroliberin in the central nervous system. They showed thyroliberin potentiation of the stimulant properties of L-dihydroxyphenylalanine in paragyline-treated mice. Furthermore this action of thyroliberin was reported to persist in both hypophysectomised and thyroidectomised animals, suggesting a non-involvement of the pituitary-thyroid axis in its behavioural effects. Since this major discovery, thyroliberin has been shown to elicit numerous centrally mediated behavioural effects in man and animals (Yarbrough,1979). For example, thyroliberin can reverse narcotic-induced sedation via activation of neurons in the septo-hippocampal area (Kalivas and Horita, 1980), this effect may involve local changes in the cholinergic system. Thyroliberin can also reverse naturally-induced

sedation, in the form of hibernation, in *Citellus lateralis* , through a hippocampal site of action (Stanton et al.,1980). The tripeptide can stimulate locomotor activity in rats, through the mesolimbic dopamine system, an effect which is mediated via the nucleus accumbens (Heal et al.,1983). Its effects on thermoregulation are more complex, producing both hyper- and hypothermia induced by a variety of pharmacological agents and neuropeptides including ß-endorphin, bombesin and neurotensin (Nemeroff et al.,1984).

Thyroliberin can induce shaking behaviour (sometimes described as 'wet-dog shakes') seen in opiate withdrawal (Griffiths et al.,1982). There can also be induction of forepaw tremor in rodents (Nemeroff et al.,1984). The central administration of thyroliberin has profound effects on the cardiovascular and respiratory systems, with an increase in blood pressure induced in rat, rabbit and goat (Nemeroff et al., 1984). In relation to the gastrointestinal system, thyroliberin administered centrally will induce an increase in gastrointestinal motility via activation of central vagal systems, as well as a marked increase in gastric secretion (consisting of increased volume and decreased pH) which also appears to be vagally-mediated (Nemeroff et al., 1984). The potential interactions between thyroliberin and central cholinergic neurons suggest an involvement in the spinal cord, midbrain, cortex and septum/hippocampus (Yarbrough, 1983). Allied to the gastrointestinal effects described above, there is evidence that thyroliberin is an anorexic agent, suppressing both food and water intake (Nemeroff et al.,1984).

In all these actions, there is an apparent interaction of thyroliberin with a variety of classical neurotransmitters and other neuropeptides, from acetylcholine, dopamine and serotonin(5-HT) through the opioid peptides and neurotensin. The complexity of these interactions makes the definition of the exact physiological significance of thyroliberin in the brain more difficult. However, the overall impression of a stimulant action does vindicate the belief that thyroliberin functions as a net 'endogenous ergotropic substance' in the central nervous system. (Metcalf, 1982).

1.4 Thyroliberin receptors

Although thyroliberin is widely distributed in various tissues , the presence of high-affinity stereospecific binding of thyroliberin to membranes is limited to relatively few tissues, including brain, (Burt and Snyder,1975) pituitary, (Banerji and Prasad,1982) retina, (Burt, 1979) and spinal cord (Prasad and Edwards,1983). Burt and Snyder (1975) were the first to demonstrate the stereospecific binding of [^{3}H]thyroliberin to rat brain membranes and to establish the presence of both high- and low-affinity binding sites. Subsequent studies revealed a close similarity between the thyroliberin-receptor from the central nervous system (cortex, nucleus accumbens and retina) and that in the anterior pituitary gland (Burt and Snyder,1975; Banerji and Edwards, 1982; Burt and Taylor, 1980). Banerji and Prasad (1982) demonstrated that following subfractionation of the

mitochondrial fraction, there was an increase in the specific binding of thyroliberin in the synaptic plasma membrane. Although the precise number of thyroliberin receptors could not be determined (the detergent used for synaptic junction preparation inactivated the thyroliberin receptor), as many as 30% of the synaptic plasma-membrane associated receptors may reside at the synaptic junction. The presence of a significant number of thyroliberin receptors in the light synaptic plasma and microsomal membranes suggests the existence of extrajunctional receptors (Table 1.3). In the human, a single class of high affinity binding sites has been characterised in the hippocampus (Eymin et al., 1993).

The many locations of thyroliberin and its receptors in brain and spinal cord and the many diverse effects associated with its administration into specific brain sites strongly support the contention that it serves as an endogenous regulator of neuronal function. It appears that many of the extrapituitary effects of thyroliberin are mediated by other neurotransmitters, notably acetylcholine and the monoamines, so that this peptide is delegated the role of a neuromodulator of those systems.

Table 1.3 Subcellular distribution of thyroliberin receptors in rat cerebellum (Banerji and Prasad,1982)

Fraction	Thyroliberin (fmol bound/mg protein)
Nuclear Fraction (P1)	3.57+/- 1.37
Crude Mitochondrial (P2)	24.24 +/- 5.51
Synaptic Plasma Membrane	44.83 +/- 3.32
Light Synaptic Plasma Membrane	29.96 +/- 6.26
Myelin	13.75 +/-4.95
Mitochondria	4.05 +/- 0.32
Microsomal Membrane (P3)	20.90 +/- 4.37

1.4.1 Cloning and functional characterisation of the thyroliberin receptor

Much investigation has recently centred on the cloning and functional characterisation of the thyroliberin receptor. Knowledge of the thyroliberin receptor (thyroliberin-R) structure would facilitate the study of ligand/receptor interactions, which could lead ultimately to the design of more clinically effective analogues.

In 1990 Straub et al. successfully cloned the cDNA encoding the mouse pituitary thyroliberin receptor. The 3.8 kilobase mouse thyroliberin-R cDNA was found to encode a protein of 393 amino acids which showed similarities to other guanine nucleotide- binding regulatory proteins (G-proteins)- coupled receptors (GPCR). The thyroliberin-R cDNA was also isolated from rat pituitary and brain (Sellar et al., 1993, Zabavnik et al., 1993). The rat protein sequence closely resembles that reported for the mouse (Straub et al., 1990), the main difference being an extra 18 amino acids at the carboxyl-terminal end. The nucleotide sequence which encoded for these extra 18 amino acids matches a sequence which occurs in the 3'-untranslated region of the published mouse sequence, some 230 bases beyond the stop codon which terminates the main open reading frame. The differences between the 2 sequences in the two species may result from differential splicing.

The human thyroliberin-R was shown to encode a 398 amino acid protein, (Duthie et al.,1993), compared to 393 for the mouse thyroliberin-R (Straub et al., 1990) and 412 (de la Pena et al., 1992a), 387 (de la Pena et al., 1992b) and 411 (Sellar et al.,1993) for the rat thyroliberin-R. Comparison of the human/ mouse/ rat thyroliberin-R amino acid sequences indicate high homology between all isoforms, except for the COOH terminal tail where the major variation occurs. This region was shown to be functionally important and has been implicated in receptor desensitisation of the ß-adrenergic receptor (Hausdorff et al., 1991). All forms of the thyroliberin-R isolated so far are functional and the reason for the existence of these alternative COOH tails is not yet known. They could possibly play a part in coupling the receptor to different second messenger systems with the same cell or be involved in receptor stability. It is also possible that the COOH tail variation could be evolutionarily neutral rather than functional.

1.4.2 Internalisation of the thyroliberin receptor follows binding

G-protein coupled receptors (GPCRs), like other plasma membrane receptors, are rapidly lost from the surface of cells, ie they are internalised, after binding agonist. Internalisation via coated pits and recycling to the plasma membrane of receptors has been delineated for low density lipoproteins and epidermal growth factor. Specific amino acid motifs in these receptors have been shown to mediate this process. Although loss from the cell surface of GPCRs has been studied in several cell systems, no general amino acid sequence for internalisation had been identified until recently. The ability of thyroliberin to cause both a rise in total inositol phosphate production and $[Ca^{2+}]_i$ confirms that the human thyroliberin-R behaves as a functional GPCR (Duthie et al., 1993). The thyroliberin-R complex undergoes rapid transformation in cells, to an acid-resistant form which appears to represent internalised agonist-receptor complex. Since residues in the carboxyl terminus of other G-protein- coupled receptors appear to be involved in internalisation, Nussenveig et al., (1993)

studied that region using site-directed mutagenesis and transfection into mammalian cells. A mutant thyroliberin-R, C335Stop, missing the last 59 residues including two cysteine residues, underwent minimal transformation to an acid-resistant form even though it bound agonist with equal affinity and activated inositol phosphate second messenger formation as effectively as wild type thyroliberin-R. Two distinct domains within the carboxyl terminus between residues 335 and 368 were shown to affect transformation equally.

Although thyroliberin-promoted transformation of the agonist-thyroliberin-R complex into an acid/salt resistant form has not been shown formally to represent the internalisation via coated pits and vesicles, this appears to be the most plausible explanation for this phenomenon (Von Zastrow and Koblinka, 1992). The functional significance of receptor internalisation is not completely understood. It has been considered one component, with receptor G-protein uncoupling and receptor down-regulation, of the mechanism whereby GPCRs desensitise. Thus, receptor internalisation is seen as a way to redistribute receptors from the plasma membrane to an intracellular compartment in which signalling may not occur. This reduces the number of available receptors on the cell surface where they could interact with agonist and activate signalling. It is possible that thyroliberin signalling is in part desensitised by this process, because thyroliberin signalling in pituitary cells appears to occur only at the cell surface and not by internalised receptors (Ramsdell and Tashjian, 1986). Recycling of receptors back to the plasma membrane may be a component of the process of resensitisation.

1.4.3. Localisation of thyroliberin receptor mRNA

The localisation and distribution of thyroliberin-R mRNA in the rat pituitary gland and brain have been studied by means of *in-situ* hybridisation (Sellar et al.,1993). In the anterior lobe of the pituitary, the hybridisation signal was evenly distributed throughout the tissue, which probably shows the presence of thyroliberin-R mRNA on thyrotrophs, lactotrophs and perhaps on somatotrophs (cell types in the anterior lobe of the pituitary). No expression was found in the posterior or neurointermediate lobes of the pituitary.
Specific hybridisation was also found in discrete areas of the brain. These areas included distinct regions of the olfactory system, septal area, amygdaloid complex, cerebral cortex, hippocampus, hypothalamus, basal ganglia and the motor nuclei of cranial nerves in the brain stem (Zabavnik et al., 1993).

The existence of thyroliberin in different regions throughout the brain coupled with receptor binding sites in these regions, reinforces the suggestion that thyroliberin has important CNS effects. Zabavnik et al., (1993) were able to demonstrate that in virtually every area where there was thyroliberin receptor mRNA expression, prohormone or tripeptide thyroliberin immunoreactivity has also been described (Lechan and Jackson, 1982). Segerson et al., (1987) , demonstrated the presence of thyroliberin prohormone mRNA in regions which Zabavnik et al. had shown to contain

thyroliberin receptor mRNA. These regions included the amygdala, the diagonal band of Broca, bed nucleus of the stria terminals, periolivary region and the dorsal motor nucleus of the vagus. The presence of thyroliberin receptors in the same regions as those which synthesise thyroliberin precursor suggests a local action of thyroliberin.

1.4.4. Regulation of thyroliberin receptors

The number of thyroliberin receptors is regulated by thyroid hormones, but the affinity of the receptors for thyroliberin is unaltered (Perrone and Hinkle, 1978). But how does thyroliberin regulate the level of its own receptors? Thyroliberin causes the level of endogenous thyroliberin-R mRNA to decrease in rat pituitary GH_3 cells, at least in part, by stimulating the rate of its turnover (Fujimoto et al., 1992). This effect of thyroliberin could have been caused by decreasing the stability of thyroliberin-R mRNA (or increasing its accessibility to an RNase), or by increasing the activity of an RNase that degrades thyroliberin-R mRNA, or both. Nayaranan et al. (1992) used a cell free system in which the RNA-degrading enzyme from lysates of thyroliberin-stimulated and unstimulated GH_3 cells was used to determine whether thyroliberin affected the activity of a RNase that degrades thyroliberin-R mRNA. They found that lysates derived from GH_3 cells stimulated by thyroliberin regulate the activity to degrade transcribed RNAs *in vitro*. The mechanism of regulation by thyroliberin of RNase activity is unknown. It could be caused by regulation of the synthesis of an RNase or of post-translational modifications that lead to changes in the activity of a latent RNase. An effect to decrease the action of an RNase inhibitor, which could also explain stimulation of RNase activity, appears less likely. The observation using the cell-free system that increased RNA degrading activity occurs as rapidly as within 10 mins of stimulation by thyroliberin of GH_3 cells, may provide an insight into the mechanism of this effect (Nayaranan et al.,1992). It is likely that this effect occurs via a post-translational mechanism affecting the RNase system rather than involving induction of RNase synthesis. Because thyroliberin signals via a mechanism that activates protein kinase C (Gershengorn, 1986), and it has been shown that protein kinase C mediates enhanced mRNA degradation caused by thyroliberin (Fujimoto et al., 1991), it is possible that this effect may be mediated by phosphorylation of a regulatory protein in the RNase system. It is also possible that in GH_3 cells stimulated by thyroliberin, some mRNAs, such as that for prolactin, are protected from increased degradation during thyroliberin action by effects on other components of the RNA degrading system. Thyroliberin stimulation of GH_3 cells appears to lead to degradation of thyroliberin-R mRNA by increasing RNase activity and simultaneously affecting a specific factor that allows for degradation (or destabilises) thyroliberin-R mRNA. Further work is necessary to define the molecular details of thyroliberin-stimulation of RNase activity and of thyroliberin-induced destabilisation of thyroliberin-R mRNA.

1.5 Thyroliberin Biosynthesis

Many other aspects of the physiology and pharmacology of thyroliberin have been extensively studied and although investigations following the structural characterisation of thyroliberin in 1969 have provided considerable information on the localisation, release, mechanism of action and inactivation of the tripeptide, the mechanisms of biosynthesis were amongst the last features of thyroliberin to be investigated.

Three possible methods of producing thyroliberin were suggested:

1. By non-ribosomal enzymatic synthesis,
2. By ribosomal production of a high-molecular weight thyroliberin precursor and subsequent processing to the tripeptide, and
3. By the uptake and reamidation of one of its metabolites, acid thyroliberin (<Glu-His-ProOH).

There was difficulty in elucidating the precise means of thyroliberin biosynthesis possibly because relatively small quantities of thyroliberin (nanogram) are detectable in brain and the actual rate of biosynthesis appears to be low (Griffiths and Millar, 1983).

The possibility of non-ribosomal thyroliberin biosynthesis was initially suggested by Mitnik and Reichlin, 1972) because of the short tripeptide sequence of this peptide, but the concept was not widely accepted (McKelvy, 1983; McKelvy and Epelbaum, 1978). Bauer and Lipmann (1976) did not find any biosynthetic activity and observed that the tripeptide amide was rapidly degraded in hypothalamic homogenates.

The concept of ribosomal thyroliberin biosynthesis stems from the mechanisms of biosynthesis of other polypeptide hormones, notably insulin and somatostatin. Here a high molecular weight precursor of the polypeptide is produced and packaged in the endoplasmic reticulum and golgi apparatus of the cell, which is then transferred to its site of release (Chretien and Seidah, 1981). During these steps, the precursor is modified either by specific proteolytic cleavage, addition of groups to the peptide structure eg glycosylation, phosphorylation or both, until the mature, biologically active peptide is present in the secretory granules ready for release.

Although the majority of evidence favoured the idea of the synthesis of a prohormonal form of thyroliberin and its subsequent activation to <Glu-His-ProNH_2, another interesting possible mechanism of thyroliberin biosynthesis was suggested by McKelvy (1983). Acid thyroliberin (<Glu-His-ProOH), formed by the action of prolyl endopeptidase on thyroliberin, is relatively stable to further degradation and could potentially be taken up at nerve terminals for reamidation to thyroliberin. There is virtually no evidence of neuropeptide uptake and reamidation because of the relatively small contribution of this process to the overall thyroliberin pool. However improbable this scheme may seem, the obvious similarity of this uptake/reamidation process to that occurring for classical neurotransmitters can only add weight to its possible significance in thyroliberin biosynthesis.

The long standing controversy concerning the mode of thyroliberin biosynthesis was only solved in 1979 (Rupnow et al., 1979). It was shown that thyroliberin, like other hypothalamic releasing factors such as Lutenising Hormone Releasing Hormone (LHRH), arises from the post-translational cleavage of a large precursor protein and not by soluble non-ribosomal enzymatic mechanisms. Richter et al. (1984), isolated mRNA from the skin of the frog *Xenopus laevis* (a rich source of thyroliberin), and were able to obtain a cDNA clone with an insert of 478 nucleotides coding for a portion of the preprohormone precursor of thyroliberin (preprothyroliberin). The deduced thyroliberin 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 flanking basic amino acids are potential cleavage sites in thyroliberin peptide biosynthesis. Thyroliberin would then arise from enzymic amidation at the carboxyl terminus with the glycine residue acting as an amide factor while glutamine would undergo a cyclisation to form the mature hormone.

Jackson et al. (1985) raised an antiserum against a synthetic peptide hypothesised to represent a portion of the mammalian thyroliberin prohormone. Immunocytochemical studies indicated that this antiserum recognised the rat thyroliberin prohormone but not the fully processed peptide. From this they were able to identify a cDNA that encoded the thyroliberin precursor from a rat hypothalamic lambda-gt11 bacteriophage expression library. Because of the small size of the thyroliberin sequence and the degeneracy of the codons representing the three amino acids, the application of conventional hybridisation techniques to identify a cDNA encoding mammalian hypothalamic thyroliberin presents several problems.

1.5.1. Distribution of the thyroliberin prohormone

Lechan et al. (1986a) used immunolocalisation techniques to examine the distribution of the thyroliberin prohormone in the CNS of the rat. It was found that immunoreactive prothyroliberin is present in several hypothalamic nuclei, the medullary raphe, and regions of the telencephalon, including the diagonal band of Broca, medial septum and bed nucleus of the stria terminalis, in a distribution identical to that reported for the mature tripeptide (Lechan and Jackson, 1982). Confinement of reaction product to the cell soma or dendrites in all of these areas, however, contrasts to the widespread distribution of thyroliberin in axons and axon terminals in the CNS (Lechan and Jackson, 1982). This suggests that, in general, prothyroliberin is processed within all soma in most regions throughout the brain and is rarely transported in axons. The distinct compartmentalisation of immunoreactive prothyroliberin in the cell cytoplasm of paraventricular neurons, which is suggestive of its association with the Golgi apparatus, as opposed to the more diffuse cytoplasmic distribution in lateral hypothalamic neurons and certain other regions of the brain however, indicates that the location of thyroliberin prohormone processing may vary in different nuclear groups.

In addition to the presence of immunoreactive prohormone in regions of the CNS where thyroliberin

had been observed (Lechan et al., 1983), the prothyroliberin was located in the perikarya in several nuclear groups where no thyroliberin had previously been located by immunocytochemical methods. This included particularly prominent groups of neurons in the glomerulus of the olfactory lobes, sexually dimorphic nucleus of the pre-optic area, reticular nucleus of the thalamus, periaqueductal gray, cerebral cortex and hippocampus.

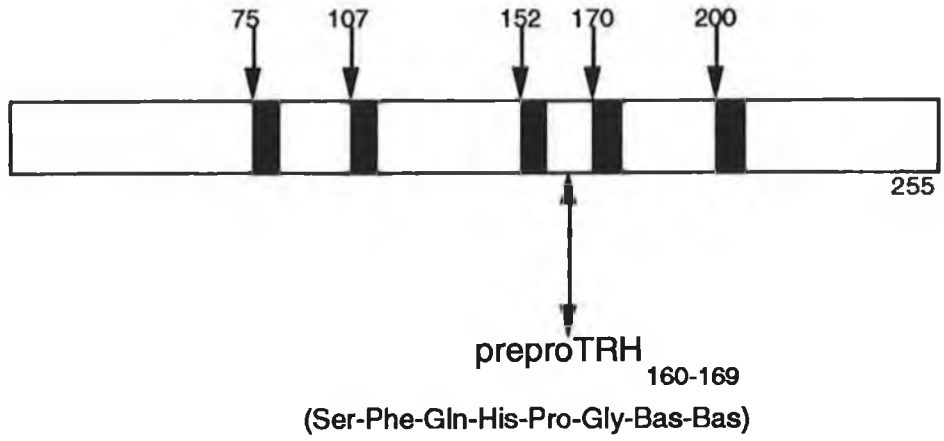
In addition to ascribing a large and varied role for the products of thyroliberin precursor in the CNS, it is intriguing to speculate that in certain regions of the brain, the thyroliberin prohormone may be preferentially processed to non-thyroliberin peptides or extended forms of thyroliberin, which may be of biological importance. This possibility is supported by the structure of pro-thyroliberin deduced from cloned cDNA derived from the rat hypothalamus (Lechan et al., 1986b). In addition to thyroliberin, this molecule of approximately 26 kDa molecular weight, includes a 22 amino acid sequence at the amino terminal portion of the molecule and a 49 amino acid peptide at the carboxyl terminal. Consequently the thyroliberin prohormone may be akin to other peptide precursors that contain more than one biologically active peptide, such as the precursors for vasoactive intestinal peptide (Itoh et al., 1983), substance P (Nawa et al., 1983) and LHRH (Seeberg et al., 1984) and the final biosynthetic products may depend on the type of processing enzymes contained within the cell.

1.5.2. Processing of the thyroliberin prohormone

Lechan et al. (1986a) also used an antiserum raised against a synthetic peptide hypothesised to represent a portion of the mammalian thyroliberin prohormone, to encode the complete sequence of the thyroliberin prohormone. The deduced amino acid sequence reveals that the prohormone is approximately 26 kDa molecular weight and contains five copies of the sequence Gln-His-Pro-Gly flanked by pairs of basic residues and separated by intervening sequences. Two of the pro-thyroliberin-connecting peptides, preprothyroliberin (160-169) and preprothyroliberin (178-199) have been detected in rat neural tissues by radioimmunoassay (Bulant et al., 1988). The connecting peptides were found in the paraventricular nucleus of the hypothalamus, and a dense plexus of immunopositive nerve terminals was observed in the external zone of the median eminence, in a distribution similar to that described for thyroliberin. Bulant et al. (1988) suggested that both the preprothyroliberin (160-169) and (178-199) were, together with thyroliberin, predominant storage forms of the thyroliberin precursor in hypothalamus and spinal cord, as they were present in molar ratios corresponding to those expected for a nearly complete processing of the prohormone molecule. The presence of pro-thyroliberin-connecting peptides in various brain regions, including the median eminence, suggests that these peptides might act as neuromodulators in the CNS and/or neuroendocrine signals at the pituitary level. In the olfactory lobes, preprothyroliberin is processed differently since a C-terminally extended form of thyroliberin, preprothyroliberin (172-199), is found as a major end product along with lower but significant amounts of preprothyroliberin (178-199) and preprothyroliberin (161-169). The striking difference

in pro-thyroliberin processing patterns among the various tissues examined suggests differential regulating mechanisms for thyroliberin and/or thyroliberin-related mechanisms.

Fig 1.3 Structure of preprothyroliberin



Bas-Bas-Gln-His-Pro-Gly-Bas-Bas (ie proTRH)
Bas = Lys or Arg

More recently Bulant et al. (1990b), continuing their research into the processing of pro-thyroliberin, found that the preprothyroliberin (160-169) (Ps4), was involved in regulating thyroliberin-induced thyrotropin secretion. Using a radiolabelled probe, iodinated Tyr-Ps4, they found that the anterior lobe of the pituitary contains a high density of Ps4 binding sites, while the posterior lobe is almost devoid of binding sites. The concentration of ^{125}I-labeled Tyr-Ps4 binding sites in the adenohypophysis was in the same range as that reported for thyroliberin receptors (Burt and Snyder, 1975). It was also found that Ps4 does not affect TSH secretion, but potentiates the action of thyroliberin on TSH release. This result suggests that the two peptides, derived from a single precursor, may act in a synergistic manner on the same target cells to modulate hormone release. The differential processing of the thyroliberin precursor may contribute an important regulatory point in modulating the biological potency of thyroliberin-connecting sequences (Ladram et al., 1992).

Carr et al (1993) found that Ps4 (thyroliberin-potentiating peptide) not only stimulated TSHß gene promoter activity (as reflected by CAT synthesis in GH_3 cells), but also stimulated both the synthesis and secretion of endogenously produced PRL. The disparate responses of synthesis and secretion to Ps4 will probably be an important tool to facilitate determination of the intracellular signalling events in thyroliberin regulation of gene expression. Ps4 was found to stimulate TSH secretion in GH_3 tumour cells, but did not directly stimulate TSH secretion in the pituitary (Bulant et al., 1990a), suggesting that different receptor and/or effector systems exist for preprothyroliberin (160-169) on lactotrophs, thyrotrophs and tumour cells.

While Ps4 stimulated TSHß gene expression as did thyroliberin, it is unlikely that Ps4 acts through the same cellular mechanism as thyroliberin. Thyroliberin and Ps4 have distinct binding sites, and a demonstration of unique binding sites for Ps4 in the anterior pituitary was shown by Ladram et al. (1992) and Carr et al. (1993). Moreover, the effect of Ps4 appears to be additive to that of thyroliberin, further emphasising the likelihood of distinct pathways. A final common pathway is possible, because the same region of the TSHß-subunit gene required for thyroliberin regulation of TSHß gene expression was also essential for Ps4 effects (Carr et al. 1993). These data supports the hypothesis that processing of the thyroliberin precursor molecule, pro-thyroliberin, gives rise to at least two peptides (ie thyroliberin and preprothyroliberin (169-169)) with biological activity. This processing may constitute an important regulatory element in modulating the biological potency of thyroliberin and maintaining constant levels of TSH.

1.6. Degradation of thyroliberin

Neuropeptides represent a class of extremely potent biological substances acting as neurohormones, neuromodulators, neurotransmitters or paracrine effector substances on a variety of target cells via binding to specific receptors. In general, the effect a hormone produces varies with its concentration at the receptor sites in its target organ or tissue. This can be influenced by a variety of factors such as peptide binding to plasma proteins, the rate of diffusion of the hormone through capillaries and irreversible biochemical processes initiated by the hormone at its target organ or tissue, but the ultimate control over a hormone's activity is through a balance between its biosynthesis (section 1.5) and degradation. As for other biologically potent substances, highly efficient degradation and/or elimination mechanisms must also exist for neuropeptides, because at inappropriate concentrations, highly active biological substances are also highly toxic. This is especially true for neuronal communication factors transmitting chemical signals and it is clear that for neuropeptides which are released with high frequency, these mechanisms not only have to be very efficient, but they also have to be very rapid.

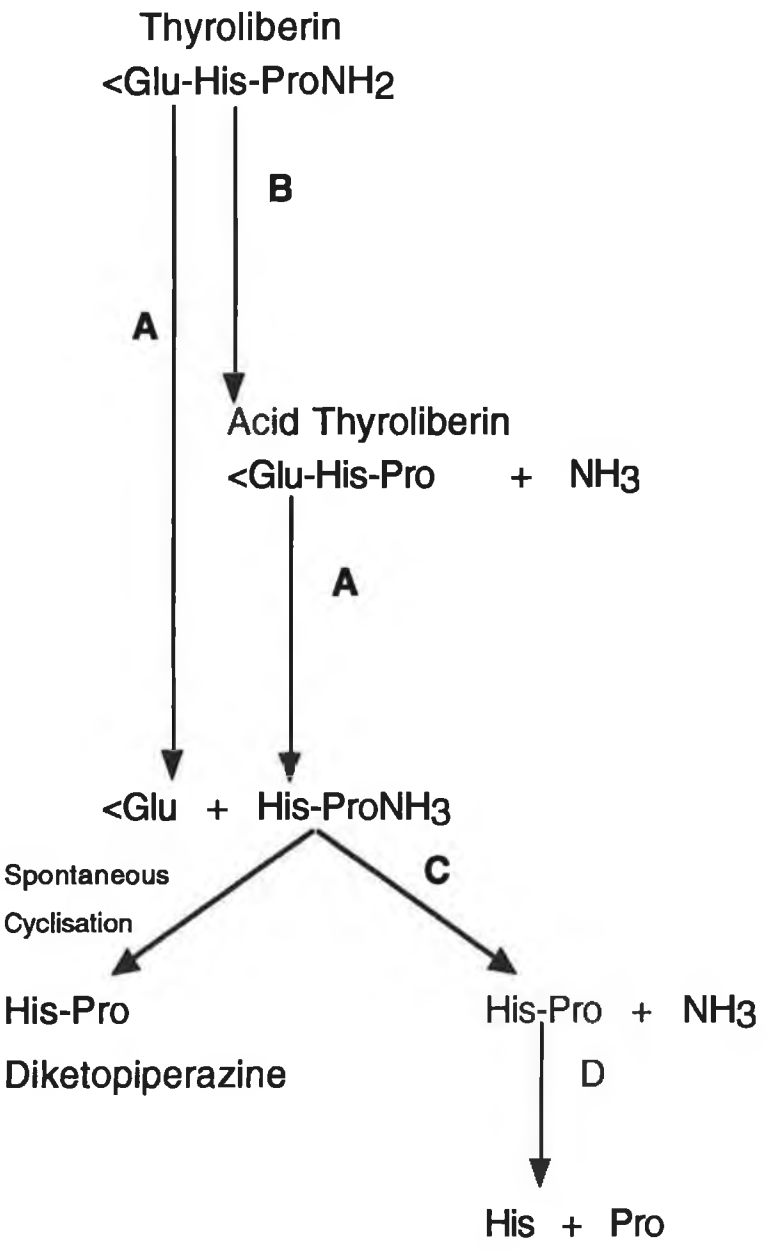
There has been much interest in the enzymatic inactivation of hypothalamic hormones and in the significance of the peptidase enzymes involved in the physiological control of their action. The study of these peptidases both *in vivo* and *in vitro* requires the ability to detect the particular hypothalamic hormone and to measure its concentration, and to identify and quantify metabolites formed as a result of enzyme action. Bioassays and radioimmunoassays were used initially, but these have been replaced more recently by the cheaper, faster and more sensitive spectrophotometric and fluorimetric assays. *In vitro*, rapid degradation by blood and tissue enzymes has been demonstrated for all other neuropeptides and the rapid inactivation of thyroliberin had already been observed long before its structure had been elucidated (Griffiths, 1976). However, thyroliberin was found to be resistant to a variety proteolytic enzymes and even against the action of very unspecific enzymes such as pepsin, papain or thermolysin (Burgus and Guillemin, 1970). This fact is explained by the structural features of the tripeptide amide. Due to the cyclised aminoterminal end and the amidated carboxyterminus, thyroliberin is resistant against the action of exopeptidases and due to the internal proline residue, thyroliberin is not even susceptible to degradation by general hydrolytic enzymes.

In order to understand the biological function of neuropeptide-degrading enzymes, it is a prerequisite to first delineate the pathways of neuropeptide fragmentation and to evaluate the biochemical properties of the individual enzymes capable of hydrolysing the neuropeptide.
Fig 1.4 (a and b) summarises the degradation pathways of thyroliberin.

Thyroliberin is unstable in serum and tissues due to the presence of both specific and non-specific peptidases. There exists 2 modes of primary inactivation:
1. a pyroglutamyl aminopeptidase (cleaving the <Glu-His bond), and
2. a proline endopeptidase (cleaving the C-terminal amide group)
These enzymes are of physiological importance in regulating various aspects of the endocrine and CNS actions attributed to thyroliberin (Griffiths,1976).
To fulfil such a function the enzymes should conform to certain criteria i.e. they should be located at the most appropriate sites in the body to perform their proposed function, they should have the correct biochemical characteristics such as substrate specificity, kinetics and products formed and finally, it should be possible to demonstrate changes in their activity with changes in physiological status(Griffiths and McDermott, 1984; Griffiths et al., 1983).

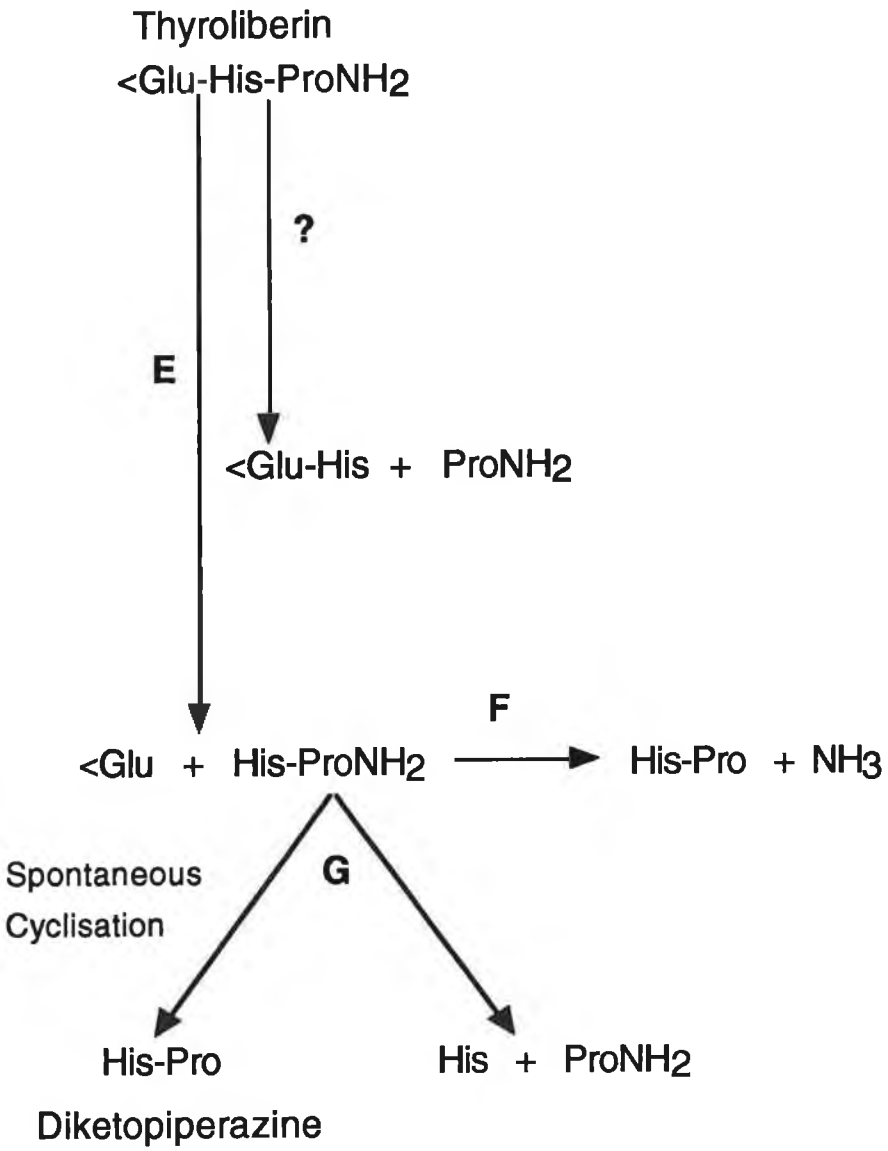
Fig 1.4 Primary degradation of thyroliberin by enzymes

a. Thyroliberin degradation by enzymes in the soluble fraction of brain cells



A= Pyroglutamate Aminopeptidase Type I
B= Prolyl Endopeptidase
C= Dipeptidyl Peptidase II
D= Proline Dipeptidase

b. Thyroliberin degradation by enzymes of the particulate fraction of brain cells



E= Pyroglutamate Aminopeptidase Type II

F= Dipeptidyl Peptidase IV

G= Imidiopeptidase

The first of the primary inactivators of thyroliberin is Pyroglutamate Aminopeptidase (PAP) (EC 3.4.19.3.) which generates His-ProNH$_2$ from brain thyroliberin. The brain contains at least 2 enzymes that exhibit this activity (Emerson and Wu, 1987).

1.6.1 Pyroglutamate Aminopeptidase (EC 3.4.19.3.)

PAP was first described in *Pseudomonas fluorescens* by Doolittle and Armentrout (1968) and has since been reported to be present in several bacterial, plant, animal and human tissues. In each case, this enzyme has been shown to exhibit maximal activity and stability in a highly reduced environment and to be highly sensitive to inhibition by sulphydryl reagents and heavy metal ions. Soluble PAP has been purified from several microbial sources including *Pseudomonas fluorescens* (Armentrout and Doolittle, 1969), *Bacillus subtilus* (Szewczuk and Mulczuk, 1969), *Klebsiella clocae* (Kwiatkowska et al., 1974) and *Streptococcus faecium* (Sullivan et al., 1977). It has also been partially purified from bovine adenohypophysis (Mudge and Fellows, 1973), bovine brain (Hersh and McKelvy, 1979), rat adenohypophysis (Bauer and Kleinkauf, 1980), chicken liver (Tsuru et al.,1982), human cerebral cortex (Lauffart et al., 1988) and human kidney (Mantle et al., 1990).

The soluble PAP (PAP I) purified from rat adenohypophysis (Bauer and Kleinkauf,1980) and guinea pig brain (Browne and O'Cuinn,1983) was found to be capable of cleaving the N-terminal <Glu residue from various pyroglutamyl peptides, such as Neurotensin, LHRH and Bombesin - the exceptions being those commencing with the sequence <Glu-Pro. PAP I cleaves the thyroliberin tripeptide to yield free glutamic acid and the dipeptide His-ProNH$_2$. This dipeptide spontaneously and non-enzymatically undergoes a cyclisation reaction to form His-Pro diketopiperazine (Cyclo(His-Pro)). Guinea-pig brain PAP I was shown to have a molecular weight of 24 kDa (Browne and O'Cuinn, 1983).

This enzyme has also been reported to be inhibited by diazomethyl ketone, an active site-directed irreversible inhibitor (Wilk et al., 1985) and by 5-oxoprolinal, an active site-directed reversible inhibitor (Ki=26nM) (Friedman and Wilk, 1985) .

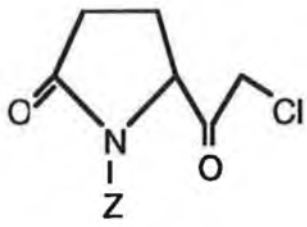
There appear to be conflicting reports regarding the role of PAP I in the metabolism of thyroliberin and LHRH in the brain and CNS. The role of soluble PAP in the metabolism of LHRH and thyroliberin in the brain was addressed by Mendez et al. (1990). By exposing hypothalamic cells or median eminences in culture to pyroglutamyl diazomethyl ketone (a specific PAP I inhibitor) they found that the thyroliberin content or recovery of released thyroliberin did not vary. Even though the activity of PAP I is regulated in GH$_3$ cells by 5-oxoprolinal (Friedman and Wilk, 1986), 3,5,3'-Triiodo Thyronine(T$_3$) (Suen and Wilk,1987b) and sodium butyrate (Suen and Wilk,1989a), Mendez et al. concluded that there does not appear to be a direct role for PAP I in thyroliberin or LHRH degradation. However a more recent study by Svoboda and Currie (1992) suggests the opposite. By chemically synthesising a series of chloromethyl ketone analogues of thyroliberin

which had previously been shown to possess thyroliberin-like activity (Bhargava et al.,1982) in the CNS, and which were also found to be good inhibitors of PAP I, they suggested that thyroliberin-like activity could derive indirectly from inhibition of endogenous thyroliberin degradation of PAP I.

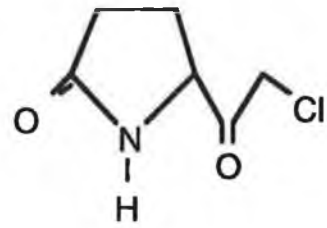
1.6.1.1. Inhibitors of the soluble PAP

PAP I is primarily a cytosolic enzyme with a widespread tissue distribution. Within the brain, the enzyme is fairly uniformly distributed (Torres et al.,1986). Active site-directed inhibitors of the enzyme were synthesised by Fujiwara et al. (1981) and included Z-pGlu-chloromethylketone, Z-pGlu-diazomethyl ketone and pGlu-chloromethyl ketone (Wilk, 1989).

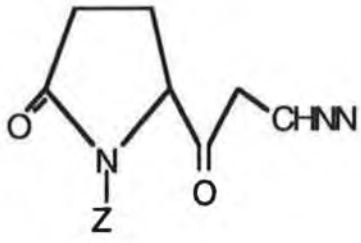
Fig 1.5. Structures of inhibitors of the soluble PAP



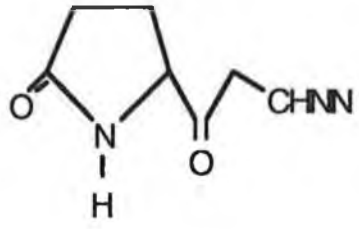
1. Z-pGlu-chloromethyl ketone



2. pGlu-chloromethyl ketone



3. Z-pGlu-diazomethyl ketone



4. pGlu-diazomethyl ketone

The chloromethyl ketone derivatives were found to be excellent inhibitors but difficult to evaluate, because they interacted with thiol-reducing agents which were in the assay mixture to stabilise the enzyme. Z-pGlu-diazomethyl ketone was found to be relatively inert toward thiol reagents, but it was 10-fold less potent an inhibitor than pGlu-chloromethyl ketone. Wilk (1989) synthesised pyroglutamyl diazomethyl ketone (PDMK). This compound, lacking the Z-group, had a dramatically

the particulate PAP.

There are no such doubts about the role of the second pyroglutamyl aminopeptidase activity involved in the primary degradation of thyroliberin. Several authors had reported PAP activity to be, at least in part, particulate in the brain tissue of several species (Schock,1977; Griffiths et al.,1979, 1980, 1982; Joseph-Bravo et al.,1979; Krieder et al.,1981). However the relationship between the soluble and particulate activities was not clear. Browne et al. (1981) first proposed that there were two pyroglutamyl aminopeptidase activities present in guinea-pig brain: one a soluble enzyme requiring DTT and EDTA for expression of its activity, and the second a particulate activity that was inhibited by EDTA. This finding was confirmed by O'Connor and O'Cuinn (1984) who located the particulate PAP activity to synaptosomal membrane preparations of guinea-pig brain from which it could be solubilised by papain treatment, thus confirming an earlier suggestion by Greany et al. (1980) that the particulate activity was located on synaptosomal membranes. Similarly, two PAP activities, one soluble and one particulate, were demonstrated in rat brain (Charli et al.,1984) , and further studies involving subcellular preparations (Garat et al.,1985; Torres et al.,1986; Vargas et al.,1987). A particulate PAP was also reported in rat adenohypophysis (Bauer,1987) and it was found to be involved in a biologically important control function within feedback-regulatory mechanisms.

1.6.2. The particulate PAP

The particulate PAP (EC 3.4.19.- designated as PAP II to differentiate it from the cytoplasmic PAP I by McDonald and Barrett,1986) was purified from synaptosomal membranes of guinea-pig brain and shown to be more restricted in its substrate specificity than the soluble PAP I . PAPII has been shown to only hydrolyse tripeptides containing N-terminal pyroglutamate and with histidine in the penultimate position (O'Connor and O'Cuinn, 1985). In marked contrast to the nonspecific soluble activity, the particulate PAP of guinea-pig brain was found to have its active site oriented toward the extracellular space where thyroliberin would be expected to be located when involved in neurotransmission (Charli et al.,1988). Therefore PAP II should be considered a true ectoenzyme. It is primarily located in the synaptosomal region (O'Connor and O'Cuinn,1984; Garat et al.,1985; Torres et al.,1986). Using primary cultures of neuronal, glial and adenohypophyseal cells from rat, Bauer et al. (1990) found a heterogeneous distribution of PAP II. Relatively high enzymatic activity was found on neuronal cells while glial cells were almost devoid of it. Low, but significant, activities were associated with pituitary cells as measured in aggregates consisting of the total pituitary cell population. This extremely heterogeneous distribution indicates that the thyroliberin-degrading ectoenzyme is not a general constituent of plasma membranes and thus strongly supports the hypothesis that it may serve very specialised functions for the inactivation of thyroliberin after its release. While peptidases associated with glial cells very likely only carry out general scavenger functions, peptidases on neuronal target cells potentially could be involved in turning off peptidergic signals, provided they are localised in the vicinity of the receptors or the site of

neuropeptide release.

PAP II has been found to be a metalloenzyme of molecular weight 230 kDa (Czekay and Bauer, 1993), with a Km of 40μM for thyroliberin (O'Connor and O'Cuinn,1985). It has a broad pH optimum in the neutral range, is sensitive to chelating agents such as EDTA, 8-hydroxyquinoline and 1,10-phenanthroline, and it shows no sensitivity to sulphydryl reagents (O'Connor and O'Cuinn, 1984). Moreover, pyroglutamyl diazomethyl ketone, an active site-directed inhibitor of PAP I does not inhibit synaptosomal PAP II (Friedman and Wilk,1986).

Perhaps the most significant difference between the soluble and the particulate PAP activities was the finding that the particulate activity, purified from the synaptosomal membranes of guinea-pig brain, cleaved the N-terminal <Glu from only thyroliberin or very closely related peptides (O'Connor and O'Cuinn,1985; Elmore et al., 1990). In these studies, various N-terminal pyroglutamyl peptides of varying lengths were used, and with the exception of <Glu-His, each peptide competitively inhibited the hydrolysis of thyroliberin by PAP II. The inhibition by LHRH (Ki=20μM) is of specific interest, as LHRH, although not hydrolysed by PAP II, showed a greater affinity for the enzyme than thyroliberin (Ki =42μM) itself (O'Connor and O'Cuinn, 1985). Substitution of <Glu or His by either Phe or N-Val in the thyroliberin sequence abolished the ability of the peptide to be hydrolysed, although some tolerance to substitution of the Pro-NH_2 residue was observed (Elmore et al.,1990). These observations and the non hydrolysis of thyroliberin analogues that possessed modified <Glu or His residues (Elmore et al.,1990) suggest that PAP II has an absolute requirement for an amino-terminal <Glu-His sequence for hydrolysis action, as opening the pyroglutamate ring, substitution of the histidyl residue or addition of any amino acid to the amino end of the molecule alter the molecules so that the enzyme cannot cleave them (Wilk and Wilk,1989).

The enzyme also appears to have a preference for substrate molecules which have a carboxy-terminal NH_2 group. This was seen when the peptide amides tested which contained carboxy-terminal NH_2 groups had lower Ki values (though they may not have been hydrolysed) than the corresponding molecules without the NH_2 group . Studies on the active site of PAP II indicate that tyrosine, histidine, arginine and possibly lysine residues are necessary for catalytic activity and that the Tyr, His and Arg are probably within the enzyme's active site (O'Connor and O'Cuinn, 1987). Czekay and Bauer (1993) confirmed the presence of tyrosine, histidine and arginine residues as catalytically important elements of PAPII and also identified zinc as the catalytically-active metal of the enzyme- a feature which confirms the enzyme as a metalloprotease.
In an attempt to establish the inactivation mechanism of pyroglutamate aminopeptidase activity, Suen and Wilk (1989b) identified a 48kDa subunit of the PAP and showed that the inactivation mechanism was likely due to phosphorylation and not due to internalisation or dissociation. The regulation of the PAP activity will be discussed further in section 1.6.4.
Trypsin-treatment of rat brain cells or rat brain intact synaptosomes has been found to destroy 70-

80% of PAP II without affecting LDH or PAP I activity. Moreover, pretreatment of intact synaptosomes with saponin did not lead to an increase in PAP II activity (Charli et al.,1988). These findings suggest that PAP II is an ectoenzyme that is involved in the metabolism of thyroliberin in the synaptic cleft.

1.6.2.1. The distribution of PAP II activity

The highest PAP II activity is found in brain tissue (Friedman and Wilk, 1986), with the cortex being richest in enzyme activity (Suen and Wilk, 1989b; Bauer, 1988). Significant activity is also found in spinal cord, cerebellum and posterior pituitary, with lower levels detected in liver, retina and anterior pituitary (Wilk et al., 1988). Vargas et al. (1987) also reported a wide variation in levels of specific activity of membrane-bound PAP in different regions of rat brain, but was unable to find a correlation between specific activities of the enzyme and thyroliberin levels or density of thyroliberin receptors. However, Wilk et al. (1988) observed that membranes of rat retina, a tissue known to contain relatively high levels of immunoreactive thyroliberin (Martino et al., 1980) and of thyroliberin receptors (Taylor and Burt, 1982) exhibits a specific activity for membrane-bound PAP II that exceeds specific activity values for all non-CNS tissues thus far investigated. They suggested a functional relationship between PAP II and thyroliberin, which was supported by Bauer (1988), who reported rapid degradation of thyroliberin by PAP II of neuronal cells, while noting an absence of this activity in astroglial cells. These findings suggest that this enzyme is only associated with neuronal cells in brain. Wilk (1986) proposed that the membrane-bound PAP II be considered the first characterised neuropeptide-specific peptidase.

Table 1.5 The distribution of PAP II in rabbit and rat brain regions and hypophysis (Vargas et al., 1992a)

Region	Rat PAP II Specific Activity (Thyroliberin/min/mg protein)	Rabbit PAP II Specific Activity (Thyroliberin/min/mg protein)
Olfactory Bulb	6.58	6.33
Posterior part of Cerebral Cortex	6.50	
Hippocampus	4.98	5.05
Cerebellar vermis	4.64	
Cerebellar hemisphere	3.78	
Nucleus accumbens-lateral septum	2.20	2.80
Mesencephalon	1.90	
Median Eminence	1.53	
Hypothalamus (minus ME)	1.25	
Pons	1.13	
Medulla oblongata	0.90	1.12
Adenohypophysis		0.50
Hypophysis	0.23	
Serum	0.02	0.11

Bauer et al. (1990) studied the cellular localisation of PAP II in primary cultures of neuronal, glial and adenohypophyseal cells and found the the thyroliberin-degrading ectoenzyme (PAP II) is specifically located on PRL cells. This preferred localisation of PAP II on PRL cells strongly suggests that the hormonally regulated thyroliberin-degrading activity plays an important and specific role in thyroliberin signalling to this cell type and might provide a rational explanation for the differential response of distinct pituitary cell types to the hypothalamic thyroliberin signal. As known, thyroliberin stimulates PRL release *in vitro* and has been implicated as a physiologically relevant secretagogue *in vivo*. It is also known that secretion of PRL is strongly modulated by peripheral hormones such as thyroid, glucocorticoid, and sex hormones. However, apart from the species-dependent luteotrophic action of PRL in rodents (McNeilly ,1987), which may give rise to production of oestrogens, these hormones are not produced by PRL-responsive organs and consequently, the secretion of PRL is not regulated by peripheral hormones via a classical feedback mechanism comparable to the well-known feedback action of thyroid hormones on the release of TSH. Obviously other mechanisms acting centrally or peripherally are required for balancing PRL secretion. The observed hormonal regulation of the enzyme by T_3 and estradiol may determine the availability and/or the duration of action in the vicinity of PRL cells and thus may modulate the stimulatory effect of thyroliberin on PRL release according to the needs of the body (Bauer et al., 1990).

1.6.3. Serum PAP

The presence of a potent thyroliberin-degrading activity in serum was first observed by Redding and Schally in 1969 and confirmed by Vale et al. (1971). The characterisation of the enzyme (Taylor and Dixon, 1978; Bauer and Nowak, 1979; Bauer and Kleinkauf,1980) showed that the enzyme present in serum degrades thyroliberin into <Glu and His-ProNH_2 in a manner similar to the tissue pyroglutamate aminopeptidase. However, this enzyme of molecular weight 260 kDa, exhibited chemical characteristics distinctly different from the previously characterised PAP I. It was inhibited by EDTA and DTT (agents which were known to activate PAP I), but not by 2-iodoacetamide and N-ethylmaleimide. It was shown to be inhibited by metal chelators such as 1,10-phenanthroline and 8-hydroxyquinoline and was characterized as a metalloprotease (Bauer et al., 1981). In contrast to the broad specificity of PAP I, the serum enzyme caused rapid, stereo-specific cleavage only of the <Glu-His bond of thyroliberin and very closely related analogues. <Glu-Ala, <Glu-His and <Glu-ß-naphthylamide are not effectively degraded by the serum enzyme and inhibit it poorly. Pyroglutamyl-containing peptides such as LHRH (<Glu-His-Trp...), neurotensin (<Glu-Leu-Trp...) or gastrin (<Glu-Gly-Pro....) and thyroliberin-analogues such as LLD-thyroliberin, <Glu-His-Pro-GlyNH_2 or <Glu-Gly-ProNH_2 are not hydrolysed by the serum enzyme (Bauer et al., 1981). It was not until the discovery of a particulate PAP in 1984 (O'Connor and O'Cuinn,1984) which resembled the serum thyroliberin-degrading activity in its size, chemical characteristics and substrate specificity that the serum enzyme (designated a 'Thyroliberinase' rather than a PAP due to its strict substrate

specificity, (Bauer,1983)) and the PAP II were suggested to be products of the same gene code (Bauer,1988).

1.6.4. PAP regulation

The possibility that the rapid inactivation of thyroliberin by serum, may be related to thyroid status was first suggested by Redding and Schally (1969) when they observed that degradation of thyroliberin was reduced in hypothyroid rats and the effect could be reversed by the administration of thyroid hormones. Similar studies by Vale et al. (1971) however, could not confirm these findings and human studies detected no difference in thyroliberin-degrading activity in patients with varying thyroid status (Jeffcoate and White, 1975). In 1976 a study by White et al., confirmed the relationship between thyroid status and thyroliberin inactivation originally suggested by Redding and Schally. They found that thyroliberin degradation is reduced in hypothyroid rat serum and increased in hyperthyroid rat serum. They went on to suggest that the mechanism by which this regulation was exercised most likely involved enzyme synthesis and degradation. Bauer (1976) compared the activity of serum from control rats to that of T_3- (triiodothyroxine) and PTU- (propylthiouracil, a mild goitregenic agent) treated cells. The study reported that enzymatic degradation contributes to the balance of thyroliberin secretion and its degradation is controlled by thyroid hormone-induced negative feedback mechanisms.

1.6.4.1. PAP regulation by thyroid hormones

With the characterisation and localisation of the thyroliberin-degrading enzymes, PAP I and II and the serum thyroliberinase in the 1980s, came a further surge of insight into regulation of thyroliberin levels by thyroid hormones. The ability to isolate and purify the specific enzyme fractions from brain homogenates allowed more exclusive research into the effect of thyroid status on thyroliberin degradation via regulation of PAP I and II.

The regulation of PAP I and II and the serum thyroliberinase has been studied both *in vivo* and *in vitro*. *In vivo* studies have been performed with T_3 (triiodothyroxine) or T_4 (thyroxine) to induce hyperthyroidism, or with PTU to induce hypothyroidism (Wolf et al., 1984; Aratan-Spire et al., 1984; Bauer, 1987; Scharfmann et al., 1990). The *in vitro* approach to investigation of the regulation of thyroliberin degradation by thyroid hormones has also been widely used (Suen and Wilk, 1987a; Bauer et al.,1990).

There is a conflict of reports regarding the effect of thyroid status on PAP I activity. Suen and Wilk (1987a) demonstrated that low concentrations of T_3 induce PAP I in the thyroliberin-target GH_3 cell. Later they found elevations in the PAP I activity in a number of brain regions and in the pituitary

following chronic but not acute T_3 treatment (Suen and Wilk, 1989b). Changes in the activity of PAP I are not necessarily related to thyroliberin, as other pyroglutamyl peptides such as LHRH and neurotensin are substrates of PAP I. However they felt it was possible to suggest that the increased levels of the enzyme in pituitary and hypothalamus may contribute to the negative feedback regulation of thyroid status by T_3 (Suen and Wilk, 1987a). Scharfmann et al. (1990) found that there were no changes in soluble rat liver PAP I with thyroid status. This report agreed with earlier reports from brain (Emerson and Wu, 1987) and adenohypophysis (Bauer, 1987) that PAP I is thyroid-hormone insensitive.
Emerson and Wu (1987) also found that that thyroid status influences only rat serum PAP, but has no effect on the cytosolic or membrane-bound PAPs of brain, even at increasing T_4 concentrations. In the case of serum thyroliberinase, activity significantly increased in the hyperthyroid group and correspondingly decreased in the hypothyroid group. This evidence supported Bauer's findings (Bauer, 1976) that thyroid hormones modulate thyroliberin degradation in rat serum.

A study by Bauer (1987) indicated that thyroid hormones exerted a direct effect on the activity of the membrane-bound adenohypophyseal enzyme, unlike the serum enzyme whose activity increases significantly only when thyroid hormones are injected repeatedly for long periods of time (Bauer,1976). This study agreed with Emerson and Wu (1987) that while the activity of the brain PAP II is not controlled by thyroid hormones, the adenohypophyseal PAP II is controlled by thyroid hormones. This suggests that the enzyme itself could serve as a regulatory control element to influence the extent and duration of the endocrine activities of thyroliberin at adenohypophyseal target sites. Hence the induction of the enzyme and the rapid decline in its activity within physiologically significant time periods indicate a high turnover of the enzyme, and thus it supports the concept of specialised functioning of the membrane-bound thyroliberin-degrading enzyme of the adenohypophysis.

The detection of increased activity of PAP II in the pituitary in response to acute T_3 treatment (Suen and Wilk, 1989b) firmly supports the earlier report of a 550% increase on control levels of PAP II activity when T_3 was acutely administered (Bauer,1987). This finding indicates that the biological activity of thyroliberin at the pituitary is terminated by PAP II and that the increased enzymatic activity contributes to the negative feedback regulation of thyroid status by T_3. This increase is no longer seen in the chronically treated animals where other mechanisms such as induction of PAP I may come into play. The increased activity of PAP in rat serum following chronic T_3 treatment is a well documented phenomenon and has already been discussed (White et al., 1976; Bauer,1976). Suen and Wilk (1989b) also provided evidence to suggest that PAP II activity in particulate brain regions is also modulated by thyroid hormone levels. This possibility had been rejected by previous studies (Emerson and Wu, 1987; Bauer, 1987). The fractionation of the brain into particular areas allowed the study of hormone effects on PAP in localised regions.

Effects of hormones on PAP activities were possibly masked in the earlier studies by the use of whole brain homogenates. Ponce et al. (1988) found the effect of thyroid hormones to be tissue specific- affecting thyroliberin degradation only in serum and at its target site in the axis: PAP II activity localised on adenohypophyseal membranes. Their results agreed with the findings of Bauer (1987) and indicates that if PAP II is an ectoenzyme in adenohypophysis as in brain, the degradation of thyroliberin on adenohypophyseal plasma membranes plays a role in the rapid negative feedback control, more than the delayed changes in thyroliberinase (serum PAP), or in PAP I whose contact with thyroliberin in adenohypophysis has not been demonstrated (Suen and Wilk, 1987b). The picture which emerges from recent studies is that negative feedback by thyroid hormones involves multiple level of regulation: thyroliberin metabolism (biosynthesis, release and inactivation), thyroliberin receptors and TSH secretion.

1.6.4.2. Significance of regulation studies in establishing the biochemical role of PAP I, PAP II and the serum thyroliberinase

PAP regulation studies have led to increased speculation as to the underlying role of the PAP enzymes in thyroliberin degradation and consequently the importance of thyroliberin degradation in biochemical and neurological pathways. There is little doubt that PAP II of adenohypophyseal tissue is tightly controlled by thyroid hormone levels (Bauer, 1987; Scharfmann et al., 1990b). This finding is of major importance as it suggests that this enzyme in particular may serve as a regulatory control factor, to determine the extent and duration of the endocrine activity of thyroliberin at pituitary target sites. Thus control of the secretion of adenohypophyseal hormones can be effectively exercised.

Bauer (1987) recognised that the rapid induction of pituitary PAP II in response to augmented T_3 levels and its subsequent decline in activity, lies within time periods which are of significance in demonstrating a high turnover of the enzyme in the pituitary. Such a high enzyme turnover in this specialised location is desirable if PAP II is to play a role in feedback regulation of thyroliberin levels, which modulate secretion of hormones from the pituitary. The lag phase of 4 hours reported by Bauer (1987), before rapid induction of PAP II activity in adenohypophysis is interesting as it indicates a period of protein synthesis which would make large quantities of enzyme available for thyroliberin degradation. If increased PAP II activity can be explained by the induction of enzyme synthesis, then it is reasonable to suggest that the decline in activity reflects an inhibition of the existing enzyme activity and of further enzyme synthesis. Some insight into the mechanism by which such inhibition is achieved has come to light in a recently published report by Suen and Wilk (1991). The implications of this suggestion will be discussed later. The inability to produce substantial evidence that PAP II in brain regions is controlled by thyroid hormones may be significant in itself as it suggests that this enzyme in such locations serves a more specialised function in neurotransmission.

The significance of the finding that PAP I enzyme is also regulated by chronic treatment with thyroid hormones, remains unclear. The broad specificity of PAP I means that changes in its activity cannot be interpreted with confidence in terms of thyroliberin degradation alone. There appears to be a case for the regulation of adenohypophyseal PAP I by thyroid hormones due to T_3 on PAP I of brain and extrahypothalamic regions. Further studies are necessary.

The thyroliberin-degrading serum pyroglutamyl peptidase enzyme has also been shown to be up-regulated in hyperthyroidism and down-regulated in hypothyroidism (Emerson and Wu, 1987). However a later report (Yamada et al., 1990) did not find this relationship, so the true significance of the finding remains to be seen. Efforts are being made to establish the nature of the enzyme which mediates this effect in serum. Much evidence points to PAP II as the two enzymes share similar physical and biochemical properties as well as substrate specificity. The role of the serum pyroglutamyl peptidase in thyroliberin degradation will be approached again in the next section as reports have suggested that it is also affected by steroid hormones.

1.6.4.3. Regulation of Pyroglutamyl Aminopeptidases by steroid hormones

The case for control of thyroliberin degradation through the regulation of specific thyroliberin-degrading enzymes in serum and the adenohypophysis has been established. Such regulation is mediated by thyroid hormone effects on thyroliberin-degrading enzymes. Studies on PAP II and the serum enzyme have supported the notion that thyroliberin is important in the regulation of adenohypophyseal hormone secretion. Equally the inability to confirm that PAP I in pituitary, brain and extra-hypothalamic regions and PAP II of brain, are regulated by thyroid hormones, may be significant. It implies that pyroglutamate aminopeptidase (especially PAP II) may serve some special role in transmission of thyroliberin signals and may represent the peptidergic equivalent of acetyl cholinesterase, which would cause the termination of nervous signals at the appropriate time. Steroid hormones are thought to effect the degradation of thyroliberin-degrading enzymes (Bauer, 1988). Through the study of steroid hormone effects on the PAP enzymes it has been possible to lend support to the proposal that PAP II, the membrane-bound thyroliberin-degrading enzyme, has specialised and independent functions in the pituitary and brain.

A 1988 study by Bauer was carried out to determine the effect of both thyroid hormones and oestrogens on the regulation of membrane bound PAP II of rat anterior pituitary. The study reveals some interesting results regarding the difference in the response of male and female rats to T_3 at the level of the membrane bound PAP in brain, pituitary and serum. The study then investigates the effect of injection of estradiol and testosterone on the induction of enzyme activity by T_3. Significant and appropriate findings resulted for both serum and adenohypophyseal (membrane-bound)enzyme. Activities were influenced by the sex of the animal and by estradiol administration but brain PAP II enzymes were not affected. For the serum enzyme, euthyroid females showed only 85% enzyme activity of males. It has been demonstrated that this enzyme is controlled by thyroid hormones and also that its activity alters with developmental changes (Neary et al., 1976).

With regard to PAP II of rat brain, no alteration in activity in response to thyroid status and moreover, no sex difference could be determined. Conversely, the activity of adenohypophyseal PAP II enzyme from male rats showed 75% reduction with PTU treatment and this activity was recovered following injection of thyroid hormone. A significant sex difference was noted for this enzyme, only 25% of the enzyme activity was found in female rat pituitary tested. Studies of ovariectomised females showed a three-fold increase in pituitary PAP II activity which was reduced with estradiol benzoate but injection of testosterone propionate could not counteract the effect of T_3. Injection of T_3 into intact females caused a rapid increase in PAP II adenohypophyseal enzyme (about 10-fold on control levels), an increase which could be counteracted by injection of estradiol benzoate.

Thus the experiments have shown that estradiol exerts a negative effect on the feedback regulation of PAP II by thyroid hormones. The results confirm a role for oestrogen in the regulation of PAP II enzymes by thyroid hormones and weaken the possibility that PAP II from brain sources may be affected by sex differences. The activity variations of the serum enzyme, brain PAP II and pituitary PAP II can be interpreted to contend with postulated specialisation of these enzymes to a particular function. For the membrane-bound thyroliberin-degrading brain enzyme, it could be demonstrated that it preferentially associated with synaptosomal membranes and represents a true ectoenzyme which is almost exclusively associated with neuronal cells. Therefore, this enzyme may exert a biologically important function for the inactivation of synaptically released thyroliberin in order to clear the target site for the transmission of the next thyroliberin signal. For such static functions, the activity of the thyroliberinase should not be a limiting factor and, therefore, it is not surprising that the activity of the brain enzyme was found not to be influenced by the hormonal manipulation of the animals (Bauer, 1988).

Adenohypophyseal PAP II has been shown to be dually controlled by steroid and thyroid hormones (Bauer, 1988). This finding should also be regarded in terms of the proposed function of pituitary PAP II. All evidence appears to point to a regulatory role for the enzyme in the release of adenohypophyseal hormones (mediated by hypothalamic thyroliberin). Such an effective regulation system would be expected to be finely tuned to respond to the levels of both thyroid and steroid hormones at target sites. The dual and inverse control of PAP II in the pituitary by oestrogens and thyroid hormones, is desirable if the stimulation and duration of thyroliberin action at its target sites is to be maintained in accordance with thyroid and steroid hormone levels.

The serum thyroliberinase has also been found in many studies to be controlled by thyroid hormones and to alter with developmental changes. This suggests that the enzyme functions in a regulatory system. There has been a suggestion that thyroliberin degradation by hypophyseal portal blood may represent a functional control element within the regulatory mechanism. Perhaps by limiting thyroliberin levels in serum and thus restricting the hormones availability to the trophic cells of the pituitary, the production of hormone by the cells could be limited (Bauer, 1988). This

possibility however requires further study, particularly of the enzyme's source and exact relationship with PAP II of the pituitary as some identity between the two enzymes is suspected. Bauer's findings (Bauer, 1988) , have provided firm evidence to support the proposed roles of the individual pyroglutamyl aminopeptidases in brain, adenohypophysis and serum. *In vitro* studies carried out (Bauer et al., 1990) arrived at very similar results for the dual effect of thyroid hormones and estradiol on PAP II of pituitary cells which demonstrate that the observed effect is a result of a direct action of T_3 on the cells rather than an indirect effect via other T_3 -responsive systems.

1.6.4.4. Possible mechanisms of PAP enzyme regulation

The study of regulation of thyroliberin-degrading PAP enzymes has uncovered a vast quantity of information which allows the pathways of thyroliberin degradation to be examined, with a view to ascertaining the tuning of the endocrine system to hormone levels. Such studies have established that thyroid hormones control the activities of some of the thyroliberin-degrading enzymes, in an effort to regulate hormone levels in accordance with bodily needs. There has however been little insight into establishing the mechanism which allows enzyme activity to be turned on and off.
In 1991 Suen and Wilk suggested that PAP II is subject to short term regulation and that the protein kinase C (PKC)- mediated phosphorylation is the most likely mechanism for this effect (Suen and Wilk, 1991). Earlier studies had shown that phosphorylation of hormone-responsive peptides was mediated *in vitro* by a Ca^{2+}-dependent phospholipid-activated protein kinase C (Drust and Martin, 1984). Furthermore PKC has been shown to be stimulated by diacylglycerol and phorbol ester *in vitro* (Drust and Martin, 1985). Thus it is reasonable to suggest that thyroliberin rapidly initiates both Ca^{2+} and lipid-dependent pathways of protein phosphorylation.

Suen and Wilk's studies are based on the inhibition of PAP II in Y-79 retinoblastoma cells by phorbol ester 12-o-tetradecanyl-phorbol-13 acetate (TPA). This tumour-promoting phorbol ester dually inactivates Ca^{2+} and phospholipid-dependent PKC, which in turn causes a variety of biological effects in a fashion very similar to hormonal treatment. In their 1990 study, Suen and Wilk observed that the time course of TPA-mediated effects paralleled the time course of translocation and activation of protein kinase C in this cell line. They demonstrated a decrease of PAP II activity to 10% of control levels within 15 mins, followed by a return to 70% of control levels within 1 hour. They reasoned that the initial decrease in activity resulted from enzyme phosphorylation via a TPA-activated protein kinase C. In a 1991 report, Suen and Wilk further investigated the TPA treatment of Y-79 retinoblastoma cells and reported a second phase of inactivation which occurs following a long exposure to TPA. After 1 hour enzymatic activity slowly declined and by 7 hours 15% of the control levels remained. It was Suen and Wilk's aim to explain this biphasic inactivation of PAP II in terms of enzyme phosphorylation mediated by PKC. Proof of a protein kinase C involvement in the decreased enzyme activity, observed on thyroliberin treatment, came from the pre-treatment of the Y-79 cells with a PKC inhibitor, H-7 or sphingosine. This was seen to prevent the inactivation of PAP

II. Using immunoblotting techniques they showed that the amount of PAP II was not changed following TPA treatment. Neither was the decreased activity due to dissociation or internalisation of PAP II (Suen and Wilk, 1991). By incubating Y-79 cells for varying times with TPA, they showed that TPA treatment causes the phosphorylation of a 48 kDa subunit of PAP II thereby causing the inhibition of PAP II activity.

How is the second phase of PAP II inactivation accomplished? A possible insight into the mechanism has come from Suen and Wilk's 1991 study, where the amount of PAP II enzyme in the Y-79 cell membranes was determined by immunoblotting. Following incubations with TPA, the membrane proteins were immunoblotted with a PAP II polyclonal antibody. The amount of the 48 kDa subunit of PAP II was seen to decrease with increasing time of exposure to TPA.

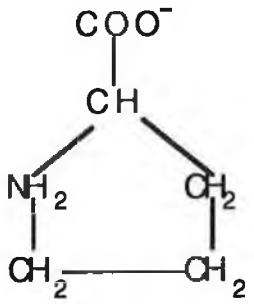
PKC was thus shown to be targeted by phorbol esters *in vitro* and *in vivo*. The activation of PKC stimulates dual signals which can stimulate or inhibit biological responses (Suen and Wilk, 1991). Recovery of PAP II activity in the current case, following the initial decrease in activity is no doubt due to a reversal of phosphorylation, restoring the enzyme to its active state. The second inactivation, from the above experiment by Suen and Wilk, also appears to involve PKC. The mechanism for this second phase of inactivation was seen not to be due to dissociation from the membrane or internalisation of PAP II, as no activity could be detected in the cytosol or in the medium.

The 1990 and 1991 experiments have elucidated the mechanism by which thyroliberin regulates PAP enzymes. It has been established that thyroliberin somehow activates protein kinase C. The mechanism by which protein kinase C activates the PAP II enzyme, to degrade thyroliberin, when thyroid hormone levels are in excess of requirements, is so far speculative but it most likely involves protein phosphorylation or dephosphorylation.

1.6.5. Prolyl endopeptidase activity

The unique cyclic aliphatic structure of proline represents a characteristic target for specific endopeptidases and exopeptidases (Kato et al., 1980). Proline differs from the other amino acids in the basic set of twenty in that it contains a secondary rather than a primary amino group. Strictly speaking proline is an imino acid rather than an amino acid. The side chain of proline is bound to both the amino group and the α-carbon which results in a cyclic structure as shown in Fig 1.6. Enzymes hydrolysing peptide bonds involving proline are of particular interest because of the significance of this amino acid in determining the conformation of peptide chains. Enzymatic hydrolysis of peptide bonds in which either the carboxyl group or the imido group of proline are involved have been known for years.

Fig 1.6 The unique cyclic aliphatic structure of proline (Kato et al., 1980)



1. Iminopeptidase (Proline dipeptidase EC 3.4.13.9.), hydrolyses dipeptides containing a free imino group of proline or hydroxyproline
2. Prolinase (Prolyl dipeptidase EC 3.4.13.8.), hydrolyses dipeptides containing a substituted imino nitrogen of proline
3. Dipeptidyl Aminopeptidases (DAP II EC 3.4.14.2. and DAP IV EC 3.4.14.5.), can both act as post proline cleaving enzymes on a number of tripeptides which have proline in the central position
4. Aminopeptidase P (EC 3.4.11.9.), is an exopeptidase which cleaves the N-terminal residues linked to proline in oligopeptides of low and high molecular weight (Holtzman et al., 1987)
5. Proline carboxypeptidase (EC 3.4.16.2.), is a COOH terminal exopeptidase which cleaves at the carboxyl side of proline provided that the imino group is protected and the carboxyl group of the terminal is free.
6. Post Proline Cleaving Enzyme (EC 3.4.21.26), is capable of cleaving the peptides on the carboxyl side of proline residues located internally in the peptide (Walter and Yoshimoto, 1978)

In 1971, Walter et al. reported on the partial purification of an enzyme from uterine homogenates which cleaved the prolyl-leucyl bond of oxytocin:

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2

This enzyme was purified to apparent homogeneity from lamb kidney by Koida and Walter (1976) and termed 'post-proline cleaving enzyme' based on its specificity for peptide bonds in which the carbonyl group was provided by proline residue. Oxytocin, angiotensin II and bradykinin were all cleaved at the appropriate 'post-proline' sites, although the Pro-Pro bond of bradykinin was not cleaved. The enzyme was reported to have a dimeric structure and a molecular weight of 115 kDa. Subsequent studies established PPCE as a serine protease (Yoshimoto et al., 1977). In 1976 Oliviera et al., studying the degradation of bradykinin, partially purified an enzyme from the supernatant fraction of rabbit brain homogenates which they named kinase B. This thiol-activated enzyme cleaved bradykinin at the Pro^{7}-Phe^{8} bond and was reported to have a molecular weight of

68 kDa.

In 1978 Orlowski et al. purified a brain enzyme which cleaved peptides on the carboxy side of proline residues. This enzyme had a molecular weight of 66 kDa and showed a substrate specificity similar to that of PPCE (Oriowski et al., 1979) in that it hydrolysed peptidyl prolyl-peptides and peptidyl prolyl amino acid bonds. However, because it was inactive on substrates with an unsubstituted imino group of proline, the name of prolyl endopeptidase was proposed.

The degradation of thyroliberin at the prolyl-amide group uncovered another enzyme with a similar specificity to lamb kidney PPCE (Hersh and McKelvy, 1979; Knisatschek and Bauer, 1979; Rupnow et al., 1979). This enzyme was isolated from bovine anterior pituitary and had a molecular weight of 76 kDa. It was inhibited by diisopropylfluorophosphate (DFP) and sulphydryl blocking agents. Despite the discrepancy by Koida and Walter (MW of 115 kDa, dimeric structure) for PPCE from lamb kidney, the other enzymic activities detected in different species and in a variety of tissues, that cleave peptide bonds at the carboxyl side of proline residues within a peptide chain share many common features (Table 1.6).

Table 1.6 A comparison between Prolyl Endopeptidase and other proline-cleaving peptidases

Peptidase	Source	Molecular Weight (kDa)	Inhibitors	Substrate Specificity
Prolyl Endopeptidase	Rabbit brain Cow brain Lamb brain Human serum	69 76 74 100	Thiol reactive reagents Z-Pro-prolinal	Thyroliberin, LHRH Pro9-Gly10 NT Pro7-Arg8 NT Pro10-Tyr11 BK Pro3-Gly4, Pro7-Phe8
Angiotensin Converting Enzyme (ACE)	Rat brain Pig kidney Human liver Human serum	165 180 150 140	EDTA (metallo-protease) Captopril	Ang I Phe8-His9 BK Phe5-Ser6, Pro7-Phe8 NT Tyr11-Ile12 LHRH Trp3-Ser4 Tyr5-Gly6, Leu7-Arg8
EC 3.4.24.11	Rabbit kidney Human kidney Human lung	93 90 90	EDTA Thiols Phosphoramidon	Ang I Arg2-Val3, Tyr4-Ile5, Pro7-Phe8 BK Gly4-Phe5, Pro7-Phe8 NT Pro10-Tyr11, Tyr11-Ile12
EC 3.4.24.16	Rat brain Pig brain		EDTA	NT Pro10-Tyr11 Ang I Pro7-Phe8 LHRH Tyr5-Gly6, Gly6-Leu7 BK Phe5-Ser6

NT = Neurotensin
BK = Bradykinin
Ang I = Angiotensin I
LHRH = Lutenising Hormone Releasing Hormone

The true identity of proline-specific endopeptidase activities was elucidated by Hersh in 1981 using immunochemical techniques. These revealed that a single enzyme was responsible for prolyl endopeptidase activities. An antibody to the bovine brain enzyme cross-reacted with the brain enzyme of various species and with prolyl endopeptidase-like activity from rat liver, lung and kidney (Hersh,1981). The rat brain and kidney enzymes had similar molecular weights, substrate specificities and sensitivity to thiol blocking agents. Antibodies to the rat brain precipitated the lamb kidney enzyme, and also cross-reacted with the enzyme from a number of other tissues (Andrews et al., 1982).
In view of the identity of enzymes from various tissues and different species, a unified nomenclature was proposed. The name prolyl endopeptidase was chosen, rather than post-proline cleaving enzyme, since not all post-proline bonds are cleaved (eg. Pro-Pro). The names thyroliberin-deamidase and kinase B implied restrictive specificity and were therefore misleading. The name prolyl endopeptidase (EC 3.4.21.26) was therefore adopted (Barrett, 1980).

Since its discovery in 1971 by Walter et al. in human uterus, prolyl endopeptidase has been found to be widely distributed. It has been purified from a variety of mammalian sources such as rat brain (Rupnow et al., 1979), rabbit brain (Orlowski et al., 1979), bovine brain (Yoshimoto et al., 1983), lamb brain (Yoshimoto et al., 1981),pig brain (Schonlein et al., 1990) and human tissues (Kato et al., 1980; Daly et al., 1985; Zolfaghari et al., 1986). The distribution of prolyl endopeptidase in a number of species, including rat (Fuse et al., 1990), rabbit (Orlowski et al., 1979), bovine (Tate, 1981) and human (Kato et al., 1980) has been studied.

Prolyl endopeptidase has been predominately described as a soluble, cytosolic enzyme. However, it has become increasingly clear that prolyl endopeptidase is found in both soluble and insoluble fractions of tissue homogenates. Very few studies have described the exact distribution of enzyme activity between soluble and insoluble fractions from tissue to tissue. Dresdner et al. (1982) localised the prolyl endopeptidase of rabbit brain in the cytoplasm. They noticed a sizable percentage of prolyl endopeptidase activity to be associated with the particulate fraction, but took that activity to be due to entrapped cytoplasm as they had not washed the membrane fraction.

Camargo et al. (1984) found that 10% of prolyl endopeptidase activity in rabbit brain remained attached to the membrane fraction during extensive salt washing, but it could be solubilised with detergents such as Triton X-100. They suggested that the enzyme was only loosely associated with

the membrane. They confirmed this report by immunoprecipitation of the cytosol and solubilised membrane enzyme with the anti-prolyl endopeptidase immunoglobulin, as both fractions contained an antigen immunoprecipitated by the immunoglobulin.
Dalmaz et al. (1986) also found prolyl endopeptidase activity in the membrane fractions of rat hypothalamus and localised it in the synaptosomal fraction. However, they found that within the synaptosomal fraction, the highest prolyl endopeptidase specific activity 40 times higher than the synaptic vesicle or membrane fractions. Particulate forms of the prolyl endopeptidase have been reported in the brush border of kidney (Sudo and Tanabe, 1985), in the neuroblastoma x glioma hybrid cell lines NG 108-15 (Chappell et al., 1990) and in the N1E-115 neuroblastoma cells (Checler et al., 1986).

In brain homogenates, the amount of membrane-bound activity amounts to 10% of the total activity (Dresdner et al., 1982). In the brush border of the kidney, a 10-fold greater amount of prolyl endopeptidase specific activity is membrane bound as opposed to being soluble (Sudo and Tanabe, 1985). Prolyl endopeptidase activity is also found in membrane preparations of circular, but not longitudinal muscle from canine ileum (Checler et al., 1987).
It is not known whether expression of different gene products account for insoluble and soluble forms of prolyl endopeptidase. Presently, there is no information on the prolyl endopeptidase genes and the cDNA sequence is consistent with a cytosolic or soluble enzyme (Rennex et al., 1991). It is possible that the difference between soluble and particulate forms results from a post-translational modification.

1.6.5.1 Tissue distribution of Prolyl Endopeptidase

Prolyl endopeptidase is widely distributed in various tissues (Table 1.7). The brain is one of the richest sources of the enzyme. Within the brain, prolyl endopeptidase activity is found in all regions. In rabbit brain, the highest activity is found in some cortical regions, hippocampus and striatum, and lowest activity in the pons and medulla (Orlowski et al., 1979). A difference of 2 to 3 -fold separates regions of highest and lowest activity. In human, the frontal cortex shows the highest prolyl endopeptidase activity, and activity is also found in the nucleus caudatus, the thalamus and hypothalamus (Kato et al., 1980). Yoshimoto et al. (1983) showed a similar regional distribution for prolyl endopeptidase activity in bovine brain.

Table 1.7 Tissue Distribution of Prolyl Endopeptidase (Welches et al., 1993)

Species	Kidney	Lung	Testis	Heart	Serum	Brain
Man	1.00	0.30	1.40	0.20	0.00	-
Rabbit	1.00	2.20	-	-	-	-
Mouse	1.00	2.40	-	1.00	-	1.00
Rat	1.00	0.60	-	0.60	-	0.80
Rat	1.00	0.20	-	0.70	-	1.60
Rat	1.00	1.80	3.30	1.60	0.00	2.10

Levels of PE activity are expressed relative to the value found in kidney. A dash indicates that the activity was not determined.

1.6.5.2. Characterisation of Prolyl Endopeptidase

Prolyl endopeptidase is a serine peptidase with no metal ion requirement. Its molecular weight is approximately 70 kDa. However there are two reports of a human serum prolyl endopeptidase that appears to be a dimer of 70 kDa molecular weight subunits (Kato et al., 1984; Soeda et al ., 1984). It has a pH optimum of 7-8.5 depending on the source of the enzyme and the substrate used (Yoshimoto et al., 1983; Knisatschek and Bauer, 1979).

Prolyl endopeptidase has an essential thiol residue, and its levels of activity are increased by including a thiol reagent such as ß-mercaptoethanol or dithiothreitol (DTT). The typical increase in activity by thiols is from 1.3 to 1.6-fold using synthetic substrates (Orlowski et al.,1979; Kato et al.,1980; Kalwant and Porter, 1991). However, one report shows a 30-fold activation of prolyl endopeptidase by thiols using the natural peptide substrate angiotensin II (Greene et al., 1982). This result suggests that there may be differences in prolyl endopeptidase activity observed when using synthetic and natural substrates.

Rennex et al. (1991) used prolyl endopeptidase from porcine brain to isolate a cDNA clone. This clone contained the complete coding sequence of prolyl endopeptidase and encoded a polypeptide of molecular weight 80,751Da. The deduced amino acid sequence of prolyl endopeptidase showed no sequence homology with the other known serine proteases. Tritiated DFP was used to identify the active-site serine of prolyl endopeptidase. One labelled peptide was isolated and sequenced. The sequence surrounding the active-site serine was Asn-Gly-Gly-Ser-Asn-Gly-Gly. This sequence is different from the active site sequences of other known serine proteases. This difference and the lack of overall homology with the known families of serine proteases suggested that prolyl endopeptidase represents a new type of serine protease.

1.6.5.3. Substrate Specificity of Prolyl Endopeptidase

Prolyl endopeptidase was originally discovered as an enzyme which could degrade oxytocin (Walter et al., 1971). This enzyme can also hydrolyse many oxytocin and vasopressin analogues by cleaving the peptides on the carboxyl side of proline (Walter, 1976), with the exception of Pro-Pro bonds. However it will cleave at the alanine residues in oligoalanine peptides at low efficiency (Yoshimoto et al, 1978). A report from Rosen et al. (1991) showed prolyl endopeptidase cleaving at the Ala-Ser bond in which the serine is phosphorylated in an undecapeptide mimicking a phosphorylation site in proteins. What is most interesting was the failure of prolyl endopeptidase to cleave at this site of the unphosphorylated peptide because it suggests that prolyl endopeptidase's specificity and protein peptide phosphorylation could function as a method for peptide processing.

Prolyl endopeptidase is an oligopeptidase and shows no activity toward proteins as opposed to peptides (Camargo et al., 1979).

There exist significant differences in the rates of cleavage depending on the nature of the amino acid occupying the Pro-X bond. The rate of cleavage is faster when X is a lipophilic residue, slower in the case of a basic residue and slowest when an acidic residue is present in the vicinal residue (Koida and Walter, 1976). Prolyl endopeptidase from human erythrocytes have been shown to have a preference for phosphorylation in the X position (Rosen et al., 1991). Tate (1981) isolated prolyl endopeptidase from bovine brain and claimed that it had a requirement for a basic amino acid prior to the proline moiety which set it apart from prolyl endopeptidase from other sources. Andrews et al. (1982) showed that prolyl endopeptidase is the only enzyme capable of cleaving the Pro-Naphthylamide (NA) bond of the thyroliberin analogue <Glu-His-ProNA. This compound would satisfy the requirements of a limited specificity described by Tate as it has the basic amino acid residue preceding the proline residue eg thyroliberin, LHRH, α-Melanocyte stimulating hormone, substance P, neurotensin, angiotensin II and dynorphin 1-13. It is also of interest to note that the majority of the synthetic substrates used *in vitro* to quantify the prolyl endopeptidase activity do not have a basic amino acid preceding the Pro and are all readily cleaved by prolyl endopeptidase eg Z-ala-Pro-4-methoxy-2-NA (Taylor and Dixon, 1980), Z-Gly-Pro-SM (Friedman et al., 1984) and Z-Gly-ProMCA (Yoshimoto et al., 1979).

Prolyl endopeptidase clearly prefers small peptide substrates over large polypeptides. Taylor and Dixon (1980) could not demonstrate hydrolysis of albumin or myoglobin. Denatured proteins with molecular weights of approximately 16 kDa were not cleaved. Camargo et al. (1979) prepared $(Gly)_3$-Arg-bradykinin and attached this peptide to succinyl polylysine polymers (MW 3-180kDa) and to Affi-Gel 10. Trypsin released bradykinin from the 3 bound forms and from free $(Gly)_3$-Arg-bradykinin. On the other hand, prolyl endopeptidase hydrolysed only the free bradykinin analogue. On the basis of their experiments, Camargo et al. described the enzyme as an endo-oligopeptidase with a specificity towards small peptides.

Table 1.8 Naturally occurring peptide substrates for prolyl endopeptidase (Welches et al., 1993)

Cleavage points are indicated by ↓

Peptide	Sequence
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro ↓ Phe-His-Leu
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro ↓ Phe
Bradykinin	Arg-Pro-Pro/Gly-Phe-Ser-Pro ↓ Phe-Arg
Substance P	Arg-Pro-Lys-Pro ↓ Gln-Gln-Phe-Phe-Gly-Leu-Met-n
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro ↓ Arg-Arg-Pro ↓ Tyr-Ile-Leu
Vasopressin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro ↓ Arg-Gly
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro ↓ Leu-Gly
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro ↓ Gly-n
Thyroliberin	pGlu-His-Pro ↓ -n

Cleavage of a number of naturally occurring neuropeptides by prolyl endopeptides was mentioned previously. These include thyroliberin, LHRH, bradykinin, neurotensin, substance P, angiotensin I and II and oxytocin. The peptides with the consistently lowest Kms for prolyl endopeptides are angiotensin I and bradykinin. Table 1.9 shows that neurotensin, substance P, vasopressin, oxytocin and LHRH have moderately low Kms. One report of prolyl endopeptidases Km for substance P is extremely high at 1000μM (Kato et al., 1980). Thyroliberin and LHRH have a variable range of Kms as low as 10μM or less (in the case of LHRH) increasing to a range of 100's or 1000's of μM. The variation in Km values seen for prolyl endopeptidase is presently unexplained and may reflect species differences or perhaps procedural differences.

Table 1.9 Prolyl endopeptidase affinity (Km) for natural peptides (Welches et al., 1993)

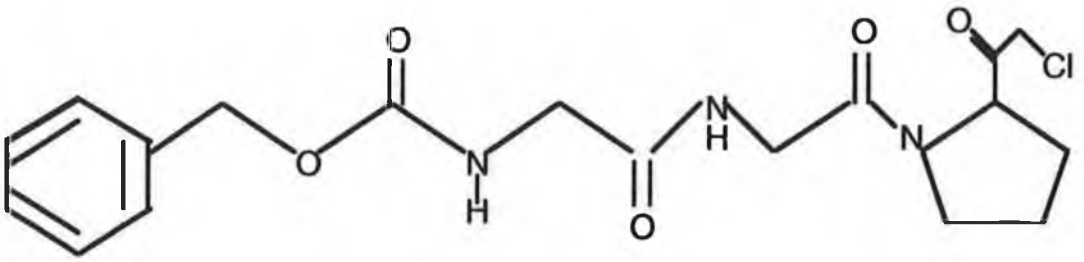
Neuropeptide	Peptidase Affinity (μM Km)	Source	Reference
Thyroliberin	540.00	Bovine Brain	Hersh and McKelvey(1979)
	18.90	Bovine Brain	Hersh (1981)
	690.00	Bovine Brain	Yoshimoto et al. (1983)
	17.10	Human Lung	Hersh (1981)
	4100.00	Rat Brain	Andrews et al. (1980)
LHRH	150.00	Bovine Brain	Hersh and McKelvey(1979)
	4.00	Bovine Brain	Tate (1981)
Neurotensin	1.80	Bovine Brain	Tate (1981)
Substance P	1.10	Bovine Brain	Blumberg et al. (1980)
	1000.00	Rat Brain	Kato et al. (1980)
	5.40	Rabbit Brain	Camargo et al. (1984)
Angiotensin I	3.20	Human Lung	Zolghari et al. (1986)
Angiotensin II	63.00	Rabbit Brain	Greene et al. (1982)
	1.20	Bovine Brain	Tate (1981)
Bradykinin	2.30	Rabbit Brain	Camargo et al. (1984)
	7.00	Bovine Brain	Tate (1981)

1.6.5.4. Prolyl endopeptidase inhibitors

As prolyl endopeptidase is distributed ubiquitously, a naturally occurring prolyl endopeptidase inhibitor is also widely distributed in various tissues. Knisatschek and Bauer (1979) found that pancreatic trypsin inhibitor was effective in inhibiting 90% of the prolyl endopeptidase activity. However, they later showed this inhibition to be due to the presence of impurities in the inhibitor preparation. Yoshimoto et al. (1982) purified a specific prolyl endopeptidase inhibitor from porcine pancreas. This inhibitor showed no inhibitory activity for trypsin, and was highly specific for proline endopeptidases, prolyl endopeptidase from mammalian organs and proline specific endopeptidase from *F.meningosepticum*. It had no effect on dipeptidyl aminopeptidase IV from lamb kidney, prolidase from bovine intestine and proline aminopeptidase form *B.megaterium*, although these enzymes have substrate specificity for proline residue. The presence of another naturally occurring prolyl endopeptidase inhibitor was investigated by Yamakawa et al. (1990). They purified a peptide inhibitor (MW=7000Da) from rat liver cytosol and found that its inhibitory activities for prolyl endopeptidase largely depends on peptide compounds having phosphate ester in its molecule. They found also that coenzyme A (CoA), its related compounds and acylcarnitine non-compepetitively inhibited the activity of prolyl endopeptidase in rat liver cytosol.

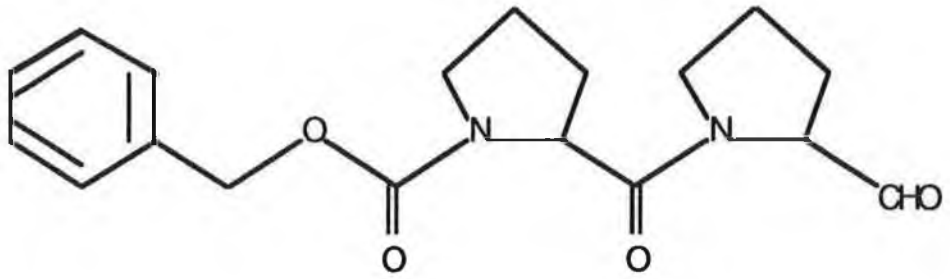
Even though there existed a naturally occurring prolyl endopeptidase inhibitor, much investigation leads to the development of specific site-directed synthetic inhibitors. Biochemical studies indicated that prolyl endopeptidase is a serine protease (Yoshimoto et al., 1977; Andrews et al., 1980), but prolyl endopeptidase is also sensitive to cysteine protease inhibitors. The first moderately potent inhibitors of this enzyme were proline-containing chloromethyl ketone derivatives (Yoshimoto et al., 1977). The most potent member of this series, Z-Gly-Gly-Pro-CH_2Cl, did not inhibit trypsin and only negligibly inhibited chymotrypsin. Such inhibitors are irreversible, and it is assumed that they alkylate a histidine residue at the active site.

Fig.1.7 Z-Gly-Gly-Pro-CH_2Cl Structure



Serine and cysteine proteases are potently inhibited by peptide aldehyde analogues of good substrates. This inhibition proceeds by the formation of a transition state analogue intermediate. N-Benzyloxycarbonyl-prolyl-prolinal (Z-pro-prolinal) was the first of the transition state analogues of prolyl endopeptidase to be synthesised (Wilk and Orlowski, 1983). This compound inhibits prolyl endopeptidase non-competitively with a Ki of 14nM. This value is three orders of magnitude lower than the Ki of the acid Z-Pro-Pro or the alcohol Z-Pro-Prolinol, consistent with its designation as a transition state analogue inhibitor. At a concentration 150-fold greater than its Ki for prolyl endopeptidase, Z-pro-prolinal does not inhibit trypsin, chymotrypsin, papain, DAP IV, endopeptidase 24.11 or the multicatalytic protease complex. Bakker et al. (1990) showed that Z-Pro-prolinal was a slow tight-binding inhibitor of both mouse brain and human brain prolyl endopeptidase activity with a Ki of 0.35nM.

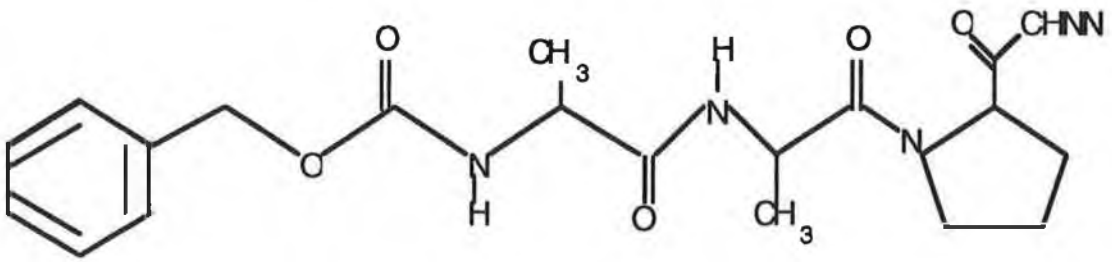
Fig. 1.8 Z-Pro-prolinal Structure



Z-Pro-prolinal is also an effective inhibitor *in vivo*. This lipophilic compound readily crosses the blood brain barrier, where it produces a long-lasting inhibition of brain prolyl endopeptidase (Friedman et al., 1984). Friedman et al. showed that a dose of 0.5mg/kg, intraperitoneally administered, inhibited the brain prolyl endopeptidase more than 50%, and 5mg/kg significantly inhibited the brain enzyme for as long as 6.5 hours. It is clear that Z-pro-prolinal is an excellent tool for exploring the biological significance of prolyl endopeptidase.

As seen previously, prolyl endopeptidase is also sensitive to cysteine protease inhibitors. Green and Shaw (1983), treating prolyl endopeptidase as a cysteine protease, synthesised a series of diazomethyl ketone inhibitors. Z-Ala-Ala-Pro-CHN_2 rapidly inactivated prolyl endopeptidase from mouse peritoneal macrophages at a concentration of $4x10^{-7}M$. It is also able to cross the blood-brain barrier and appears specific for prolyl endopeptidase, because other cysteine proteases were inhibited only at concentrations many orders of magnitude greater.

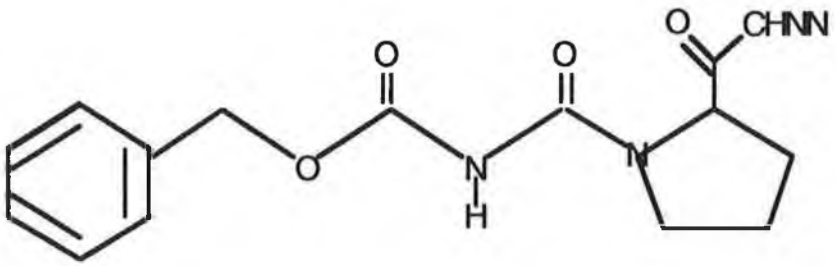
Fig 1.9 Z-Ala-Ala-Pro-CHN_2 Structure



A similar approach was taken by Knisatschek and Bauer (1986) who synthesised Z-Gly-ProCHN_2. Exposure of the bovine brain enzyme to $2.8 \times 10^{-7}M$ inhibitor for 5 minutes resulted in total

inactivation. This compound was assessed by demonstrating that at a concentration of 6 x 10^{-7}M, Z-Gly-Pro-CHN_2 did not inhibit trypsin, PAP I or dipeptidyl peptidase IV.

Fig. 1.10 Z-Gly-Pro-CHN_2 Structure



However in cell culture studies such specificity was no longer apparent (Faivre-Bauman et al., 1986). In these studies inclusion of the inhibitor not only caused a decrease in prolyl endopeptidase activity, but also a corresponding decrease in PAP I activity. 50% inhibition of both enzymes was found at an inhibitor concentration of 10^{-8}M. Apparently in purified preparations of PAP I, DTT added to the assay mixture served to protect the enzyme from inactivation. When the DTT concentration was decreased from 2 to 0.02 mM, Z-Gly-Pro-CHN_2 at a concentration of 6μM inhibited PAP I by 88.7%.

Tsuru et al. (1988) discovered that the inhibitory effect of Z-pro-prolinal can be increased by the introduction of a sulphur atom into the pyrolidine. By the replacement of pyrrolidine with thiazolidine or thiazolidine aldehyde (thioprolinal) and the conversion of an L-proline to an L-thioproline residue, an increase in the inhibitory activity resulted. They showed that Z-Thiopro-thiazolidine and Z-L-Thiopro-L-thioprolinal showed Ki values of 0.36 and 0.01nM respectively, for prolyl endopeptidase from bovine brain, both values being significantly lower than that of Z-Pro-prolinal (Ki=3.7nM).
Yoshimoto et al. (1991) continued to examine the potential of Z-Pro-Pyrrolidine derivatives as potent prolyl endopeptidase inhibitors. They found that replacement of L-proline by the D-isomer in the inhibitor almost completely abolished the inhibitory activity. This is consistent with the stereospecificity found in these enzyme-substrate interactions, confirming that the inhibitor binds to the substrate binding region of the enzyme.

Bakker et al. (1990) studied the inhibitory characteristics of two novel Z-pro-prolinal derivatives, Z-Cyclohexyl-Prolinal and Z-Indolinyl-Prolinal, *in vitro* and *in vivo*. These compounds were shown to be slow-tight binding inhibitors of prolyl endopeptidase (like the naturally occurring inhibitor discovered by Yoshimoto et al.1982) and were shown to cross the blood-brain barrier *in vivo*. The half time for the recovery of enzyme activity *in vivo* was found to be 3 to 4 hours as opposed to a half

time of in excess of 5 hours for Z-pro-prolinal. This feature is important in the determination of drug efficiency, as compounds with excellent *in vitro* potency may often be poor inhibitors *in vivo*.

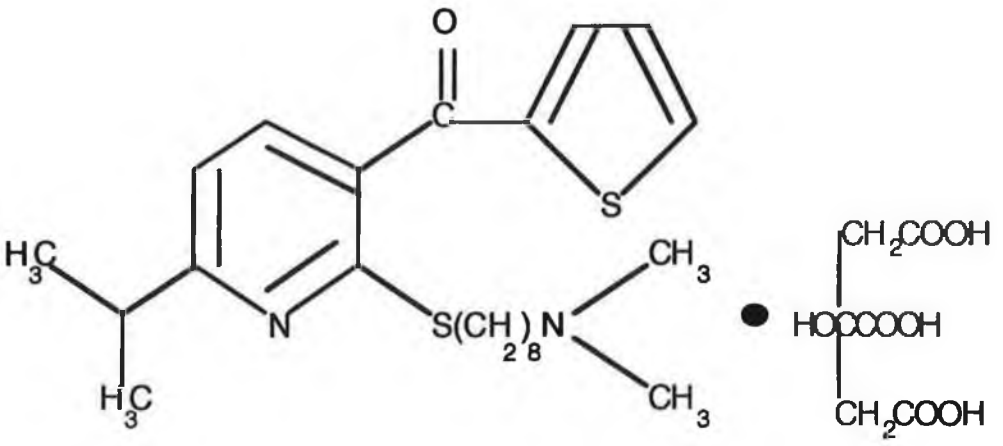
The inhibitors were also used to elucidate the kinetics of inactivation and the sites of modification. Using active site-directed choromethanes, Stone et al. (1991), showed that Ac-Ala-Ala-Pro-CH_2Cl was a competitive inhibitor of prolyl endopeptidase. The kinetic mechanism was shown to involve the formation of an initial complex between the enzyme and the chloromethane, followed by an inactivation step. The substrate was shown to compete for the formation of the initial complex, indicating that binding at the active site was a prerequisite for inactivation. After reaction of the enzyme with a radiolabelled Ac-Ala-Ala-Pro-CH_2Cl, it was possible to isolate 5 labelled peptides. Four of these peptides contained a cysteine residue as the site of modification, whereas the fifth peptide contained no cysteine and a histidine residue was identified as the site of modification. This residue (His-680) may be the active site of prolyl endopeptidase.

A non-peptide prolyl endopeptidase inhibitor was synthesised in the laboratories of Nakajima et al. (1992). This compound, Y-29794, was found to be a highly specific prolyl endopeptidase inhibitor, ie the compound was completely inactive to common serine and thiol proteases, (DAP IV, trypsin, α-chymotrypsin, elastase, leucine aminopeptidase, papain and cathepsin B) at concentrations up to 100μM. The inhibitory potency of Y-29794 to regional prolyl endopeptidase activity was unchanged in all brain regions, suggesting broad distribution of the compound in the brain. But several synthetic and naturally occurring inhibitors of prolyl endopeptidase have been described - why the need to find another? Some of the inhibitors were not as potent as Y-29794 (Nakajima et al., 1983; Yoshimoto et al., 1987) and others irreversibly interact with the enzyme (Wilk and Orlowski, 1983). Prolyl endopeptidase recently moved into the focus of interest when it was reported that the enzyme has some important role in memory process, based on the findings that nootropic aniracetam and several related pyrrolidine derivatives with anti-amnesic properties inhibit prolyl endopeptidase *in vitro* (Nakajima et al., 1983). Although known inhibitors of prolyl endopeptidase showed anti-amnesic properties in animal models (Yoshimoto et al., 1987), their bioavailability and duration of action would be limited by their peptido-mimetric structures. In contrast, the non-peptide inhibitor,Y-29794 can pass through the blood-brain barrier to exhibit long duration of action. Therefore Y-29794 is advantageous over some of the known inhibitors in potency, reversibility, oral bioavailability, brain penetrability and duration of action.

In brains of patients with Alzheimer's disease, it is well known that the cerebral ACh content decreases and the cerebral function suffers severe damage. The stimulatory effect of Y-29794 on thyroliberin-induced ACh release suggests that this compound has some positive effect on the metabolism of reduced ACh functions in the CNS. Ishiura et al. (1990) claimed that prolyl endopeptidase is a possible candidate engaged in the generation of brain ß-amyloid. The protective activity of substance P involved in the neurodegenerative effect of ß-amyloid was demonstrated *in vivo* (Kowall et al., 1991). Since substance P is known to be susceptible to prolyl

endopeptidase (Blumberg et al., 1980; Kato et al., 1980), it is expected that Y-29794 might have neuroprotective activity by inhibiting substance P degradation to elevate cerebral substance P content. If these theories are correct, use of Y-29794 alone or in combination with neuropeptides would be an effective therapy that might halt or slow the progression of the disease.

Fig. 1.11 Y-29794 Structure (Y-29794 = 2-(8-dimethylaminooctylthio-6-isopropyl-3-pyridyl 2-thienyl ketone citrate)



1.6.5.5. Prolyl endopeptidase actions on neuropeptides

Studies on the physiological role of prolyl endopeptidase have lagged behind studies on its biochemical properties. Considerable attention has been paid to its role in thyroliberin degradation (Taylor and Dixon, 1976; Orlowski et al., 1979; Tate, 1981; Knisatschek and Bauer, 1979). In fact, Busby et al. (1982) calculated that the total catalytic activity of prolyl endopeptidase in brain exceeds that of pyroglutamate aminopeptidase but they also found that the Km for thyroliberin of prolyl endopeptidase exceeds that of PAP for thyroliberin.

Prolyl endopeptidase removes a glycinamide moiety from LHRH (Wilk et al., 1979; McDermott et al., 1983a). However Advis et al. (1982) studied changes in prolyl endopeptidase activity in rat hypothalamus during the prepubertal period and during the first estrous cycle at puberty in an attempt to find changes correlating to LHRH content and degradation. They found that enzyme activity was essentially unaltered indicating that prolyl endopeptidase may not play a physiologically

relevant role in LHRH inactivation. McDermott et al. (1983b) showed that following the inactivation of LHRH with intact synaptosomes the major degradation product was LHRH(1-5) caused by the action of neutral endopeptidase (EC 3.4.24.11). LHRH(1-9) formed by the action of prolyl endopeptidase is the second most prominent product.

Neurotensin is cleaved at the Pro^{10}-Tyr^{11} bond by prolyl endopeptidase (Taylor and Dixon, 1980), producing biologically inactive fragments. Prolyl endopeptidase is by no means the most prominent neurotensin-degrading enzyme- neurotensin endopeptidase (EC 3.4.24.16) cleaves Pro^{10}-Tyr^{11} and has been purified from rat brain synaptic membranes and reported to be a metallopeptidase (Checler et al., 1986; Dauch et al., 1991; Millican et al., 1991). Hydrolysis of both the Pro^{10}-Tyr^{11} and the Tyr^{11}-Ile^{12} bonds of neurotensin is catalysed by endopeptidase 24.11 (Coquerel et al., 1986) and hydrolysis at the Arg^{8}-Arg^{9} bond has been attributed to endopeptidase 24.15 (EC 3.4.24.15) (Checler et al., 1985). Hernandez et al. (1984) found that the neurotensin fragment 1-10 formed by the action of prolyl endopeptidase on neurotensin exerts significant cryoprotective activity as measured by the ability to significantly prevent the development of cold-restraint stress(CRS)-induced gastric ulcers in rats after intra-cisternal injection.

The traditional pathway for the processing of angiotensin I involves angiotensin converting- enzyme which converts Ang I to Ang II . Ang II was long considered the physiologically active end point of this pathway. Recently prolyl endopeptidase has been found to be involved in the generation of Ang (1-7) from Ang I (Welches et al., 1991; Santos et al., 1988). The N-terminal heptapeptide, Ang (1-7) possess biological activity including activation of vasopressin secretion (Schiavone, 1988), stimulation of neuronal excitability within the vagal-solitary complex (Barnes et al., 1990), modulation of the baroreflex (Campagnole-Santos et al., 1989) and release of prostaglandins (Trachte et al., 1990). Because the formation of Ang (1-7) is from a pathway not dependent on ACE, prolyl endopeptidase and neutral endopeptidase 24.11 should all be defined as Ang I processing enzymes, ie those enzymes that act on Ang I to form biologically active end products.

Substance P interacts with prolyl endopeptidase with a high affinity and is specifically cleaved at the Pro^{4}-Gln^{5} bond to yield the C-terminal heptapeptide and the N-terminal tetrapeptide (Blumberg et al., 1980; Endo et al., 1988). Observations have been made which suggest that additional properties of substance P may be encoded in the N-terminal part of the substance P molecule. For example, it has been reported that substance P can increase intracellular cyclic AMP levels and neurite extension in neuroblastoma cells (Narumi and Maki, 1978), and in cultured dorsal root ganglion cells (Narumi et al., 1979) and that this property may be due to the N-terminal tetrapeptide. An additional activity of the N-terminal tetrapeptide which could be related to a possible role of substance P in neurogenic inflammation is the enhancement of the phagocytic activity of macrophages. Furthermore, studies of the inactivation of substance P and partial sequences of substance P by homogenates and membrane fractions of rat brain suggest a stabilisation toward

inactivation mechanisms of the substance P molecule by its N-terminal tetrapeptide sequence. These observations raise the possibility that prolyl endopeptidase plays a role in the action of substance P in the nervous system.

It has been reported that vasopressin may facilitate learning and memory. Since prolyl endopeptidase cleaves vasopressin *in vitro* it is possible that inhibition of this enzyme *in vivo* could lead to a beneficial increase of CNS vasopressin levels (Bakker et al., 1990). In passive learning avoidance tests using rats, pretreatment with Z-pro-prolinal prevented the induction of amnesia by scopolamine at a dose of 1μmol/animal. The anti-amnesic effect of this compound was found to be approximately parallel to the *in vitro* inhibitory activities of prolyl endopeptidase (Yoshimoto et al., 1987), suggesting that the anti-amnesic effects can be produced by the inhibition of prolyl endopeptidase.

The significance of prolyl endopeptidase measurements for clinical medicine is unclear. Kar and Pearson (1981) reported that the activity of the enzyme is elevated in muscle biopsies of patients with muscular dystrophies. The activities of other proteases are also elevated in muscular dystrophies. Prolyl endopeptidase activity was also measured in bronchopulmonary lavage of patients with pulmonary disease (Orlowski et al., 1981).
Pittaway et al. (1984) has suggested that a linkexists between abnormally low prolyl endopeptidase activity (22% of control) found in the caudate nucleus and Huntington's disease. A significant decrease in prolyl endopeptidase activity was also detected in the lateral globus pallidus and medial globus pallidus (37% and 40% of control respectively). Prolyl endopeptidase levels were found to be higher in knee joint synovial fluid membranes from patients with rheumatoid arthritis than in that from patients with osteoarthritis (Kamori et al., 1991). This activity increased in parallel with the increase in joint fluid volume. Thus prolyl endopeptidase may be related to immunological or inflammatory disturbance in the joint in rheumatoid arthritis. A significant factor in this case is that proline is one of the main amino acids in collagen.

1.6.5.6. Regulation of Prolyl Endopeptidase Activity

Little is known on the control of prolyl endopeptidase activity. Ohta et al. (1992) found that prolyl endopeptidase activity was higher in the extract of ovary than extracts from liver or brain in rat, pig and mouse. They found that prolyl endopeptidase activity was high at estrous and low at diestrus. In addition, treatment with oestrogen or progesterone resulted in an increase in the uterine prolyl endopeptidase activity in the ovariectomised mice compared with the oil-treated mice. Therefore they suggested that the levels of prolyl endopeptidase activity in the uterus and ovary are closely related to the circulating levels of ovarian steroids, oestrogen and progesterone. Since prolyl endopeptidase is known to cleave the physiologically active peptides which are related to the uterine myometrial contraction (oxytocin and bradykinin), PE may contribute to this primary function of the uterus, especially during pregnancy and parturition.

Further information on the physiological role of PE will likely derive from studies on the localisation of PE using immunocytochemical techniques and from studies on the effect *in vivo* of administration of the potent and selective inhibitor Z-pro-prolinal.

1.7. Further Degradation of the Primary Metabolites of Thyroliberin

The action of the soluble PAP on thyroliberin produces His-ProNH_2 , and this metabolite spontaneously and non-enzymatically cyclises to produce His-Pro diketopiperazine at neutral and alkaline pH. His-Pro diketopiperazine, which does not appear to be further degraded by any enzymatic mechanism, is itself reported to possess endocrine activity (Prasad et al.,1982). The conversion of thyroliberin to His-Pro diketopiperazine represents an instance of biotransformation of a peptide (Griffiths and McDermott, 1984) (see Fig 1.4).

Any enzyme that converts His-Pro-NH_2 to a metabolite other than His-Pro diketopiperazine must be considered as a regulator of His-Pro diketopiperazine formation by competing with the biotransformation process. A post proline dipeptidyl aminopeptidase (EC 3.4.14.2) was described in the soluble fraction of rat adenohypophysis (Bauer and Kleinkauf, 1980) and of guinea pig brain (Browne and O'Cuinn,1983) that was capable of converting His-ProNH_2 to His-Pro. This activity was found to be sensitive to puromycin (Ki = 42μM), and this suggests that it should be properly classed as dipeptidyl peptidase II (McDonald and Barrett, 1986), an activity normally located in lysosomes. Its location in cytoplasm studies may reflect its release from lysosomes in the presence of hypotonic buffers.

As in the case of soluble pyroglutamate aminopeptidase , the products of action of pyroglutamate aminopeptidase II on thyroliberin are pyroglutamate and His-ProNH_2. In the absence of further enzymatic activity, His-ProNH_2 cyclises to form His-Pro diketopiperazine. A post proline dipeptidyl aminopeptidase has been demonstrated in brain membrane preparations (Garat et al., 1985), and this enzyme has been shown to be located in synaptosomal membrane preparations of rat (Torres et al., 1986) and guinea-pig brain (O'Connor and O'Cuinn, 1986) . This enzyme could be inhibited by bacitracin (Garat et al., 1985) but not by puromycin and was shown to be capable of releasing aminoacylproline from a range of tripeptides containing a central proline residue (O'Connor and O'Cuinn, 1986). The bacitracin sensitivity of the synaptosomal enzyme differentiates it from the puromycin-sensitive post proline dipeptidyl aminopeptidase (dipeptidyl peptidase II). The synaptosomal membrane-bound post proline dipeptidyl aminopeptidase is probably correctly considered as a dipeptidyl peptidase IV activity (EC 3.4.14.5) and its synaptosomal membrane

location affords it the possibility of competing with the non-enzymatic cyclisation of His-ProNH$_2$.

Torres et al. (1986) showed the production of ProNH$_2$ by rat brain membrane preparations incubated with thyroliberin. They also demonstrated that production of this metabolite was dependent on the expression of pyroglutamate aminopeptidase II activity. ProNH$_2$ production was likely, therefore, to arise from His-ProNH$_2$ by the action of an imidopeptidase, as originally reported by Matsui et al. (1979). However, Torres et al. (1986) reported that levels of ProNH$_2$ production were very low and did not permit the definitive location of the imidopeptidase activity to a particular subcellular fraction.

1.8 Biotransformations

In recent years, an ever increasing number of polypeptides with both endocrine and neuronal activity have been identified in the CNS. It is likely that an important component in the mechanisms of action of these neuropeptides is the process by which they are inactivated and their effects limited. This has led to the identification of peptidases in the CNS and at other sites in the body capable of fulfilling this function (Griffiths et al., 1983). However, a further function of neuropeptide-metabolising enzymes may be to produce peptide metabolites with inherent biological activities distinct from those of the parent peptide, a process termed biotransformation (de Wied and Jolles, 1982). The biological activity of the product may either be related to the effects of the parent neuropeptide or it may be entirely independent. Although peptide degradation is the main route of biotransformation, other modifications to the parent peptide such as sulphonation and acetylation may serve a similar function. Rather than just considering the activity of an individual neuropeptide in its defined structure, it is now necessary to examine the mechanisms of inactivation and identify the metabolites formed with their actions when appraising the biological effects of the parent peptide.

The number of defined neuropeptides whose metabolites also have effects within the body is increasing rapidly. At present these include, adrenocorticotrophin (ACTH) and melanocyte-stimulating hormone (MSH), opioid peptides such as enkephalins, endorphins and dynorphin, oxytocin and vasopressin, the hypothalamic regulatory hormones (thyroliberin, somatostatin and LHRH), substance P, neurotensin and cholecystokinin(CCK).

Two main metabolites, acid thyroliberin (<Glu-His-ProOH) and the histidylproline diketopiperazine (cyclo(His-Pro)) are formed from thyroliberin by the actions of its two primary degrading enzymes PE and PAP (Griffiths et al., 1983).

Acid thyroliberin has been detected by radioimmunoassay in rat brain (Emerson et al., 1980) and human urine (Bhanandaru and Emerson, 1980), and induces 'wet-dog shaking' in rats in a similar way to thyroliberin after intracerebroventricular injection (Boschi et al., 1980) and direct application to

the periaqueductal grey region (Webster et al., 1983). 'Wet-dog shaking (WDS)' is described as a 'paradoxic shudder of the head, neck and trunk reminiscent of the purposeful movement seen in dogs' (Boschi et al., 1980). This property may reflect the relative stability of acid thyroliberin in brain tissue, since it is only slowly degraded to its constituent amino acids (Griffiths et al., 1983), and perhaps its ability to be reamidated to thyroliberin itself (Webster et al., 1983).

His-Pro diketopiperazine or Cyclo(His-Pro) has been shown to have many effects on the brain where it is present in much higher concentrations than thyroliberin and has several properties, both related and unrelated to thyroliberin (Peterkofsky et al., 1982; Prasad et al., 1982). The cyclical dipeptide has the ability to stimulate the activity of the cortical neurons directly (Stone et al., 1983), and has also been detected in blood (Mori et al., 1982a), the gastrointestinal tract (Mori et al 1982 b) and urine (Perry et al., 1965).

Table 1.10 Regional distribution of thyroliberin, PAP activity and cyclo(His-Pro) in Rat Brain (Prasad et al., 1982)

Region	Thyroliberin (fmols Peptide/ mg protein)	PAP Activity (fmols/min/mg protein)	Cyclo(His-Pro) (fmols Peptide/ mg protein)
Hypothalamus	2208.50	641.00	832.60
Cortex	78.10	431.00	601.60
Hippocampus	50.50	449.00	571.70
Striatum	126.00	432.00	500.50
Mid Brain	351.20	500.00	478.30
Cerebellum	31.50	650.00	414.90
Pons-Medulla	269.10	530.00	397.90

Table 1.10 shows the distribution of cyclo(His-Pro) in rat brain. Thyroliberin is enriched in the synaptosomal fraction. In contrast, cyclo(His-Pro) is not concentrated in this fraction. One of the criteria for neurotransmitter function is enrichment of an agent in synaptosomes. On the basis of the above data, cyclo(His-Pro) does not meet this standard, while thyroliberin does. Rat brain contains 3.2 times as much cyclo(His-Pro) as thyroliberin. There is considerable variation in the relative concentrations of thyroliberin and cyclo(His-Pro) in different brain regions. The highest concentrations are found in the pituitary and hypothalamus. This suggests that various parts of the brain differ in their capability to metabolise thyroliberin to acid thyroliberin, to form cyclo(His-Pro) from thyroliberin or to store the peptides.

A summary of the effects of thyroliberin and cyclo(His-Pro) on various biological activities is shown in

Table 1.11. The detailed mechanism by which cyclo(His-Pro) produces these activities remains unknown. Although bioactive effects of thyroliberin occur through its specific receptor binding sites in target tissue, there is no report concerning the specific binding sites of cyclo(His-Pro) in any tissues except for the adrenal gland, in which a binding affinity and capacity of cyclo(His-Pro) were observed to be low and high respectively (Battaini et al., 1983; Prasad and Edwards, 1984b). Mori et al. (1986) showed specific binding of cyclo(His-Pro) in the rat liver plasma membranes with high affinity (Kd=59nM). The specific binding of cyclo(His-Pro) in liver membranes was significantly reduced *in vivo* by injection of the dipeptide, but not thyroliberin. A number of receptor bindings of bioactive peptides and hormones have been shown to have either down-regulation or up-regulation of themselves (Roth, 1979). The results from Mori et al. (1986) showing the down-regulation of cyclo(His-Pro) binding sites indicated the existence of a receptor-like characteristic of cyclo(His-Pro) binding in the rat liver plasma membranes.

Table 1.11 Biological activities ascribed to thyroliberin and cyclo(His-Pro)

I. Thyroliberin-related activities of Cyclo(His-Pro)
   1. Thyroliberin-like activities
      a. Antagonism of ethanol narcosis
      b. Elevation of brain cGMP levels
      c. Inhibition of food intake
      d. Inhibition of cholesterol synthesis
      e. Inhibition of abstinence syndrome in opiate dependent mice
      f. Attenuation of ketamine-induced anaesthesia

   2. Thyroliberin-opposite activities
      a. Hypothermia in rats
      b. Inhibition of *in vitro* prolactin secretion

II. Thyroliberin-unrelated activity of Cyclo(His-Pro)
      a. Inhibition of dopamine synthesis

III. Cyclo(His-Pro)-unrelated activities of thyroliberin
      a. Stimulation of thyrotropin secretion
      b. Interaction with thyroliberin-receptor
      c. Behavioural effects, including piloerection, body tremor, and tail lifting
      d. Inhibition of pentabarbital-induced sleep

It has been clear for several years, that there is an ubiquitous distribution of thyroliberin, cyclo(His-Pro) and PAP in a variety of tissues and body fluids from rodents, primates, amphibians and man (Parker Jr et al., 1983; Mori et al., 1982a; Prasad et al., 1986). If all the cyclo(His-Pro) were derived from thyroliberin by the action of the PAP enzyme, one would expect a strong correlation between the levels of cyclo(His-Pro) and thyroliberin or PAP activity. However Prasad et al. (1987) found this not to be the case. They found no obvious correlation between the normal adult levels of endogenous cyclo(His-Pro) and thyroliberin or PAP in most cases (human and CSF, rat and monkey brain). However, there are two exceptions: rat gut and the maturation of rat brain and human CSF, where endogenous levels of cyclo(HIs-Pro) correlate positively with either thyroliberin or PAP (Mori et al., 1982a, Prasad et al., 1983).

Based upon the ubiquitous distribution of thyroliberin, cyclo(His-Pro) and PAP, Prasad et al. (1987) proposed a precursor/product relationship between thyroliberin and cyclo(His-Pro) (Prasad, 1984; Prasad and Edwards, 1982). The known primary sequence of the prohormone of thyroliberin has already been discussed (Section 1.5). However, depending on the extent and the site of processing, prepro thyroliberin can lead to the formation of not only thyroliberin, but also extended and reduced forms of thyroliberin. From these data , it was proposed that cyclo(His-Pro) is synthesised via multiple biochemical pathways (See Fig 1.12).
A precursor common to all three pathways (A,B and C) is His-ProNH_2, which then cyclises to cyclo(His-Pro). His-ProNH_2 could be derived from:

1. thyroliberin by the action of PAP,
2. Gln-His-ProNH_2 after cleavage of Gln by an aminopeptidase type enzyme, or
3. by the de novo synthesis from amino acid histidine, proline and glycine.

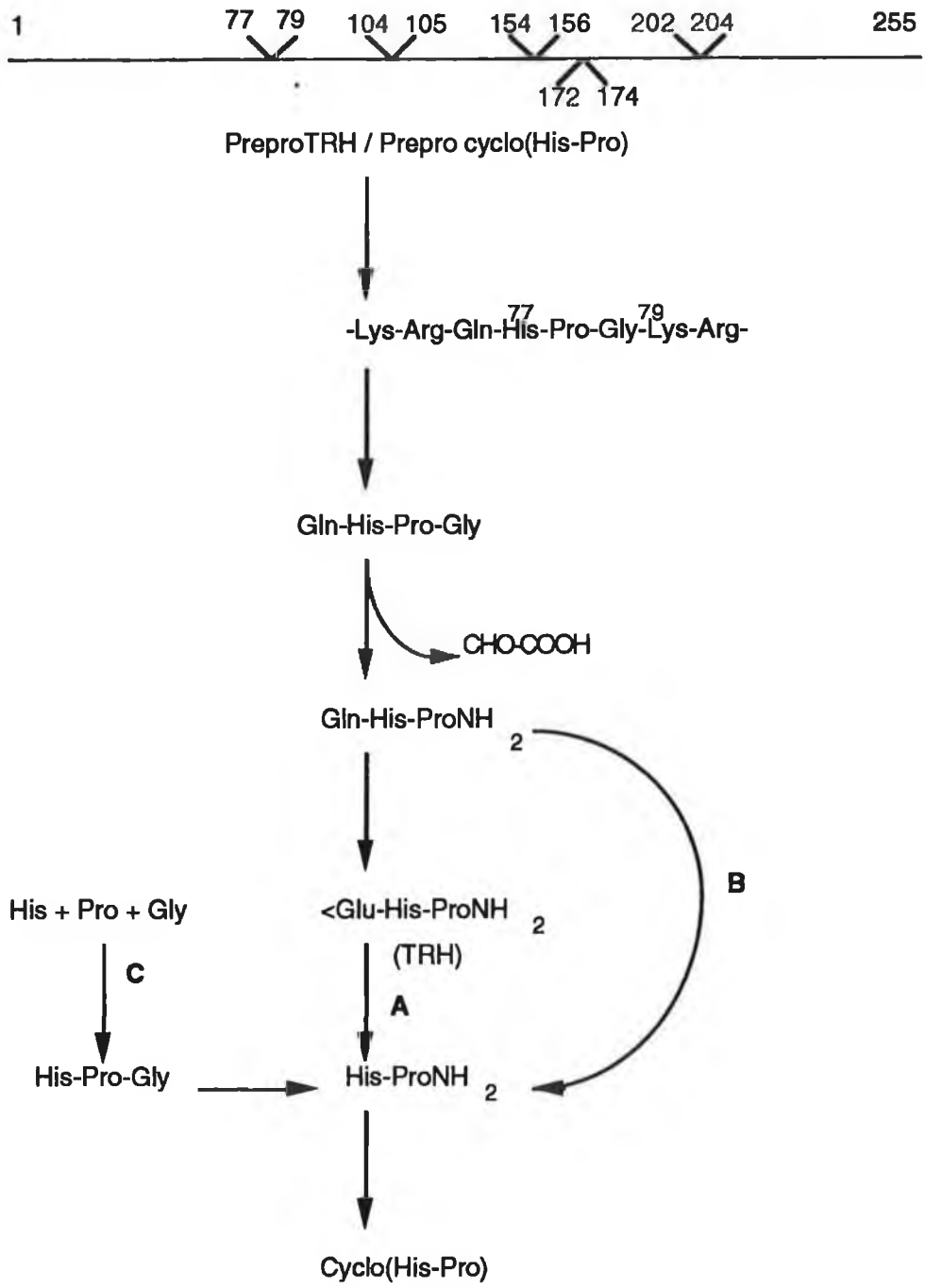
They concluded that the three pathways may not contribute equally toward the formation of cyclo(His-Pro), but may also be regulated differentially in various tissues, or during different physiologic/pharmacologic changes (Prasad , 1988).

The lack of correlation between the levels of cyclo(His-Pro) and thyroliberin or PAP was also addressed by Salers et al. (1991). In the RINm 5F cell line, they actually found thyroliberin content to be very low, barely detectable. The low thyroliberin content observed was likely to be related to a very low expression of the thyroliberin gene as was suggested by the undetectable preprothyroliberin mRNA levels in this cell line (Sevarino et al., 1988). They found that thyroliberin content did not increase following incubation with the inhibitor, Z-Gly-ProCHN_2, under conditions which inhibit PE and PAP I activities. In cultured rat hypothalamus cells, the addition of Z-Gly-ProCHN_2 induces a significant increase in thyroliberin content of these secreting cells (Faivre-Bauman et al., 1986).

The cyclo(His-Pro) and acid thyroliberin contents of RINm 5F cells are approximately 10 and 13 times higher than those of thyroliberin, respectively. These data and the lack of change in thyroliberin, cyclo(His-Pro) and acid thyroliberin after the addition of the PE (and PAP I) inhibitor, suggest that thyroliberin can be hydrolysed by other unknown peptidases, or thyroliberin may not be the sole precursor for these peptides- as suggested by Prasad et al. (1987).

Griffiths et al. (1989) suggested that the most likely peptide sequence to form cyclo(His-Pro) is -His-Pro-Gly-. Such a sequence is found in more than one copy on several proteins. The tripeptide may be released and cleaved enzymatically, then spontaneously cyclised in order to give cyclo(His-Pro). Miyashita et al. (1993) also provided evidence that cyclo(His-Pro) can emanate from a thyroliberin precursor thyroliberin-Gly, by the action of pyroglutamate aminopeptidase, and bypass the formation of thyroliberin.
So far there is no chemical evidence to suggest that acid thyroliberin may occur through the degradation of thyroliberin-unrelated peptides. There is no indication that the -Gln-His-Pro- sequence is contained in proteins other than thyroliberin and its precursor.

Fig 1.12 Three possible pathways of cyclo(His-Pro) biosynthesis

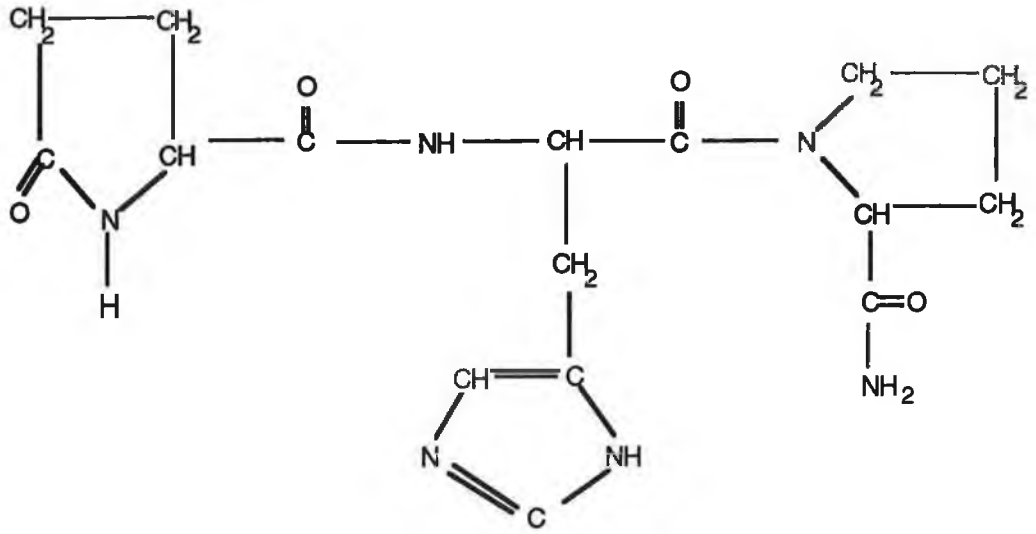


A = Cyclo(His-Pro) from TRH
B = Cyclo(His-Pro) from proTRH
C = Cyclo(His-Pro) from de novo synthesis

1.9 Thyroliberin analogues

In the experimental evaluation of thyroliberin and its therapeutic potential for certain clinical conditions, one major problem has been the tripeptide's relatively short half-life, partly resulting from rapid enzymatic degradation. Attempts have been made to synthesise thyroliberin analogues with greater stability to the major degrading enzymes, pyroglutamate aminopeptidase and prolyl endopeptidase, and hence with a more prolonged duration of action, to capitalise on the neurotransmitter/ neuromodulator effects of thyroliberin.

Fig. 1.13 Structure of thyroliberin



The thyroliberin analogue [3MeHis]thyroliberin is the most potent analogue in stimulating the release of TSH from the pituitary. However, although [3MeHis]thyroliberin has a high very affinity for central thyroliberin receptors (Ward et al., 1987), it is experimentally less active in the brain than thyroliberin. By contrast, several analogues that have a much lower affinity for thyroliberin receptors, but that are much more stable to degradation by brain peptidases show enhanced central action over thyroliberin. RX77368, the most enzymatically stable analogue, is excreted 60% unchanged and would be expected to have the greatest activity by virtue of this fact. In some tests though, RX77368 is more active than thyroliberin within the latter's half-life of inactivation; RX77368 generally varies in potency between 2 and 220 times that of thyroliberin (Metcalf, 1982).

Fig. 1.14 Structure of [3MeHis]thyroliberin

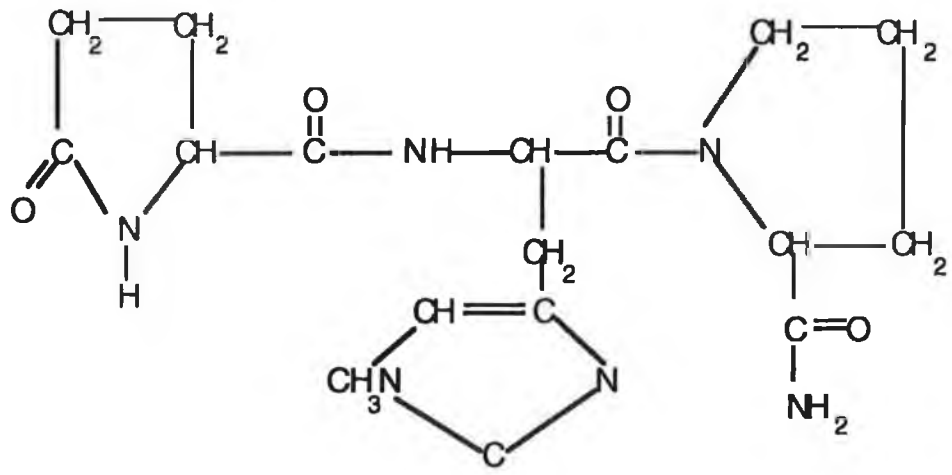
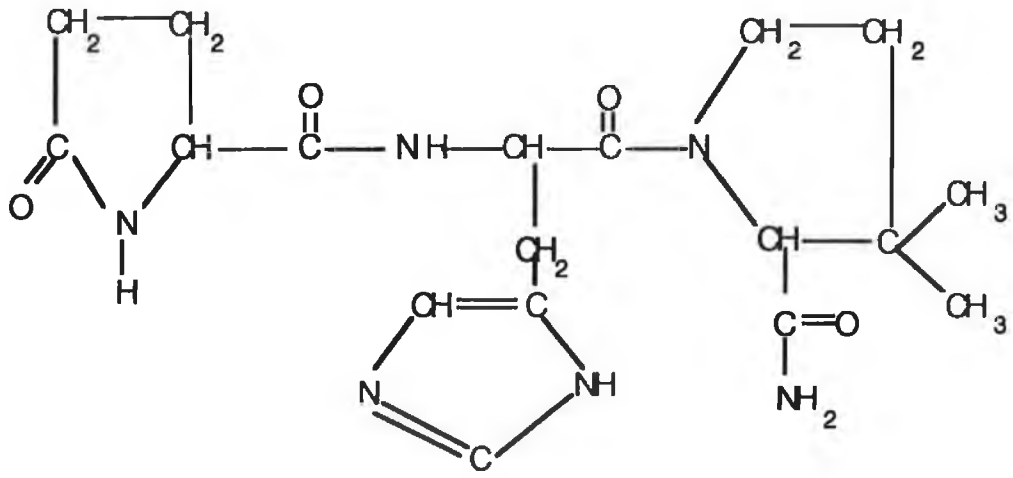


Fig. 1.15 Structure of RX77368



[3MeHis]thyroliberin bound to receptors in rodent, rabbit and dog brain and spinal cord, and in sheep, rat , bovine and dog anterior pituitary (PIT) glands, with high affinity (Sharif et al., 1991). Bungaard and Moss (1990) suggested that the problems in using thyroliberin as a potentially effective drug were two fold - firstly its rapid degradation and secondly the low lipophilicity of thyroliberin may also be a reason for the limited ability of the peptide to penetrate the blood-brain barrier. They suggested that a solution to the delivery problems was derivitization of the peptide to produce prodrugs or transport forms (Bungaard, 1986), that are markedly more lipophilic than the parent peptide and resistant toward the thyroliberin-degrading serum enzyme, but remain cleavable by chemical or nonspecific enzyme-catalysed hydrolysis to release the parent thyroliberin *in vivo*.

By forming N-alkoxycarbonyl derivatives at the imidazole moiety of thyroliberin, the great susceptibility of the pyroglutamyl peptide bond in thyroliberin to cleavage by pyroglutamate aminopeptidase is abolished. The modification is readily bioreversible, as the parent thyroliberin is formed quantitatively from the derivatives by spontaneous hydrolysis or by enzymatic hydrolysis effected by plasma enzymes not attacking the pyroglutamyl peptide bond. The increased lipophilicity of the derivatives relative to the thyroliberin may render it feasible to deliver thyroliberin to the brain in the form of the prodrugs. Further, the N-alkoxycarbonyl moieties are hydrolysed to innocuous products (carbon dioxide and alcohols).

1.10 Age dependent changes in thyroliberin-degrading activity

The role of thyroliberin regulation of thyroid status remains a major area of investigation and because in most mammals thyroid function matures rapidly in the early days of life, it is of interest to study the thyroliberin-degrading activity during this period. Unique differences in thyroliberin-physiology occur during the perinatal period in the developing animal. At birth the rat pancreas contains higher concentrations of thyroliberin than neonatal or adult hypothalamus; pancreatic levels fall throughout the first weeks of life as hypothalamic concentrations increase (Aratan-Spire et al., 1983; Salers et al., 1992). Extra-hypothalamic brain thyroliberin is lower than levels in gut tissues during the first week of life, and brain levels gradually increase to adult values by the end of the fourth postnatal week. The developmental patterns of the specific activity of the two soluble enzymes involved in the primary degradation of thyroliberin *in vitro*, PAP I and prolyl endopeptidase, in mouse hypothalamus and cerebral hemispheres of rat brain (Prasad et al., 1983), show that in whole brain PE activity increased throughout the first two weeks of life, with levels decreasing after this time, while values for PAP I were highest on the first day of life in rats, and decreased over the next two weeks to adult levels (Fuse et al., 1990). This developmental pattern is clearly different from the development of thyroliberin and most markers of differentiation and synaptogenesis. The low specificity and wide localisation of these enzymes, their low proportion in synaptosomes and their

inability to degrade thyroliberin or LHRH in intact cells (Mendez et al., 1990), suggests a general role in cellular metabolism (Vargas et al., 1992b).

In contrast PAP II activity shows a different pattern, increasing from birth to postnatal days 8-22 depending on the brain region. It appears before or during axon terminals and synapses formation in the brain. In the hypothalamus, PAP II increased from day 16 prenatal to 8 postnatal. The ontogeny of adenohypophyseal PAP II activity correlates well with that of thyrotropin (peak serum and pituitary TSH levels between postnatal days 8 and 28) and thyroid hormones (peak serum values between postnatal days 12 and 28) (Vargas et al., 1992b). The parallelism between PAP II and T_3 or T_4 ontogenesis suggests that these hormones are involved in the development of PAP II activity in this region because of their known stimulatory action on enzyme activity in adult rats (Ponce et al., 1988).

Bauer et al. (1990) reported that PAP II is preferentially, if not exclusively, localised on prolactin (PRL) cells. Vargas et al. (1992) found that not only did PAP II activity and PRL cells appear at approximately the same time, but their developmental patterns coincide in agreement with PAP II being synthesised in lactotrophs (Bauer et al., 1990). Furthermore, the ontogenetic pattern of PAP II activity follows that of thyroliberin receptors, which peak between postnatal days 5 and 21, and that of PRL cell physiology. The appearance of PAP II activity and the PRL response to thyroliberin show a good correlation with both neurogenesis of the median eminence and the ontogeny of the hypothalamo-adenohypophyseal portal system, which does not develop until the post-natal days 4-5 (Vargas et al., 1992). Thyroliberin-like immunoreactive nerve fibres and terminals in median eminence are first detected on 0.5th day after birth; from the second day onwards, the stage where thyroliberin-like immunoreactive nerve fibres appear localised around the basal lamina of portal vessels in the external layer of the median eminence, the number and intensity of terminals and positive granules increase at least up to 20 days (Bauer et al., 1990). All these observations suggest that PAP II develops together with the hypothalamic-adenohypophyseal axis (Vargas et al ., 992).

PAP II and the serum thyroliberin-degrading PAP activity (thyroliberinase) both follow the same developmental pattern in the adenohypophysis (Scharfmann and Aratan-Spire, 1991). Thyroliberinase is absent from the plasma at birth and during neonatal periods of both rats and humans (Aratan-Spire and Czernichow, 1980; Neary et al., 1978).
It has been shown that the development of PAP II activity in the brain is anticipating or associated with synaptogenesis (Vargas et al., 1992). Early appearance of PAP II coincides with rapid expression of PAP II in foetal brain cells in culture (Cruz et al., 1991). This suggests that regional distribution of PAP II attained in adult animals is a consequence of events occurring during postnatal

development. In the adenohypophysis, PAP II activity is closely related to the development of hypophysiotropic thyroliberin neurons as well as that of its target cells (Vargas et al., 1992). These data further contribute to the hypothesis that PAP II is a specific peptidase responsible for thyroliberin extracellular inactivation in the brain and adenohypophysis.

2. Materials and Methods

2.1 Materials

Sigma Chemical Company

Poole, Dorset, England.

N-Acetylimidazole, Acid TRH (TRH-OH), Ammonium Sulphate, Ammonium Persulphate, Angiotensin II, 7-Amino-4-MethylCoumarin (MCA), Blue Dextran, Bombesin, Bradykinin, Bromophenol Blue, BSA, Benzamidine, Bacitracin, Coomassie Brilliant Blue, Cyclo(His-Pro), Dithiothreitol, Dimethylformamide, EDTA, N-Ethylmaleimide, Glycine, <Glu-His-Gly, Gly-ProMCA, <Glu-MCA, 8-Hydroxyquinoline, DL-Histidine, p-Hydroxymercuribenzoic Acid (PCMB), HEPES, Iodoacetamide, Iodoacetate, p-Iodonitrotetrazolium Violet (INT), Lauryl Sulphate, Lithium Chloride,Luteinising Hormone-Releasing Hormone (LHRH), Molecular Weight Markers (Gel Filtration), Molecular Weight Markers (SDS-PolyAcrylamide Gel Electrophoresis), MES, N,N'-Methylene-Bis-Acrylamide, Neurotensin, Ninhydrin, ß-Nicotinamide Adenine Dinucleotide (ß-NADH), Potassium Phosphate (monobasic), Potassium Phosphate (dibasic), DL-Pyroglutamic Acid, DL-Proline, 1,10-o-Phenanthroline, Phenylmethylsulfonyl Fluoride (PMSF), Puromycin, Pyruvic Acid, Papain, Sodium Chloride, Sucrose, Sodium Acetate, Sulphanilic Acid, Substance P, Triton X-100, Triton X-114, Trizma Base (Tris), TEMED, Thyrotropin Releasing Hormone (TRH), Trypsin

BDH Chemicals Ltd.

Poole, Dorset, England

Acrylamide, Ammonia, Acetic Acid, Acetone, Biuret Reagent, Butan-1-ol, Cadmium Acetate, Cobalt(II) Chloride 6-Hydrate, Chloroform, Citric Acid, Calcium Chloride, Dimethylsulphoxide(DMSO), Glycerol, Glycine, Hydrochloric Acid, Iron(III)Chloride 6-Hydrate, Magnesium Chloride, Methanol, Sodium Hydrogen Carbonate, Sodium Hydroxide, Sulphuric Acid, Sodium Succinate, Sodium Tartrate, Trichloroacetic Acid, Zinc Chloride, Zinc Sulphate

Bachem Feinchemikalein AG.

Bubendorf, Switzerland

<Glu-His-ProMCA, <Glu-(Me)His-ProNH_2, Z-Gly-ProMCA ,

Merck Chemical Co.

Frankfurt, Germany

Iron(ii) Sulphate (Heptahydrate), Manganese Sulphate, Polyethyleneglycol 6000,Sodium Carbonate (Anhydrous), di-Sodiumhydrogen Phosphate, TLC Plates (Silica Gel 60)

Riedal-de-Haen AG.

Germany

Ammonium Molybdate, Ferrous Sulphate, Potassium Chloride, Silver Nitrate

Aldrich Chemical Co.

Poole, Dorset, England

Ammonium Sulfamate, Sodium Nitrite, Trifluoracetic Acid (HPLC grade).

Pierce Chemical Company

Illinois, USA

BCA reagent

Romil Chemicals Ltd.

Loughborough, Leicestershire, England.

Acetonitrile (HPLC grade), Ethyl Acetate

Calbiochem-Novabiochem (UK) Ltd.

Nottingham, England

<Glu-ProNH_2

Penninsula Laboratories

Belmont, Ca., USA

<Glu-His

New England Nuclear

Dreiechenhain, Germany

[*Pro*-2,3,4,5,-^{3}H]thyroliberin

2.2. Determination of enzyme activities

2.2.1. Thyroliberin specific Pyroglutamate Aminopeptidase activity

The thyroliberinase activity of the pyrogiutamate aminopeptidase (PAP) was determined by a modification of the method of Bauer and Kleinkauf (1980). [Pro-^{3}H] thyroliberin was diluted with unlabelled thyroliberin (0.42mM) to give a specific radioactivity of 0.125 Ci/mmol. 10µL aliquots of each sample to be assayed were added to 10µL of diluted [Pro-^{3}H] thyroliberin (0.5µCi/4mmol) and 10µL of 100mM potassium phosphate buffer pH7.4. Following incubation at 37°C for 30 min the reaction was terminated by the addition of 10µL methanol. 10µL aliquots were spotted onto prewashed cellulose phosphate paper which was then developed in 1.0M acetic acid. Under these conditions the weakly basic thyroliberin moves up the paper while the highly basic His-ProNH$_2$ remains at the start segment. The start segment was then cut out and placed in scintillation vials to which were added 1mL of 2M ammonia and 10 min later, 10mL of toluene-based scintillation fluor containing: 66.7%(v/v) Toluene, 33.3%(v/v) TritonX-100, 0.05%(w/v) PPO and 0.01%(w/v) POPOP.
The scintillation vials were counted in a Packard Tri Carb Liquid Scintillation Spectrometer (model 3330) using the tritium [^{3}H] channel. Having calibrated the instrument and determined its percentage efficiency with the above scintillation cocktail (Table 2.1) the conversion of <Glu-His-[^{3}H]-Pro-NH$_2$ to His-[^{3}H]-ProNH$_2$ by the action of the pyroglutamate aminopeptidase could be monitored quantitatively.

Table 2.1 Calculation of the % efficiency of Packard Tri Carb liquid scintillation spectrometer (model 3330) using the scintillation cocktail given in section 2.2.1.

[Pro-2.3.4.5-^{3}H(N)] Thyroliberin (Stock)

Specific Activity	100 Ci/nmol (100µCi/nmol)
Concentration	10µM (10 nmol/mL)
Volume	0.25µL
Amount	2.5nmol (250µCi)

Method

Take 50µL Stock (50µCi) and dilute 1 in 20 with 0.42mM unlabelled thyroliberin - 50µCi/400nmol/1000µL (stock solution) .
Take 10µL (0.5µCi/4nmol) of that solution .
Read in Packard Tri Carb Liquid Scintillation spectrometer (model 3330)

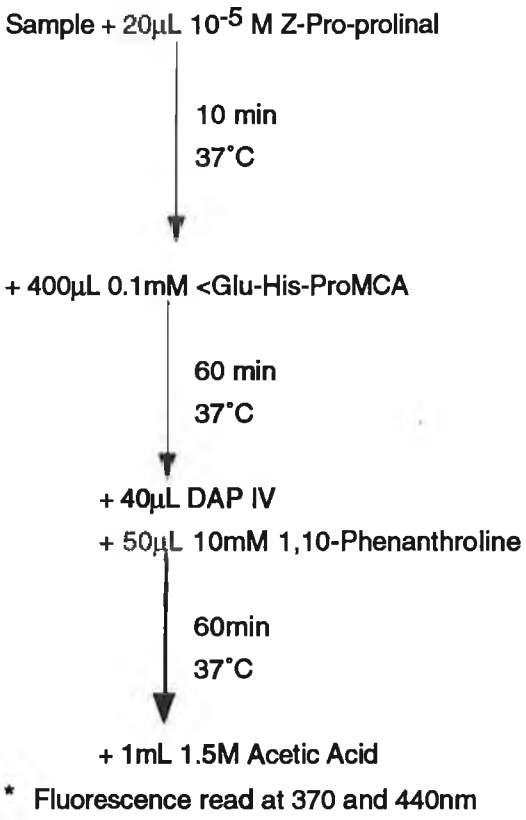
0.5µCi	1,110,000	D.P.M.
Reading	444,000	C.P.M.
% Efficiency	40%	

2.2.2. Development of two new assays for particulate Pyroglutamate Aminopeptidase activities

2.2.2.1. A fluorimetric coupled enzyme assay for synaptosomal PAP

Synaptosomal PAP activity was detected using a coupled enzyme assay developed in our laboratory based on a modification of the spectrophotometric assay of Friedman and Wilk (1986) (see Fig 2.1). 100µL of sample was incubated with 20µL of 10^{-5} M Z-Pro-prolinal, a specific prolyl endopeptidase inhibitor, for 10 min at 37°C before the addition of 400µL of 0.1mM <Glu-His-ProMCA in 100mM potassium phosphate buffer, pH 7.4. The assay mixture was incubated at 37°C for 60 min, after which time 40µL of DAP IV and 50µL of 10mM 1,10-phenanthroline was added. The 1,10-phenanthroline was added to terminate the synaptosomal PAP activity and the DAP IV cleaves the His-ProMCA to give Cyclo(His-Pro). The free 7-amino-4-methyl-coumarin (MCA) was then measured fluorimetrically in a Perkin Elmer fluorescence spectrophotometer (model LS-50) using excitation and emission wavelengths of 370 and 440nm respectively and slit widths of 10 and 5nm respectively. Blanks were prepared by substituting 100µL of buffer for sample. The fluorescence was converted into nanomoles of 7-amino-4-methyl coumarin by using a standard curve prepared with 7-amino-4-methyl coumarin under corresponding control conditions.

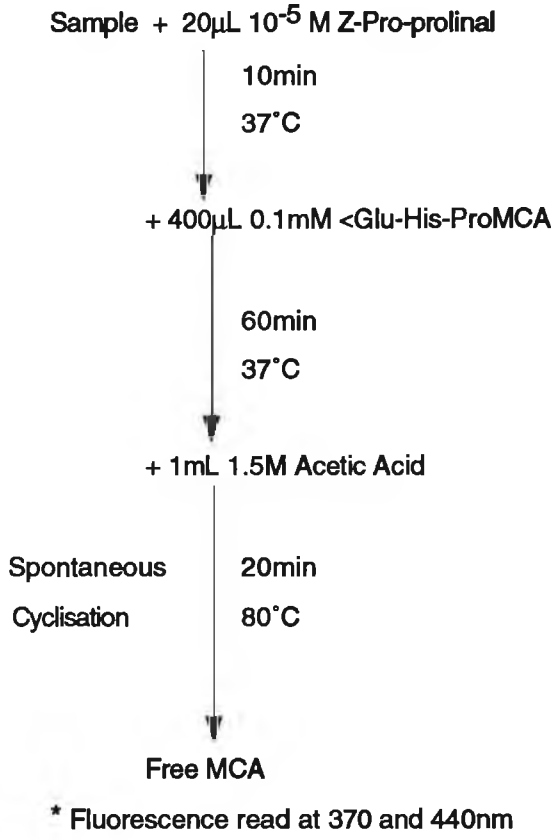
Fig 2.1 Coupled Enzyme Assay for Synaptosomal PAP Activity



2.2.2.2. Spontaneous cyclisation assay for particulate Pyroglutamate Aminopeptidase activity

Synaptosomal Pyroglutamate Aminopeptidase activity was also determined by a modification of the coupled enzyme assay described in section 2.2.2.1 (see Fig 2.2). 100µL of test solution were preincubated with 20µL of 10^{-5} M Z-Pro-prolinal for 10 minutes at 37° C. This solution was then incubated with 400µL of 0.1mM <Glu-His-ProMCA (in 100mM potassium phosphate buffer, pH7.4) at 37° C for 60 minutes. The reaction was terminated by the addition of 1mL of 1.5 M acetic acid. The mixture was then heated to 80°C for 20 min to encourage the cyclisation of the His-ProMCA to Cyclo(His-Pro) and the release of free MCA. The MCA was quantitated as described in section 2.2.2.1.

Fig 2.2 Spontaneous Cyclisation Assay for Synaptosomal PAP Activity



2.2.3. Prolyl Endopeptidase (PE) activity

The presence of prolyl endopeptidase was detected using a specific prolyl endopeptidase substrate, Z-Gly-ProMCA. The substrate was prepared by dissolution in 2% (v/v) DMSO and making up to a final concentration of 0.1mM in 100mM potassium phosphate, pH 7.4 which was warmed to 37° C. 100µL of sample was incubated with 400µL of Z-Gly-ProMCA at 37° C for 60

minutes. The reaction was terminated by the addition of 1mL of 1.5 M acetic acid. The enzymatic release of MCA was measured fluorimetrically by reference to a standard curve (excitation and emission wavelengths at 370nm, 440nm respectively). Suitable blanks were prepared in which the buffer was added to the substrate instead of enzyme.

2.2.4. Soluble Pyroglutamate Aminopeptidase activity

<Glu-MCA was used as a substrate for the detection of soluble pyroglutamate aminopeptidase (PAP I) as described by Fujiwara and Tsuru(1978) as modified by Browne and O'Cuinn (1983). 0.1mM <Glu-MCA was prepared in 100mM potassium phosphate buffer, pH7.4 with 1% (v/v) DMSO. 100µL of sample was added to 400µL of substrate and incubated at 37° C for 60 minutes. The reaction was terminated by the addition of 1mL of 1.5M acetic acid and the resulting fluorescence was measured at excitation and emission wavelengths of 370 and 440nm respectively. The enzymatic release of MCA was measured with reference to a standard curve (0-50µM) range. Suitable blanks were prepared where the enzyme sample was replaced by buffer.
Soluble pyroglutamate aminopeptidase activity could also be detected using <Glu-His-ProMCA as a substrate as described in section 2.2.2.1.

2.2.5. Assessment of the subcellular fractions from bovine brain

To localise the thyroliberin-degrading activities in bovine brain, various enzymes were assayed as markers for specific subcellular components.

2.2.5.1. Assay for Succinate Dehydrogenase Activity

Succinate Dehydrogenase was used as a marker enzyme for mitochondria. Succinate dehydrogenase activity was measured according to the method of Pennington (1961) as modified by Porteous and Clark (1965), in which iodonitrotetrazolium was used as an artificial electron acceptor.

The assay mixture consisted of :
0.5mL of 50mm potassium phosphate buffer, pH7.4,
50mM sodium succinate,
2mM EDTA,
2mM sucrose, and
0.1%(v/v) Iodonitrotetrazolium

0.5mL of assay mixture were pre-equilibrated at 37°C for 10 min. 0.5mL of sample was then added and the mixture was incubated for a further 10min at 37°C. The reaction was terminated by the addition of 1mL of 10%(w/v) TCA. 4mL of ethyl acetate was added and the mixture was well shaken. The sample was centrifuged for 10min at 2,000rpm in a Hereaus Christ bench-top

centrifuge to separate the two phases. The formazan-containing layer was removed and its absorbance was read at 490nm.

2.2.5.2. Assay for 5'-Ribonucleotidase activity

5'-Ribonucleotidase activity was used as a marker assay for synaptosomes and was assayed according to the method of Michell and Hawthorne (1965), and the released phosphate was determined by the method of King (1932).

Incubation mixture :
100mM KCl
10mM $MgCl_2$
50mM Tris/HCl, pH7.4
5mM AMP
10mM Sodium Tartrate

Phosphate Colour Reagent:

1g of ammonium molybdate in 50mL of 2.3N H_2SO_4
25mL of d.H_2O
4g Ferrous sulphate
The total volume was brought up to 100mL with d.H_2O

1mL of each sample was incubated with 1mL of incubation mixture for 30 min at 37°C. 1mL of phosphate colour reagent was added and the incubation was allowed to proceed at 37°C for a further 30min. The tubes were then spun in a bench-top centrifuge at 2000rpm for 5 min. The absorbance of each sample was read at 660nm. A standard curve of phosphate in the range of 0.2-1µmoles Pi/mL (mM KH_2PO_4) was prepared each time the assay was performed.

2.2.5.3. Assay for Lactate Dehydrogenase Activity

Lactate dehydrogenase activity was used as an enzyme marker for cytoplasm and was measured according to the method of Bergmeyer and Brent (1974).

Incubation Mixture:
100mL of 50mM potassium phosphate, pH 7.5
0.63mM sodium pyruvate

Reduced NADH:
14mg of NADH-Na_2 (working concentration = 11.3mM ßNADH)

15mg $NaHCO_3$

1.5mL d.H_2O

100µL of sample was added to 3mL of incubation mixture and incubated for 10min at 37°C. 50µL of NADH solution was added. The solution was mixed quickly and the change in absorbance was read every 10 seconds for 3 min at 340nm.

2.3. Protein determination

Two methods were employed to determine the protein concentrations as no single method was suitable for all the samples, some of which contained interfering substances such as detergents and sulphydryl reagents.

2.3.1. Biuret Protein Assay

The Biuret assay was used for the protein determination of the crude homogenate, the supernatant fraction (S_1), the particulate fraction (P_2) and the Triton X-100 solubilised sample (S_T). 50µL of sample was incubated with 200µL of Biuret reagent at room temperature for 30 minutes, and then the O.D. was read at 560nm. A standard curve using BSA (0-10mg/mL) was constructed each time the assay was performed. The Biuret assay is most accurate in the range 0-10mg/mL of protein.
Sulphydryl agents such as DTT cause interference with the Biuret assay and so the BCA assay was used if DTT was present in the sample.

2.3.2. BCA Protein Assay

A modification of the method for protein determination using Bicinchoninic Acid (BCA), (Smith et al., 1985), which may be used in the presence of thiol reagents (Hill and Straka, 1988) was used to determine protein concentration in the post-gel filtration samples. This assay is particularly useful for low protein concentration samples as is it most sensitive in the 0-2mg/mL range. 10µL of each sample was added to the appropriate microtitre-plate wells. 200µL of the BCA working reagent was added to each well and the plate was shaken well on a microtitre plate shaker for 10 seconds. The plate was then incubated at 37°C for 30 minutes. The plates were allowed to cool to room temperature before measuring the absorbance of each well at 560 nm. A standard curve using BSA (0-2mg/mL) was constructed each time the assay was performed.

2.4. Isolation of soluble rat liver PAP and Prolyl Endopeptidase activities

Rat liver was used as a source of soluble PAP and prolyl endopeptidase which were in turn used

as positive controls in the development of the assay systems, as there were no reliable pyroglutamate aminopeptidase or prolyl endopeptidase enzyme activities commercially available.

2.4.1. Preparation of a Sephadex G-100 column

4g dry weight of Pharmacia Sephadex G-100 was added to 60 mLs of 100mM potassium phosphate buffer, pH 7.4 and was allowed to swell at 90° C for 3 hours. It was then left at room temperature overnight. By swelling the gel at such high temperatures the gel was degassed. The swollen gel was stored at 4°C prior to pouring into the 1.5cm x 20cm Bio-Rad column (volume=35.5mL). The gel was poured in one step using a glass rod to ensure even sedimentation. The gel was allowed to settle under gravity before applying buffer to the column at a flowrate of 0.5mL/min (a higher flowrate than at which the column was to be run). The column was equilibrated with 3 column volumes of 100 mM potassium phosphate buffer, pH 7.4 with 0.15M KCl, 1mM EDTA and 1mM DTT. 1mL of a 2mg/mL solution of Blue Dextran was applied to the top of the column to calculate the void volume. Buffer was applied at a flowrate of 0.16mL/min and 0.5mL fractions were collected. The absorbance of each fraction was measured at 620nm and the void volume was calculated.

2.4.2. Preparation of the rat liver

7g of rat liver were homogenised on ice in 20 mL of 0.32M sucrose in 100mM potassium phosphate buffer, pH7.4 with 1mM DTT and 1mM EDTA for 15 seconds using a Sorvall Omni-Mixer. The homogenate was centrifuged for 60min at 15,000 rpm and the supernatant was decanted and stored in 1mL aliquots at -20°C.

2.4.3. Partial purification of the soluble PAP and Prolyl Endopeptidase activities from rat liver

A 1mL aliquot of rat liver supernatant was applied to the drained bed of the Sephadex G-100 column and was allowed to drain into the column before applying buffer to the top of the of the column. The running buffer was 100mM potassium phosphate buffer, pH 7.4 with 0.15M KCl, 1mM DTT and 1mM EDTA which was degassed and vacuum-filtered before applying to the column. The column was run at a flowrate of 0.16mL/min at 4°C and 0.5mL fractions were collected. Each fraction was assayed for enzymatic activity using the PAP I substrate <Glu-MCA and the specific prolyl endopeptidase substrate Z-Gly-ProMCA. The soluble rat liver PAP and prolyl endopeptidase activities were used as positive controls in the development of the spontaneous cyclisation assay for the measurement of particulate pyroglutamate aminopeptidase activity (section 2.2.2.2.).

2.5. Thyroliberin-degrading enzymes in different locations of bovine brain

2.5.1 Demonstration of two PAP activities in different subcellular fractions of bovine brain.

25g of freshly isolated bovine brain were homogenised in 100mL of ice cold 0.32M sucrose in 100mM potassium phosphate, pH 7.4 using a Sorvall Omni-Mixer. The tissue was disrupted by two 5-second pulses, and the crude homogenate (CH) was centrifuged at 15,000rpm for 60min at 4°C in a Sorvall SS-34 rotor. The resulting supernatant was stored at 4°C while the pellet was resuspended in 50 mL of ice-cold 100mM potassium phosphate buffer,pH7.4. Both the supernatant (S_1) and the resuspended pellet (P_1) were assayed for synaptosomal PAP activity, (section 2.2.2.1.) both in the presence and the absence of DTT and EDTA.

2.5.2. Demonstration of two prolyl endopeptidase activities in different subcellular fractions of bovine brain

The brain was treated exactly as above and the fractions, S_1 and P_1 were assayed for prolyl endopeptidase activity in the presence and absence of DTT and EDTA (section 2.2.3.).

2.6 Osmotic shock of the particulate fraction

The membrane fraction was subjected to a 0.5M salt wash to remove any loosely and non-specifically bound soluble enzyme activity from the fraction. The fraction was then resuspended in distilled water with a gentle hand rehomogenisation step. This was in order to lyse any entrapped vesicles formed by membranes following the initial homogenisation step. Following resuspension, the membrane fraction was then centrifuged at 15,000 rpm for 60min at 4°C. Both the supernatant and the particulate fractions were assayed for particulate PAP and PE activities (section 2.2.2.1. and 2.2.3.).

2.7. Salt washing of the particulate fraction

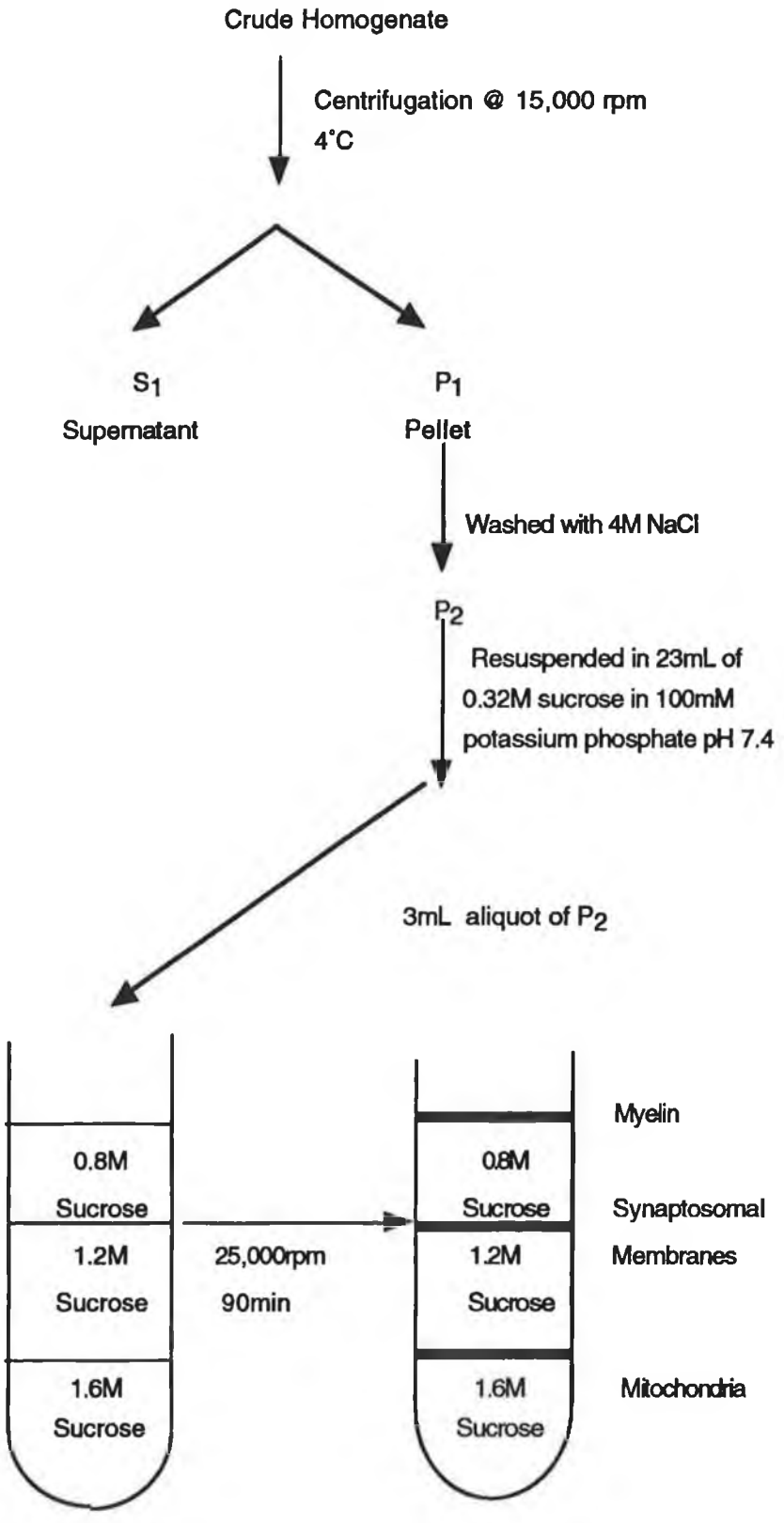
The osmotically-shocked particulate fraction, prepared as in section 2.6., was subjected to a range of salt washing steps to remove any loosely bound proteins from the membranes and to ensure that the enzyme activities were associated with the membrane fraction.
The pellet was resuspended in a range of salt concentrations (0-4M NaCl in 100mM potassium phosphate buffer, pH 7.4 at 4°C) and the mixture was gently inverted for 10 minutes. Following this washing step, the preparation was centrifuged at 15,000 r.p.m. for 60 minutes at 4°C. Half the samples were subjected to a second salt washing step of either 2 or 4M NaCl, and following centrifugation were resuspended in 100mM potassium phosphate buffer, pH 7.4, at 4°C. Each

sample was assayed for pyroglutamate aminopeptidase and prolyl endopeptidase activity as described in sections 2.2.2.1. and 2.2.3. respectively.

2.8. Subcellular localisation of the particulate PAP and prolyl endopeptidase activities

25g of freshly isolated bovine brain were homogenised and washed as described above. The subcellular fractionation procedures were based on the methods of Marchbanks (1967) and of Whittaker and Barker (1972). Aliquots of each fraction generated were removed and stored at -20°C. The crude homogenate was centrifuged at 15,000 rpm at 4°C for 60 min in a Sorvall SS-34 rotor. The resulting supernatant (S_1) was removed and the pellet was salt-washed, and centrifuged again. The pellet was then resuspended in 23 mL of 0.32M sucrose in 100mM potassium phosphate, pH 7.4 (P_2). 3mL aliquots of P_2 were placed on discontinuous sucrose gradients consisting of 9mL of 1.6m sucrose, 10mL of 1.2M sucrose and 9mL of 0.8M sucrose. Following ultracentrifugation at 25,000rpm for 90min, 3 bands of material were observed in the gradient- one on top of the 0.8M sucrose, one in the 1.2M sucrose and one in the 1.6M sucrose. Each fraction was diluted up to 60 mL with 100mM potassium phosphate, pH 7.4 buffer , centrifuged at 25,000rpm for 90min and resuspended in 0.32M sucrose in 100mM potassium phosphate, pH7.4 to give fractions A, B, and C respectively. Each fraction was stored for enzyme marker studies as described in section 2.2.7.

Fig 2.3 Subcellular Localisation of the Particulate PAP and PE Activities



2.9. Phase-partitioning study on the synaptosomal membrane-bound PAP and Prolyl Endopeptidase activities

Using the method of Bordier (1981), it was possible to determine the location of the membrane-bound enzymes - whether they were integral or peripheral membrane proteins.
2%(v/v) of Triton X-114 was added to 2.5mg/mL of membrane protein in 10mM potassium phosphate buffer, pH7.4, with 150mM NaCl,1 mM EDTA and 1mM DTT. The mixture was shaken gently for 1 hour on ice. The sample was centrifuged at 25,000rpm for 60min at 4°C (100,000g) in a Beckman UltraCentrifuge, using an SW28 rotor. The resulting supernatant was decanted and was layered over a 6%(w/v) sucrose solution (prepared in 10mM potassium phosphate buffer, pH7.4 with 1mM DTT and 1mM EDTA). The samples were incubated at 30°C for 15min and were then centrifuged at 1000rpm at 25°C for a further 10 min in a Sorvall centrifuge using an SS-34 rotor. Both the aqueous and the oily hydrophobic layers were assayed for both synaptosomal PAP and prolyl endopeptidase activity using <Glu-His-ProMCA and Z-Gly-ProMCA as substrates respectively (sections 2.2.2.1. and 2.2.3.). An intramembrane enzyme activity is found in the oily hydrophobic layer at the bottom, while a peripheral membrane enzyme activity is found predominately in the upper aqueous layer.

2.10 Release of the particulate thyroliberin-degrading enzymes from the synaptosomal membranes

2.10.1. Release of the thyroliberin-degrading enzymes from the synaptosomal membranes

The synaptosomal membranes were prepared as described above except the pellet was washed once in a 4M salt solution to remove any loosely bound proteins from the membranes. The fraction was centrifuged at 15,000 rpm for 60min and the resulting pellet was resuspended in 100mM potassium phosphate, pH 7.4.

2.10.2. Solubilisation of the particulate PAP and Prolyl Endopeptidase activities from the synaptosomal membranes

2.10.2.1. Triton X-100 Solubilisation

A Triton X-100 solubilisation step was used to remove the particulate PAP and prolyl endopeptidase from the Synaptosomal Membranes.
Aliquots of the resuspended salt-washed synaptosomal membrane fraction were treated with a range of concentrations of Triton X-100 (0-1% v/v) in 100mM potassium phosphate, pH7.4, to find the optimum Triton X-100 concentration. Each sample was incubated for 60 minutes on a shaking ice bath.

2.10.2.2. Trypsin-Treatment of the Synaptosomal Membranes

A salt-washed synaptosomal membrane fraction was incubated with a Trypsin solution (prepared in 1mM $CaCl_2$ in 0.1mM potassium phosphate, pH 7.4) to give a final concentration of 1040 units of trypsin activity/10 mg membrane protein. The mixture was then incubated at 30°C for 60 minutes in a shaking water bath.

2.10.2.3. Papain-Treatment of the Synaptosomal Membranes.

A synaptosomal membrane preparation was treated with a Papain solution (prepared in 100mM potassium phosphate buffer, pH7.4.) to give a final concentration of 1.57units of Papain activity/10 mg membrane protein. The mixture was then incubated at 37°C for 60 minutes in a shaking water bath.
Equal volumes of 100mM potassium phosphate, pH 7.4 were added to synaptosomal membrane fractions and incubated both at 4° and 30°C for 60 minutes to act as controls.

2.10.2.3. Sonication of the Synaptosomal Membranes

5mLs of a synaptosomal preparation were sonicated on ice for 10 seconds. Sonication was found not to be a suitable method of releasing the enzyme activities from the membranes as it was too harsh and very little enzyme activity survived the sonication procedure.

After treatment all samples were centrifuged at 15,000 r.p.m. for 60 minutes. Supernatants and resuspended pellets were assayed for synaptosomal PAP activity using <Glu-His-ProMCA (section 2.2.2.1.) and for prolyl endopeptidase activity using Z-Gly-ProMCA (section 2.2.4).
The most active synaptosomal PAP samples were pooled and stored in 5mL fractions at room temperature. The most active prolyl endopeptidase samples were pooled and stored in 5mL aliquots at -20°C.

2.11. Further purification of the Triton X-100 solubilised synaptosomal PAP by column chromatography

2.11.1. Purification of the particulate PAP by gel filtration

3mL of 0.4% (v/v) Triton X-100 solubilised particulate PAP was applied to a 2.5cm x 50cm (Vol=245mL) column of Sephacryl HR S-200 which was equilibrated with 100mM potassium phosphate buffer, pH7.4, 0.15M KCl, 1mM DTT 1mM EDTA and 0.3% (v/v) Triton X-100 at room temperature. Flow was set at 0.75 mLs/min using a Gilsen pump and 3 mL fractions were collected using a Bio-Rad fraction collector. Fractions were assayed for particulate synaptosomal PAP activity (section 2.2.2) and the fractions with the highest activity were pooled and stored at -20°C. The protein concentration was monitored by assaying the fractions using

the Biuret Assay (section 2. 3.1)

2.11.2 Ion exchange chromatography

A 25mL DEAE-Sepharose ion exchange column was equilibrated with 3 column volumes of 50mM potassium phosphate, pH7.4, with 1mM DTT, 1mM EDTA and 0.3%(v/v) Triton X-100. A 4mL TritonX-100-solubilised sample (S_T) was applied to the column. The column was washed for 3 column volumes with the equilibration buffer at a flow rate of 1mL/min. A linear gradient of 0.2 to 0.5M NaCl in 50mM potassium phosphate , with 1mM DTT, 1mM EDTA and 0.3%(v/v) Triton X-100 was applied. 3mL fractions were collected and were assayed for synaptosomal PAP activity (section 2.2.2). The fractions with the highest activities were pooled and stored at 4°C.

2.11.3. Concentration of the post-ion exchange synaptosomal PAP activity

Following ion exchange, the synaptosomal PAP activity was concentrated by reverse osmosis. Dialysis tubing was boiled in 30mM EDTA for 15 min. 5mL of post-ion exchange synaptosomal PAP was placed in the dialysis tubing and sealed at both ends. The dialysis tubing was placed on a bed of polyethylene glycol 6000 (PEG) for 30 min or until it was reduced in volume to 2mL. The sample was then stored at 4°C until the next purification stage.

2.11.4. Hydrophobic interaction chromatography

A 2mL Phenyl-Sepharose column was equilibrated with 2M$(NH_4)_2SO_4$ in 100mM potassium phosphate, pH 7.4, with 1mM DTT, 1mM EDTA and 0.3%(v/v) Triton X-100 for 10 column volumes. A 2mL concentrated post-ion exchange fraction was applied to the phenyl-sepharose column and was washed with 10 column volumes of equilibration buffer. The flow rate was set at 1mL/min and 2mL fractions were collected. A linear gradient of 0 to 25% glycerol (25mLs of each) in 100mM potassium phosphate with 1mM DTT, 1mM EDTA and 0.3% (v/v) TritonX-100 was applied. Each fraction was assayed for synaptosomal PAP activity (section 2.2.2.1.) and the most active fractions were pooled and stored at 4°C.

2.12. Further purification of the particulate Prolyl Endopeptidase from the synaptosomal membranes by column chromatography

2.12.1. Preparation of a Sephacryl S-200HR column using the Pharmacia FPLC Biopilot System

600mL of Sephacryl S-200 HR were allowed to settle before the fines and supernatant were decanted. The gel was resuspended in 100mM potassium phosphate buffer, pH 7.4. to give a

final concentration of 75% gel. The column (Pharmacia XK100/26, 2.6. x 100cm) was cleaned with distilled water and ethanol and allowed to dry before pouring the gel. The gel was degassed for 1 hour before pouring . The gel was poured into the column with the aid of a reservoir and a flow of buffer was applied immediately at a flowrate of 3mL/min for 3 hours, followed by 5mL/min for 1 hour. The reservoir was removed and the adaptor was placed onto the column and packing was continued for a further 30 min. 5mL of a 2mg/mL solution of Blue Dextran was applied to the column at a flowrate of 2 mL/min. The absorbance of the Blue Dextran at 620nm was monitored to calculate the void volume. The entire procedure was carried out at 4°C.

2.12.2. Partial purification of the Triton X-100 solubilised synaptosomal Prolyl Endopeptidase by gel filtration on Sephacryl S-200 HR.

Synaptosomal membranes were treated with 0.4% (v/v) Triton X-100 as described in section 2.10.2.1. and the solubilised enzyme was separated from the particulate matter by centrifugation at 15,000 r.p.m. for 60 minutes at 4°C. 5mL of this supernatant (S_T) was then applied to a Sephacryl S-200 HR column (100cm x 2.6cm) which had previously been equilibrated with 3 column volumes of 100mM potassium phosphate buffer, pH 7.4 containing 0.15M KCl, 1mM DTT, 1mM EDTA and 0.3%v/v Triton X-100. This running buffer was degassed and vacuum filtered at 4°C using a Gelman Sciences filtration apparatus and Millipore Prefilters (pore size 0.45μm). The column was attached to a Pharmacia FPLC system. The buffer was applied at a flowrate of 2mL/min. 3mL fractions were collected using a Pharmacia Frac-3000 Remote fraction collector and assayed for prolyl endopeptidase activity (Section 2.2.3.). Protein concentration was automatically monitored at 280nm by the system.

2.12.3. Calcium phosphate chromatography (Hydroxylapatite column)

10mL of a post-Sephacryl S-200 sample were dialysed against 1L of 20mM potassium phosphate buffer, pH7.4 at 4°C for 3 hours prior to application to a 25mL Bio-Rad Hydroxylapatite column which was equilibrated with 3 column volumes of 20mM potassium phosphate buffer, pH7.4 with 1mM DTT and 1mM EDTA. The enzyme was eluted with a linear gradient of 20mM to 500mM potassium phosphate buffer, pH7.4 with 1mM DTT and 1mM EDTA, at a flowrate of 1mL/min. 2mL fractions were collected. Each fraction was assayed for prolyl endopeptidase activity. The fractions with the highest activity were pooled and stored at -20°C.

2.12.4. Phenyl-Sepharose hydrophobic interaction chromatography

2mL of a post-Hydroxylapatite peak was applied to a 10 mL Phenyl-Sepharose column which had been equilibrated with 2M $(NH_4)_2SO_4$ in 100mM potassium phosphate buffer, pH7.4, 1mM DTT, 0.3% (v/v) Triton X-100 and 1mM EDTA at 4°C. After the sample was applied, the column was washed for 3 column volumes with the equilibration buffer, before a linear gradient of 0-50%

glycerol in 100mM potassium phosphate buffer, pH 7.4 , 1mM DTT, 1mM EDTA and 0.3% (v/v) Triton X-100 was applied at a flowrate of 1mL/min. 2mL fractions were collected and each was assayed for prolyl endopeptidase activity. The fractions with the highest activity were pooled and stored at -20°C.

2.13. Lyophilisation

10mL aliquots of sample from each stage in the purification were dialysed against 2L of distilled H_2O at 4°C overnight to remove any salts. Each sample was lyophilised on a Hetosicc Freeze-Dryer for 12 hours and then resuspended in 500µL of 100mM potassium phosphate buffer, pH7.4. The freeze-dried samples were stored at -20°C and were used for PolyAcrylamide Gel Electrophoresis .

2.14. Electrophoresis

In order to monitor the purification scheme and to estimate the molecular weight of prolyl endopeptidase and synaptosomal PAP SDS-PAGE was carried out on all the fractions, from crude homogenate to purified enzyme. The SDS-PAGE was carried out on 7.5% and 10% polyacrylamide gels as described by Laemmli (1970) to determine the molecular weight of the purified enzymes.

2.14.1. 7.5% SDS-PAGE

Stock solutions of 0.1M Tris-HCl pH 8.8, 1.0M Tris-HCl pH 6.8, 30% (w/v) acrylamide containing 0.8% (w/v) bisacrylamide (Acryl/bisacryl), and 10% (w/v) Ammonium Persulaphate (AP) were used to prepare both the stacking and resolving gels. A 7.5% resolving gel was used for the high molecular weight PAP enzyme, while a 10% resolving gel was used for the PE. The volumes required for a 7.5% resolving gel and a 3% stacking gel were as follows:

Table 2.2 Quantities required for a 7.5% SDS-PAGE

	Resolving Gel (7.5%)	**Stacking Gel (3%)**
Solution	Vol (mL)	Vol (mL)
Water	11.5	7.8
Acryl/Bisacryl	7.5	1
Tris-HCl, pH8.8	11.2	-
Tris-HCl, pH6.8	-	1.25
Ammonium Persulphate (10%w/v)	150µL	75µL
TEMED	50µL	50µL

Table 2.3 Molecular Weight Markers for a 7.5% Gel

Molecular Weight Marker	Molecular Weight (kDa)
Thyroglobulin	669
Ferritin	440
Catalase	232
LDH	140
BSA	67

2.14.2. 10% SDS-PAGE

A 10% resolving gel was used to visualise the purification scheme of the PE. The following quantities were used to prepare a 10% resolving and a 3% stacking gel:

Table 2.4 Quantities Required for a 10% SDS-PAGE

	Resolving Gel (7.5%)	Stacking Gel (3%)
Solution	Vol (mL)	Vol (mL)
Water	14.1	9.3
Acryl/Bisacryl	10.5	1.25
Tris-HCl, pH 8.8	11.2	-
Tris-HCl, pH 6.8	-	1.25
Ammonium Persulphate (10 %w/v)	0.15	50µL
SDS (10% w/v)	0.15	50µL
TEMED	30µL	50µL

Table 2.5. Molecular Weight Markers for a 10% Gel

Molecular Weight Marker	Molecular Weight (kDa)
Myosin	205
ß-Galactosidase	116
Phosphorylase B	97.4
BSA	66
Ovalbumin	45
Carbonic Anhydrase	29

Samples were dissolved in solubilisation buffer (0.8M Tris-HCl, pH6.8 ; 10% glycerol ; 2%(w/v) SDS; 0.2% (w/v) Brilliant Coomassie Blue).

30µL of sample (containing 1mg/mL of protein) were loaded on to the gel. The gel was electrophoresed in electrode buffer, pH8.3 (0.025M Tris, 0.1% (w/v) SDS, and 0.192M Glycine) at 20mA using an Atto Vertical Electrophoresis apparatus.
Gels were stained for 30 minutes in 0.5% (w/v) Brilliant Coomassie Blue in an acetic acid: methanol: water (1:8:10) mixture and then allowed to destain overnight in the same solvent system.

2.15. Characterization studies of the synaptosomal membrane-bound PAP and Prolyl Endopeptidase activities

2.15.1. Molecular weight determinations

2.15.1.1. Molecular weight determination of the synaptosomal membrane-bound Pyroglutamate Aminopeptidase

Known molecular weight markers (Sigma Gel Filtration Molecular Weight Markers, shown in Table 2.6) were applied to the Sephacryl S-200 BioPilot Column at a flowrate of 2mL/min using 100mM potassium phosphate buffer, pH7.4 as the running buffer. The elution profiles were monitored at 280nm and a plot of elution volumes versus the log of the molecular weight was plotted. From this graph the molecular weight of the prolyl endopeptidase was estimated. It was further confirmed by SDS-PAGE.

Table 2.6 Molecular weight markers for gel filtration column

Molecular Weight Marker	Molecular Weight (kDa)
Cytochrome C	12.4
Carbonic Anhydrase	29
Albumin	66
Alcohol Dehydrogenase	150
ß-Amylase	200
Blue Dextran	2000

2.15.1.2. Molecular weight determination of the synaptosomal membrane-bound Prolyl Endopeptidase

Sigma Gel Filtration Molecular Weight Markers (Fig.2.4) were also used to estimate the molecular weight of the particulate PE. The markers were applied to the 2.5x50cm column and eluted with 100mM potassium phosphate,pH7.4. The elution profiles were monitored at 280nm and a plot of elution volumes versus the log of the molecular weights was plotted. From this the molecular weight of the particulate PE could be estimated. This was confirmed by SDS-PAGE.

2.15.2. pH profiles

2.15.2.1. pH profile of the synaptosomal membrane-bound Pyroglutamate Aminopeptidase activity

5mL aliquots of post phenyl sepharose synaptosomal PAP were dialysed against 2L of distilled water for 8 hours at room temperature. These dialysed samples were then used for the pH optimum studies. 50µL of sample were pre-incubated with equal volumes of buffer at different pH units at 37°C for 10 min. The buffers used were 100mM MES (pH5.5-6.7), 100mM potassium phosphate (pH 6.2-7.8) and 100mM Tris-HCl (pH 7.0-8.6). The samples were assayed for synaptosomal PAP activity (section 2.2.2.1.).

2.15.2.2. pH profile of the synaptosomal membrane-bound prolyl ednopeptidase activity

5mL aliquots of post-phenyl sepharose prolyl endopeptidase were dialysed against 2L of distilled water for 8 hours at 4°C. These dialysed samples were then used for the pH optimum studies as described above. The samples was assayed for prolyl endopeptidase activity using Z-Gly-ProMCA (Section 2.2.4)

2.15.3. Enzyme activity in different buffers

5mL aliquots of P_2 were resuspended in range of buffers and the Triton X-100 solubilisation step was preformed as described in 2.8.4.1. The buffers were :

100mM potassium phosphate, pH 7.4
100mM HEPES, pH 7.4
100mM Imidazole, pH 7.4
100mM Tris-HCl, pH 7.4

Following incubation for 60 min in a shaking water bath, the samples were centrifuged at 15,000rpm for 60min at 4°C. The supernatants were collected and each supernatant was assayed for both synaptosomal PAP (see section 2.2.2.1.) and PE activity (see section 2.2.3.).

2.15.4 Storage of PAP and PE activities under different conditions

2.15.4.1 PAP activity under different storage conditions

Post phenyl sepharose synaptosomal PAP was aliquoted into 1mL aliquots and was stored for 1 week under different conditions as it was found to be quite unstable.
The following conditions were employed to stabilise the synaptosomal PAP activity:

1. Temperature (-20°C, 4°C and room temperature)
2. Glycerol (10, 20 and 30% v/v)

3. BSA (1 and 2% w/v, and 1% w/v with 10% v/v glycerol)
4. Sulphydryl Reagent (DTT)
5. Different Buffers (Potassium phosphate, Imidazole, HEPES and MES)

The enzyme activity was monitored by measuring PAPII activity according to section 2.2.2.1.

2.15.4.2. PE activity under different conditions

Post phenyl sepharose PE activity was quite stable and so only a minimum number of storage conditions were used to assess their effects on the PE activity. These included:

1. Temperature (-20°C, 4°C and room temperature)
2. Different Buffers (Potassium phosphate, Imidazole, HEPES and MES)
3. Glycerol (10, 20 and 30% v/v)

The enzyme activity was monitored for 1 week by measuring the PE activity according to section 2.2.3.

2.16. Preliminary kinetic studies

2.16.1. Kinetic studies on the synaptosomal pyroglutamate aminopeptidase

Kinetic analysis of the synaptosomal pyroglutamate aminopeptidase was carried out using <Glu-His-ProMCA as a substrate (Section 2.2.2). The effect of a range of pyroglutamyl peptides on the activity of the enzyme was also investigated. A stock concentration of <Glu-His-ProMCA (0.5mM) was further diluted with 100mM potassium phosphate buffer, pH7.4 to give a range of concentrations of substrate. 400µL of each dilution of <Glu-His-ProMCA was added to 50µL of peptide inhibitor in 100mM potassium phosphate buffer pH7.4. The reaction was initiated by the addition of 50µL of post phenyl sepharose pyroglutamate aminopeptidase. Separate blanks were used for each concentration of <Glu-His-ProMCA. The reaction was allowed to proceed for 60 min and was then stopped by the addition of 1mL of 1.5M acetic acid. The samples were read at 370 and 440 nm, excitation and emission wavelengths respectively. Lineweaver Burk plots (Lineweaver and Burk, 1934) were constructed and the kinetic constants (Km, Vmax and Ki's) determined.

2.16.2 Kinetic studies on the synaptosomal prolyl endopeptidase

Kinetic analysis of the synaptosomal prolyl endopeptidase was carried out as described above (Section 2.14.1) except that Z-Gly-ProMCA was used as a substrate in the range 0.01mM to 0.3mM concentration. A number of proline-containing neuropeptides were used as peptide inhibitors.

2.17. Substrate specificities

2.17. 1. Investigation of the substrate specificity of the synaptosomal PAP by HPLC

The specificity of the post-Ion-exchange and post-Phenyl Sepharose synaptosomal PAP was determined using a number of naturally occurring and synthetic pyroglutamyl and histidine containing peptides according to the method of Martini et al (1985) as modified by Griffiths, (1993).

200µL of the potential substrate (1mM in 100mM potassium phosphate buffer, pH 7.4) were incubated for 24 hours at 37°C with an equal volume of enzyme. The enzymic reaction was terminated by the addition of 100µL of 0.2% TriFluoroacetic Acid (TFA), pH 2.0.

The HPLC system consisted of 2 Waters Model 6000a pumps, a model 660 gradient programmer, a U6k injector, a Model 450 variable wavelength detector and a Novapak C-18 column (3.9 x 150mm).

The column was equilibrated with 0.2% TFA (freshly prepared and degassed) . Each sample was filtered through a Gelman Sciences Suporfilter (0.2µm pore size) before injection of a 25µL sample onto the column. A 20 minute gradient from 0-70% of a 70% acetonitrile solution in 0.2% TFA (i.e. 0-49% acetronitrile) at a flowrate of 1mL/min was enough to resolve the peaks. The absorbance of each peak was read at 220nm and at a sensitivity of 0.01-0.05 AUFS. Whenever possible commercially available standards (prepared in the mobile phase of 0.2% TFA) were run to compare the retention times of the peaks with those of the known possible metabolites resulting from the cleavage of the peptides by PAP.

2.17.2. Investigation of the substrate specificity of the synaptosomal prolyl endopeptidase

2.17.2.1. Substrate specificity of the synaptosomal PE by thin layer chromatography

TLC was the first of the two methods employed to investigate the substrate specificity of the synaptosomal membrane-bound prolyl endopeptidase.

Equal volumes of membrane-bound, Triton X-100 solubilised, post-gel filtration and post-phenyl sepharose prolyl endopeptidase were each incubated with substrate at 37°C for 24hrs and were then centrifuged at 12,000 rpm for 5 min in a bench top centrifuge to remove any insolubles. 40µL of the resulting supernatant was spotted onto a 20x20 cm Silica gel G, type 60 (Merck No.5721) 2.5cm from the bottom of the plate. The following standards were also spotted onto the plates: 1.2mM Thyroliberin, 1mM Thyroliberin-OH, 1mM Cyclo(His-Pro), 1mM D-L Histidine and 20mM D-L Proline. The plates were developed in vapour saturated tanks with the following solvent system: Chloroform/Methanol/33% Ammonia (60/30/5 v/v/v).

Histidine-containing peptides were visualised using Pauly's reagent (Solution A: 1% sulphanilic Acid in 1M HCl, Solution B: 5% aqueous Sodium Nitrite. Equal volumes of A and B were mixed together before spraying). Each plate was allowed to stand at room temperature for 5 minutes

before being sprayed with Solution C (15% sodium carbonate w/v in H_2O).

Amino acids were visualised by spraying the plates with freshly prepared Ninhydrin reagent (0.2% w/v Ninhydrin, 0.5% w/v cadium acetate and 2% v/v acetic acid in methanol) and by then heating the plate to 120°C for 10 min.

2.17.2.2. Substrate specificity of the synaptosomal Prolyl Endopeptidase by HPLC

The second method for the investigation of the substrate specificity of the post-Phenyl Sepharose prolyl endopeptidase was using HPLC (as described for synaptosomal PAP) except that equal volumes of sample and of 1mM of each potential substrate were incubated for 24 hours at 37°C. Following incubation, the reaction was terminated by the addition of 400µL of 0.2% TFA, pH2.0.
The peaks were resolved by the same gradient system as described in Section 2.15.1.

2.18. Inhibitor studies

2.18.1. Inhibitor studies on the synaptosomal pyroglutamate aminopeptidase

The effects of various inhibitors on the membrane-bound, the Triton X-100 solubilised, the post ion-exchange and the post phenyl sepharose synaptosomal PAP were investigated. The inhibitors were prepared as follows:

50µL of each sample were pre-incubated with an equal volume of each potential inhibitor for 10 min at 37°C. 400µL of 0.1mM <Glu-His-ProMCA was added and the reaction was allowed to proceed for a further 60 min. The fractions were assayed as described in section 2.2.2.1. Appropriate controls were prepared for each enzyme assay.

2.18.2 Inhibitor studies on the synaptosomal prolyl endopeptidase

The effects of a variety of inhibitors on the membrane-bound, the Triton X-100 solubilised, the post-gel filtration and the post-phenyl sepharose prolyl endopeptidase were assessed as described for the synaptosomal PAP (Section 2.16.1) using 0.1mM Z-Gly-ProMCA as a substrate.

2.18.3. Effect of DTT on the synaptosomal PAP activity

The effects of DTT on post phenyl sepharose synaptosomal PAP activity was assessed by dialysing a 2mL aliquot of post phenyl sepharose synaptosomal PAP against 100mM potassium phosphate , pH 7.4 for 4 hours. 250µL of dialysed sample were then incubated with a range of concentrations of DTT for 10 min at 37°C. 100µL were then assayed for synaptosomal PAP

activity according to section 2.2.2.1.

2.18.4. Effect of DTT on the synaptosomal PE activity

The effects of DTT on post phenyl sepharose PE were assessed using the same method as descrided in section 2.16.3. PE activity was asayed as in section 2.2.3.

2.18.5. Effect of EDTA on the synaptosomal PAP activity

A 2mL aliquot of post phenyl sepharose synaptosomal PAP activity was dialysed against 100mM ptassium phosphate, pH 7.4 with 1mM DTT for 4 hours. 250µL were incubated with a range of EDTA concentrations for 10 min at 37°C. 100µL sample was taken from each concentration of EDTA and was assayed for synaptosomal PAP activity as described in section 2.2.2.1.

2.18.6. Effect of EDTA on synaptosomal PE activity

The effects of EDTA on post phenyl sepharose PE activity were assessed in the same way as for synaptosomal PAP (section 2.16.5.). PE activity was measured as described in section 2.2.3.

2.19. Metal ion studies

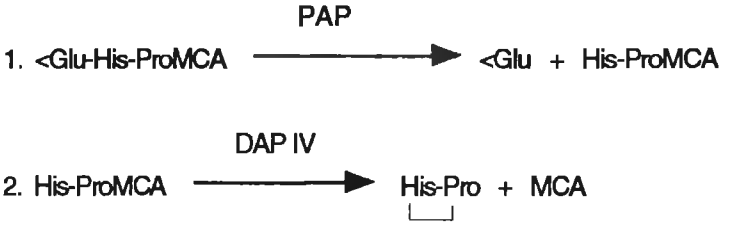
The effects of various metal ions on the synaptosomal PAP and prolyl endopeptidase were investigated by pre-incubating 50µL of enzyme sample with an equal volume of each metal ion solution (to give a final concentration of 1mM metal ion) for 10 min at 37°C. 400µL of each substrate was added and the reaction was allowed to proceed for a further 60 min. The reaction was terminated by the addition of 1mL of 1.5M acetic acid. The release of MCA was measured for each enzyme as described in sections 2.2.2 and 2.2.3. Appropriate controls were prepared for each enzyme assay.

3. Results

3.1. Development of Two New Fluorimetric Assays for the Measurement of Pyroglutamate Aminopeptidase Activity

3.1.1. Use of the fluorimetric substrate <Glu-His-ProMCA for the detection of PAP activity with a coupled enzyme assay

The use of a new fluorimetric substrate <Glu-His-ProMCA as a possible substrate for the measurement of PAP activity was assessed initially using cytosolic PAP purified from rat liver as a positive control (section 2.4). The method used was a modification of a coupled enzyme spectrophotometric assay developed by Friedman and Wilk (1986). The PAP activity was determined with <Glu-His-ProMCA in a coupled enzyme assay with excess DAP IV and in the presence of Z-Pro-prolinal, a specific PE inhibitor (Wilk and Orlowski, 1983). Z-Pro-prolinal blocks the cleavage of the Pro-MCA bond by prolyl endopeptidase. The assay is based on the following reaction sequence:



The free MCA can then be measured fluorimetrically using excitation and emission wavelengths of 370 and 440nm respectively.

Fig 3.1 shows the effect of varying the concentration of <Glu-His-ProMCA on the release of MCA by PAP. 40 nanomoles of substrate were found to be sufficient to ensure that the substrate was present in adequate quantities to be saturating without inhibiting the PAP activity.

Once it was established that the fluorimetric substrate <Glu-His-ProMCA was suitable for detecting PAP activity, the assay parameters were established using bovine brain PAP and rat liver cytosolic PAP as a positive control. The use of PAP from rat liver cytosol purified in our laboratory was preferable to the commercially available pyroglutamate aminopeptidase activity (Sigma Chemical Co., St. Louis), as this commercial enzyme was found to be inactive and unstable in several batches. This commercial enzyme preparation was also impure and required purification over a Sephadex G-100 column to separate the PAP and PE activities. For these reasons it was more convenient and reliable to use PAP activity purified from rat liver as a positive control.

In the design of this kinetic enzyme assay a number of criteria were examined:

1. The concentrations of substrate and the second enzyme (i.e. DAP IV) should all be in excess to ensure that they are not rate-limiting.
2. There should be no inhibitors of the enzyme in the reaction mixture.

Fig. 3.1 MCA Release from Increasing <Glu-His-ProMCA Concentration by PAP

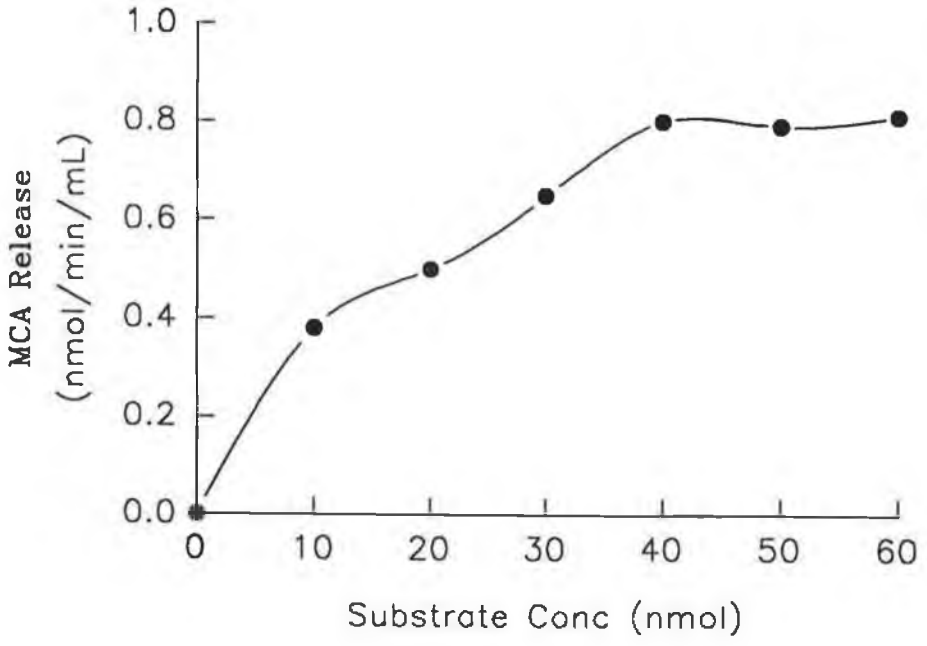
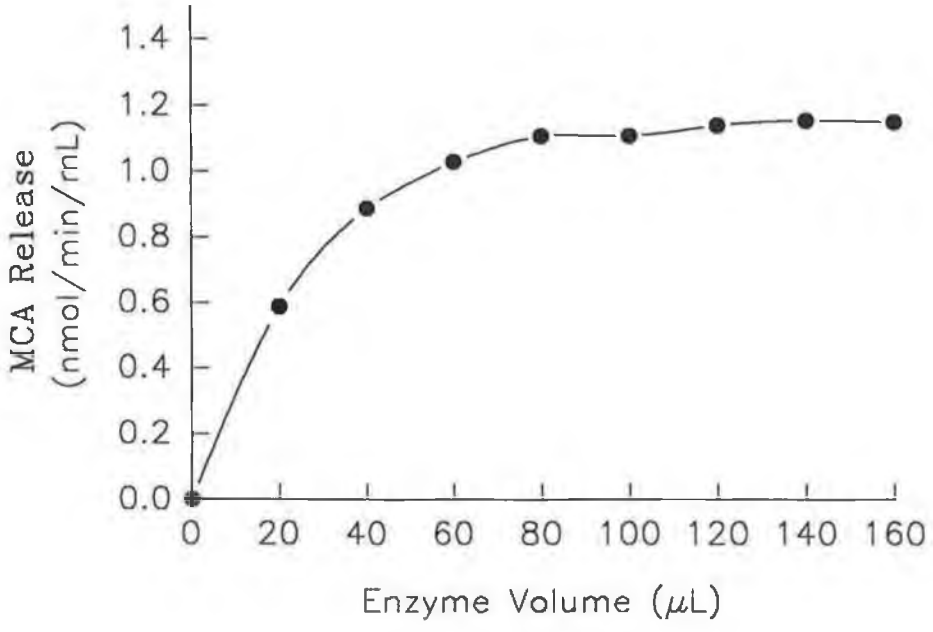


Fig. 3.2 Effect of Increasing Enzyme (PAP) Volume on MCA Release from <Glu-His-ProMCA



3. All environmental factors such as pH, temperature and ionic strength should be controlled.
The third criteria was established using the conditions as described by Friedman and Wilk (1986). All assay mixtures were prepared in 100mM potassium phosphate, pH 7.4 unless otherwise stated, and all reactions were carried out at 37°C.

3.1.1.1. The effect of pyroglutamate aminopeptidase volume on MCA release

Fig. 3.2 shows the effect of varying PAP volume on MCA released. 100µl of PAP activity with 400µl of 0.1mM <Glu-His-ProMCA ensured that the quantity of PAP was not limiting the reaction.

3.1.1.2. The effect of Z-Pro-prolinal on PAP and PE activities

Since prolyl endopeptidase can also cleave the substrate <Glu-His-ProMCA, a specific PE inhibitor, Z-Pro-prolinal was used to ensure that any MCA released from the substrate was due solely to PAP activity. A bovine brain crude homogenate (section 2.5), containing both cytosolic and particulate PAP and PE activities was assayed in the presence of a range of concentrations of the specific PE inhibitor, Z-Pro-prolinal (Fig 3.3). It was found that 20µl of 10^{-5} M Z-Pro-prolinal was sufficient to completely inhibit any PE activity in the crude homogenate or in the rat liver cytosolic fraction following incubation with both 0.1mM Z-Gly-ProMCA and 0.1mM <Glu-His-ProMCA. To ensure that this inhibition was not time dependent, the efficacy of the inhibitor at a concentration of 10^{-5} M was assessed over time (Fig. 3.4). It was also seen that this inhibitor has no effect on PAP activity.

3.1.1.3. The effect of DAP IV volume on MCA release

The coupled-enzyme assay system calls for DAP IV to be present in excess to cleave the Pro-MCA bond, following the primary cleavage by PAP of the <Glu-His bond. Without that primary cleavage by PAP, DAP IV has no activity on the substrate. A range of volumes of DAP IV (purified from bovine brain) were pre-incubated with 100µl of PAP and 20µl of 10^{-5} M Z-Pro-prolinal (to inhibit any PE activity) for 10mins at 37°C. 400µl of 0.1mM <Glu-His-ProMCA was added and the reaction was allowed to proceed as described in section 2.2.2.1. A volume of 40µl gave a sufficient excess of DAP IV (Fig. 3.5).
The purpose of the DAP IV addition to the reaction mixture is to speed up the conversion of His-ProMCA to His-Pro + MCA as the naturally-occurring cyclisation reaction is quite slow. As can be seen in Fig. 3.6 the quantity of MCA released in the absence of DAP IV is only 30% of that released in its presence.

Fig. 3.3. Effect of Different Concentrations of Z-Pro-prolinal on PAP and PE Activities

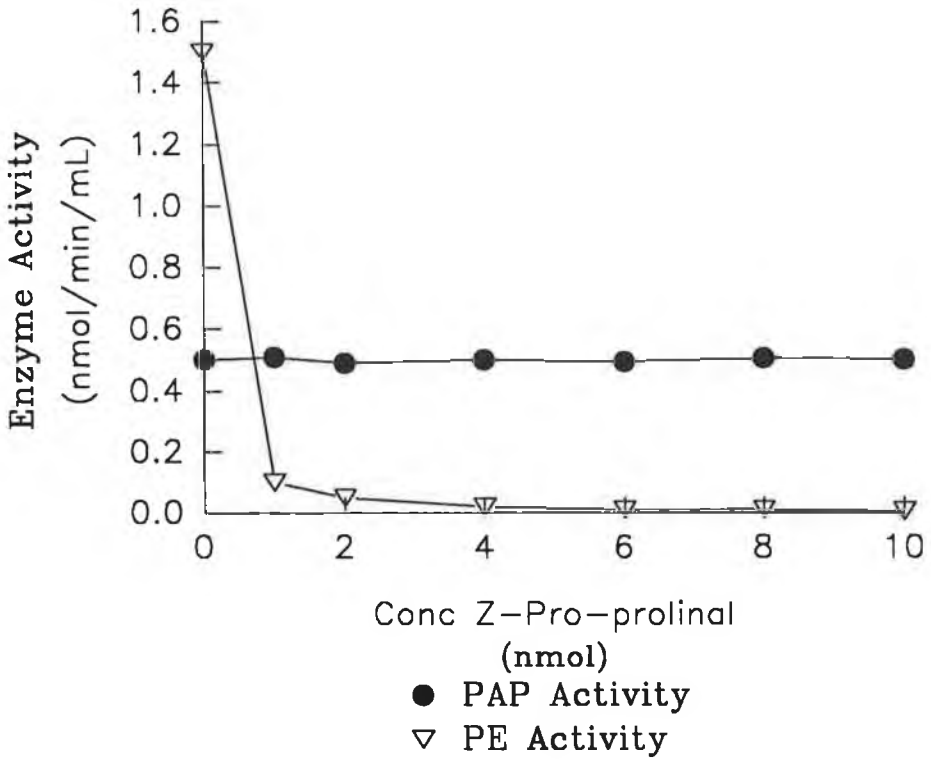


Fig. 3.4. Effect of Z-Pro-proiinai on Enzyme Activity Over Time

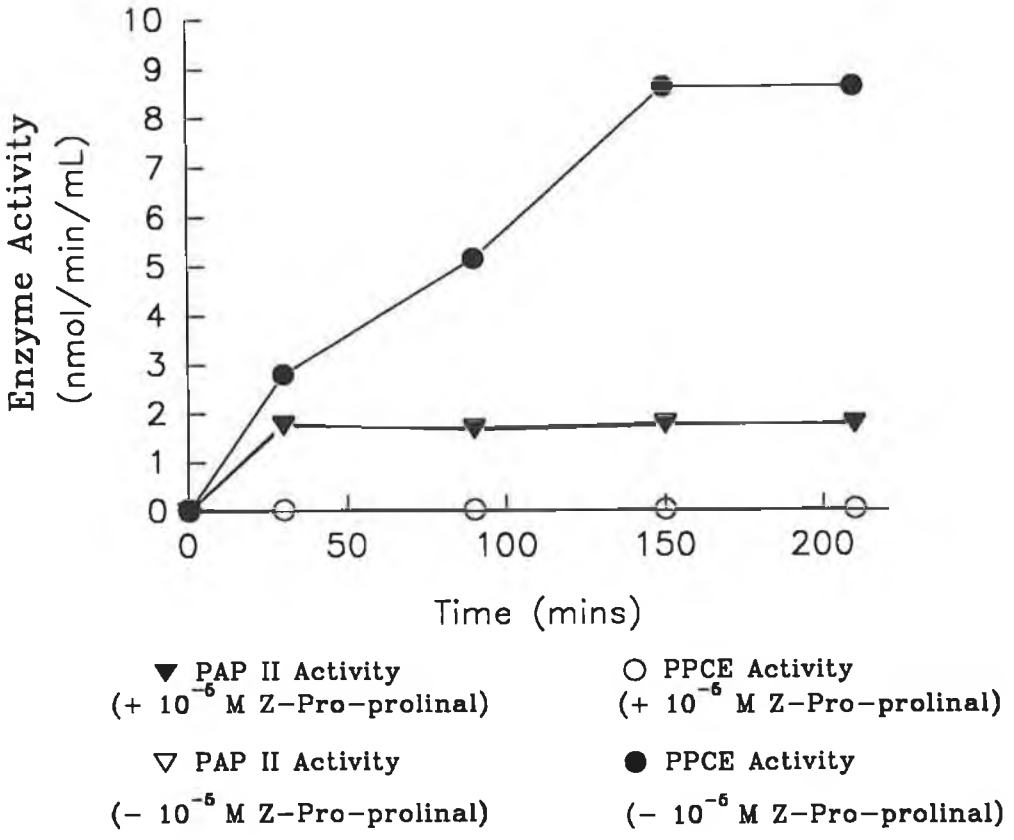


Fig. 3.5. Effect of Differing Volumes of Secondary Enzyme (DAP IV) on Release of MCA from His-ProMCA Following Primary Cleavage by PAP

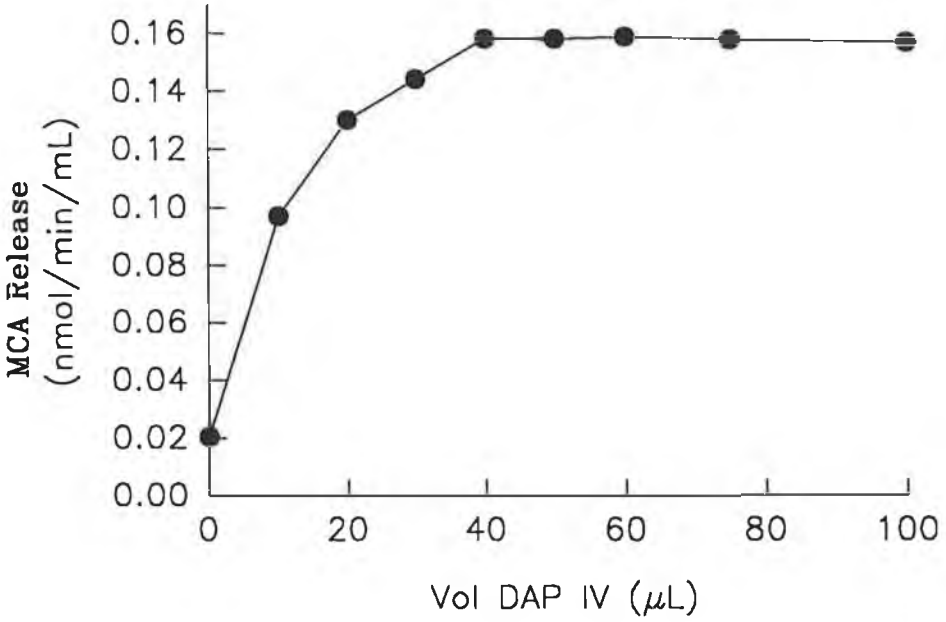
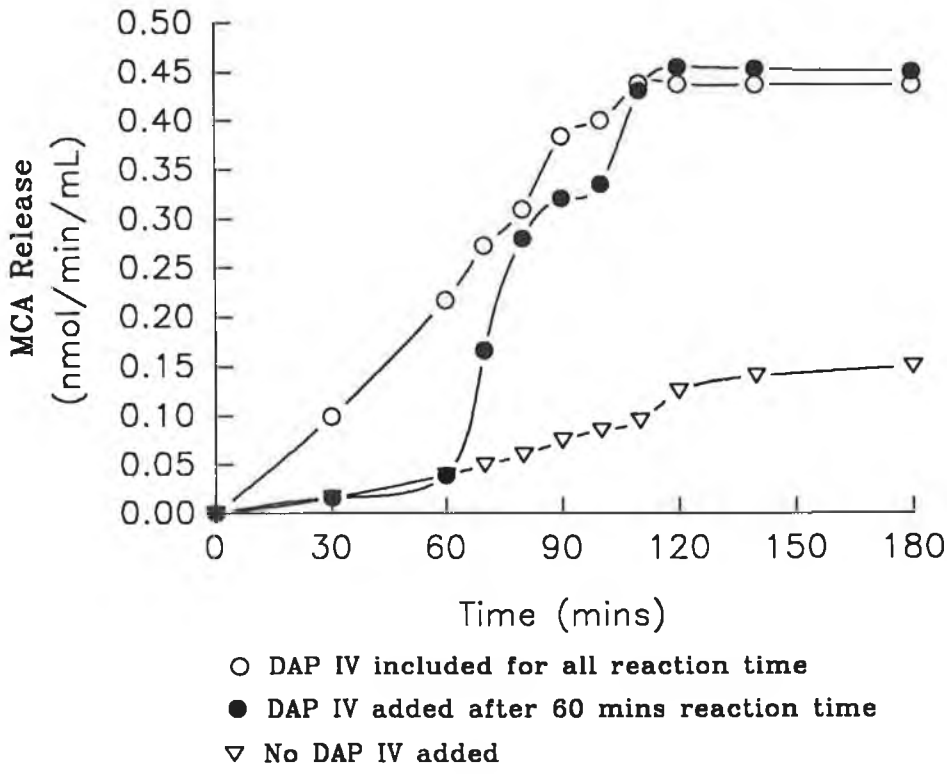


Fig. 3.6. Release of MCA from <Glu-His-ProMCA by DAP IV Over Time Following the Primary Cleavage by PAP



It was thought that the addition of DAP IV to the assay mixture at the beginning of the assay might pose a problem in the measurement of kinetic constants. It was decided to assess the possibility of allowing the reaction to proceed in the absence of DAP IV and compare the release of MCA with the rate of MCA release in the presence of DAP IV from time 0 and after its addition following 60 mins incubation.
By incubating the enzyme with the substrate for 60 mins prior to the addition of DAP IV (Fig. 3.6), it can be seen that after 120 mins the reaction has gone to completion and there is no further release of MCA. By allowing the reaction to proceed in the absence of DAP IV for the initial 60 mins, possible interference by DAP IV in the measurement of any kinetic parameters can be avoided.

However, this does not ensure that the reaction goes to completion. It is the possible that following the addition of DAP IV to the reaction, PAP can continue cleaving the <Glu-His-ProMCA, while the DAP IV will cleave the His-ProMCA produced. For the correct estimation of kinetic parameters, one must be certain that the latter reaction has gone to completion, i.e., that the DAP IV has cleaved all the His-ProMCA produced from the primary cleavage of the substrate by PAP. By adding a PAP inhibitor, 1,10-phenanthroline (which has no effect on DAP IV activity) after 60 mins at the same time as the DAP IV addition, any further PAP activity is prevented and the DAP IV is free to cleave all the His-ProMCA which was formed by PAP (Fig. 3.7).

3.1.2. Design of the Spontaneous Cyclisation Assay

The spontaneous cyclisation of His-ProNH_2 to Cyclo(His-Pro) is well documented (Bauer and Nowak, 1979; Bauer and Kleinkauf, 1980) and was found to proceed at a rate of 1.5% min^{-1} in 100mM potassium phosphate buffer, pH7.4. This corresponds to a half-life of 44 mins if first order kinetics is assumed (Moss and Bungaard, 1990). Using this feature of the His-ProMCA, it was our aim to design an assay based on spontaneous cyclisation. This assay would have the advantage over the coupled-enzyme assay of not requiring the second enzyme, DAP IV.
The reaction sequence is as follows for the spontaneous cyclisation assay:

1. <Glu-His-ProMCA $\xrightarrow{\text{PAP}}$ <Glu + His-ProMCA

2. His-ProMCA $\xrightarrow[\text{Cyclisation}]{\text{Spontaneous}}$ His-Pro + MCA

Fig. 3.8 shows the release of MCA in the presence and absence of DAP IV over time. MCA release by spontaneous cyclisation does occur, but it takes much longer to go to completion. In the presence of DAP IV, the reaction is complete in approximately 2 hours, whereas it takes over 10 hours to reach completion by spontaneous cyclisation alone.

Fig. 3.7. MCA Release from His-ProMCA Following the Addition of DAP IV (Primary Cleavage of <Glu-His-ProMCA by PAP)

(PAP II Inhibitor * Added after 60 mins)

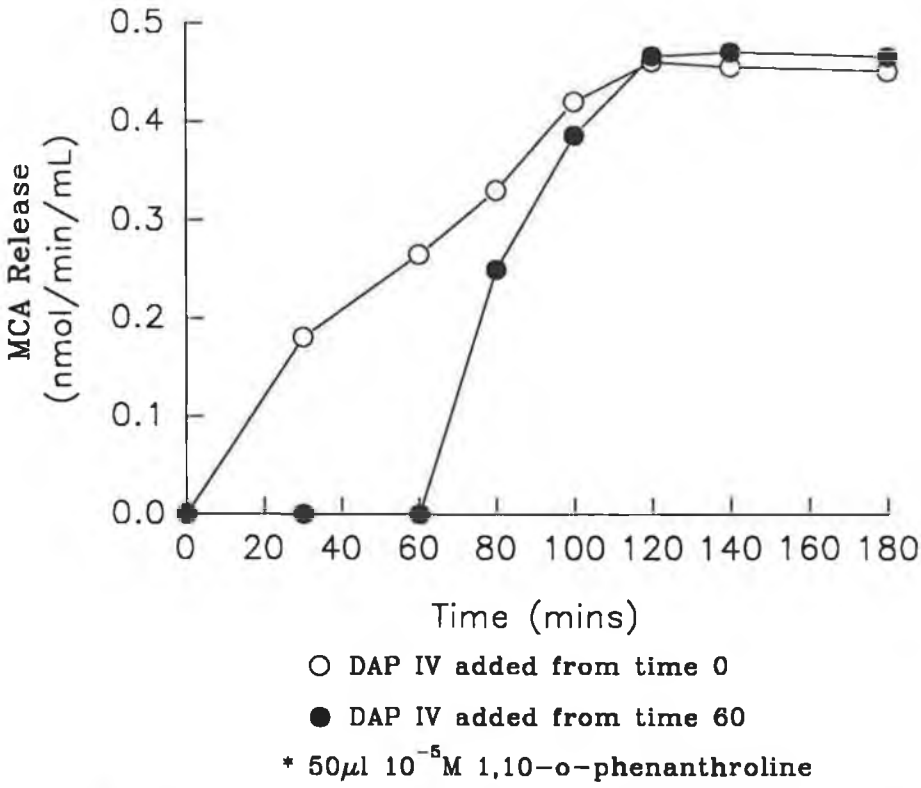


Fig. 3.8. MCA Release from <Glu-His-ProMCA in the Absence and Presence of DAP IV

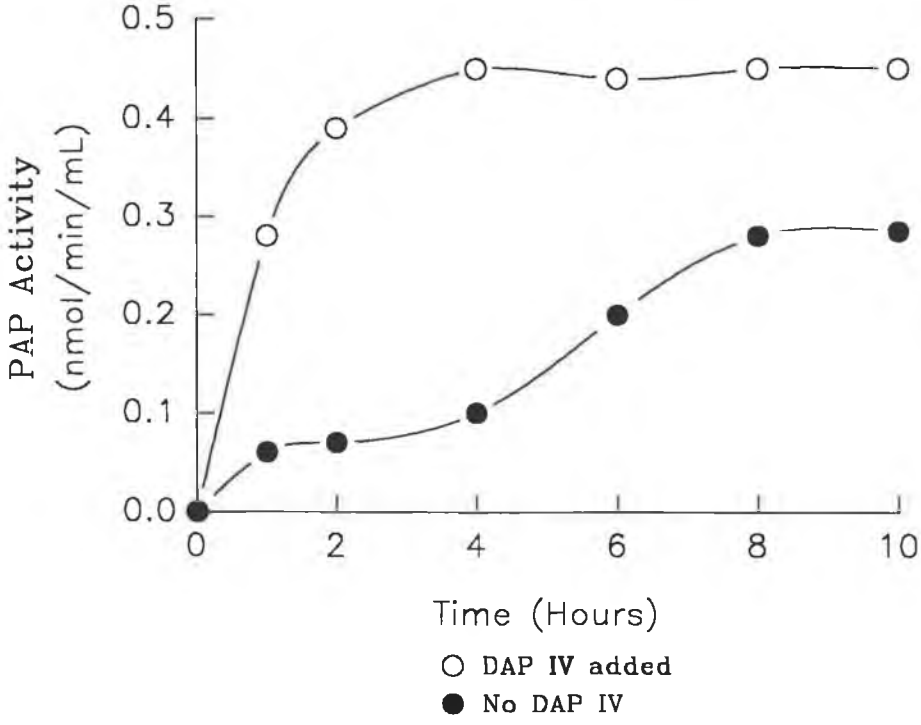


Fig. 3.9. Release of MCA from <Glu-His-ProMCA Following Incubation with PAP and Cyclisation at 80°C

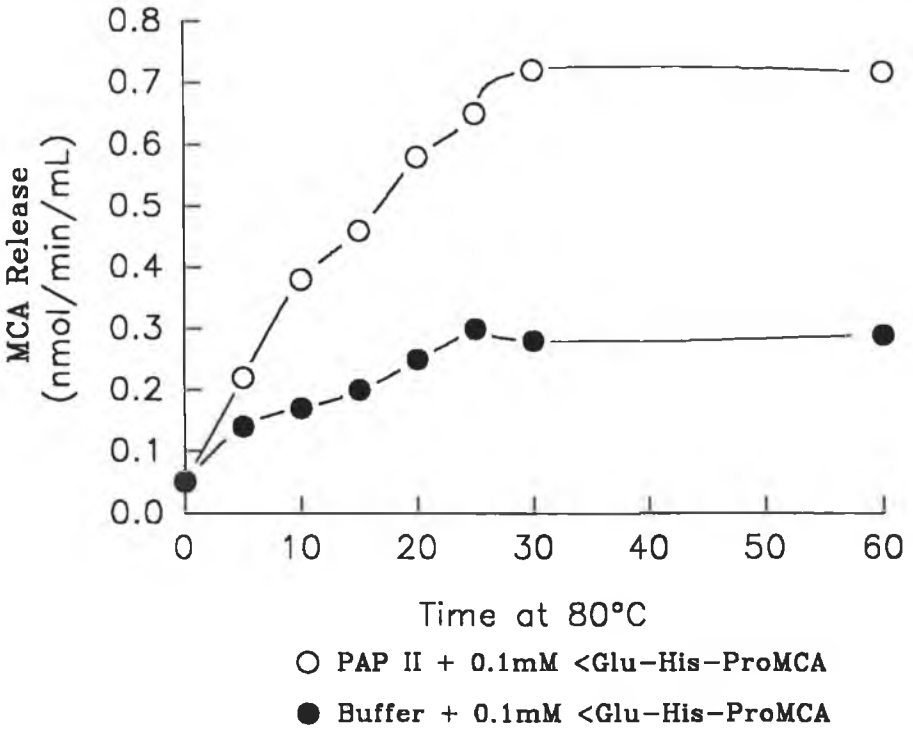


Fig. 3.10. Release of MCA from <Glu-His-ProMCA by PE Over Time

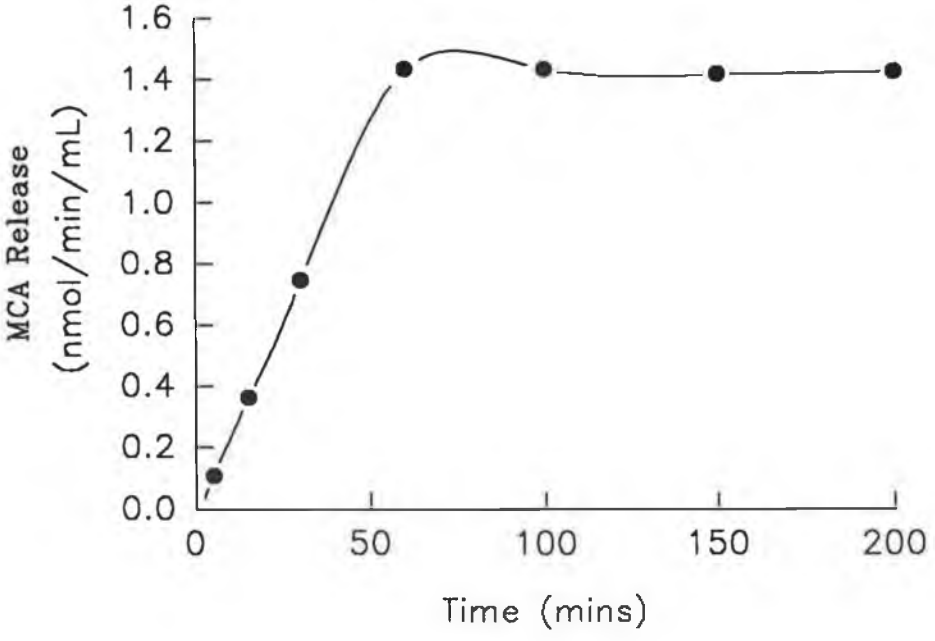
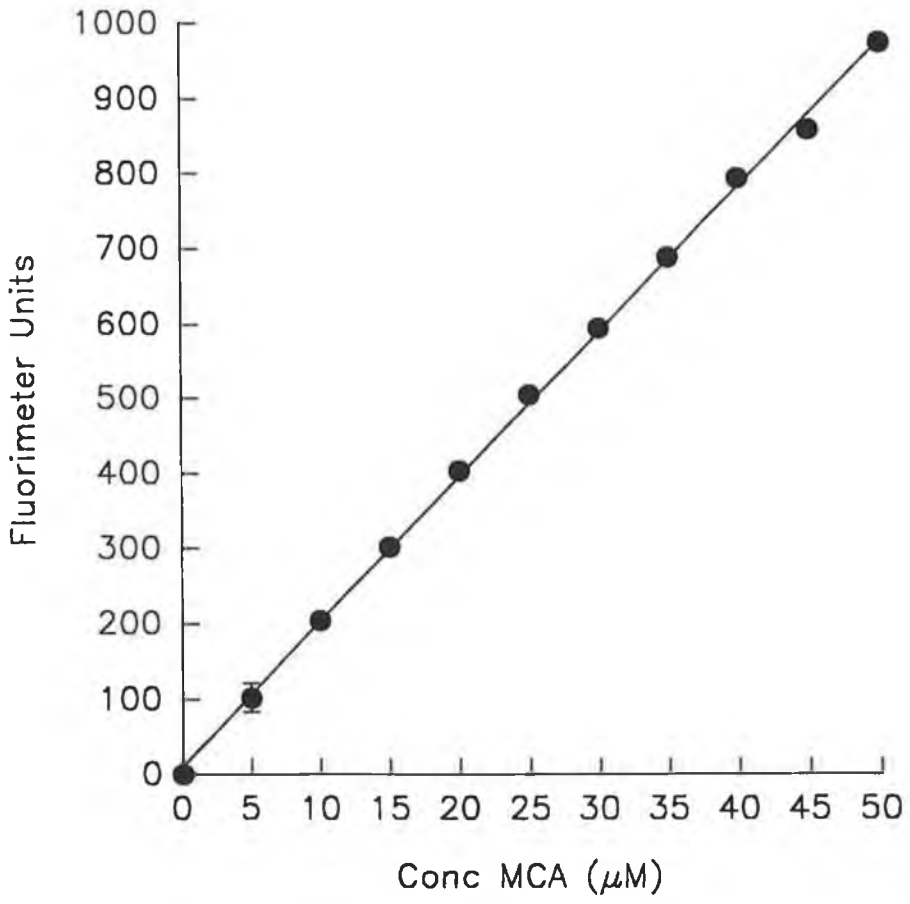


Fig. 3.11. 7-Amino-4-Methyl Coumarin (MCA) Standard Curve



Range= 0−50μM MCA

Excitation Slit Width = 10nm
Emission Slit Width = 2.5nm

Regression Coefficient = 0.99955

By varying the pH of the reaction, it was hoped to speed up the rate of cyclisation, but at alkaline pHs there is an interference with the emission of fluoresence. At acidic pHs, the fluoresence was not affected, but there was no increase in the cyclisation. Several reports have found that the rate of cyclisation was optimum at physiological pH (Peterkofsky et al.,1982; Bungaard and Moss, 1990), and so pH 7.4 was used for all the assays, with 1.5M acetic acid then used to stop the reaction.

The rate at which the cyclisation occurs was investigated by increasing the temperature of the reaction after the initial 60 mins incubation. The enzyme reaction was stopped as usual after 60 mins at 37°C, and the samples were then placed in an 80°C waterbath. The release of free MCA was monitored over time (Fig. 3.9).
The rate of release of MCA was increased, with levels apparently higher than those of the coupled enzyme assay (0.7nmol/min/mL) being achieved after 30 mins at 80°C. However a major problem lies in the degradation of the substrate at such high temperatures. Once this degradation is accounted for in any estimations of enzyme activity, the levels of free MCA are comparable to those of the coupled enzyme assay. Under these conditions the assay may be used to measure PAP activity.

Fig. 3.10 shows that <Glu-His-ProMCA can also be used as a substrate for measuring prolyl endopeptidase activity, although throughout this work Z-Gly-ProMCA was used routinely for quantifying PE activity.
A standard curve of fluorimeter units versus MCA concentration is shown in Fig. 3.11. It has a correlation coefficient of 0.99955. This curve was routinely used to calculate the enzyme activities.

3.2 Thyroliberin-Degrading Activities in Different Subcellular Locations

3.2.1. Demonstration of Two Thyroliberin-Degrading Pyroglutamate Aminopeptidases in Different Subcellular Locations of Bovine Brain.

When bovine brain homogenates were resolved into a particulate and cytosolic fraction, both fractions were assayed for PAP activity using the new fluorimetric coupled assay. The samples were assayed both in the presence and absence of DTT and EDTA, as cytosolic PAP has been reported to require a sulphydryl reagent for activity (Browne and O'Cuinn, 1983). As can be seen in Table 3.1, very low levels of PAP activity are detected in the suppernatant. However, in the presence of DTT and EDTA, 65% of the total brain PAP activity is found in the soluble fraction. The low levels of PAP activity in the cytosolic fraction in the absence of DTT and EDTA are characteristic of this enzyme requiring a thiol reagent for activity (Browne and O'Cuinn, 1983).
Increased PAP activity was also found in the particulate fraction with the addition of DTT and

EDTA, and therefore all further assays were carried out in the presence of 1mM DTT and 1mM EDTA. Overall 38% of the total brain PAP activity was detected in this particulate fraction. The rise in particulate PAP activity in the presence of DTT and EDTA is of particular note as previously identified particulate PAP activities have reported that the particulate PAP activity is inhibited by EDTA, even at 1mM concentrations (O'Connor and O'Cuinn, 1984).

Table 3.1 Demonstration of the Presence of Two PAP Activities In Different Subcellular Locations of Bovine Brain

Fraction	Total PAP Activity (nmol/min) (-1mM DTT/1mM EDTA)	Total PAP Activity (nmol/min) (+1mM DTT/+1mM EDTA)
Crude Homogenate	68.97 (100%)	182.88 (100%)
Supernatant	2.12 (3.1%)	119.85 (65%)
Particulate Fraction	26.1 (37.8%)	66.26 (35.5%)

3.2.2. Demonstration of Two Thyroliberin-Degrading Prolyl Endopeptidase Activities in Different Subcellular Locations of Bovine Brain.

The supernatant and particulate fractions were also assayed for PE activity using the substrate Z-Gly-ProMCA. To date, PE activity had only been characterised from the cytosolic fraction of brain, although it has been suggested that a particulate PE activity may be present in small quantities (Dresdner et al., 1982; Camargo et al., 1984). There was a sizeable difference in the amount of PE activity detected, even in the crude homogenate compared to the levels of PAP activity in the crude homogenate (Table 3.2).

In the presence of DTT and EDTA, nearly 50% of the total PE activity was detected in the soluble fraction, with about 42% PE activity found in the particulate fraction. This was a higher amount of PE activity in the particulate fraction than had previously been reported from other sources. Camargo et al. (1984) suggested 10% PE was membrane-associated in rabbit brain, while Dresdner et al. (1982) suggested that 35% of the PE activity could be found in the crude mitochondrial fraction from the same source. However both of these studies comment on the very low levels of PE activity present in the particulate fraction, and of the activity probably being associated with a crude mitochondrial fraction.

The addition of DTT and EDTA to the homogenisation mixture caused little variation in the levels of PE activitiy, there was a slight increase in the total PE activity, but this increase was reflected by a similar increase in the soluble and particulate PE activities.

Table 3.2 Demonstration of the Presence of Two Prolyl Endopeptidase Activities in Different Subcellular Locations of Bovine Brain.

Fraction	Total PE Activity (nmol/min) (-1mM DTT/1mM EDTA)	Total PE Activity (nmol/min) (+1mM DTT/+1mM EDTA)
Crude Homogenate	2506.7 (100%)	3003.1 (100%)
Supernatant	1267.12 (50.5%)	1467.12 (48.8%)
Particulate Fraction	1068.4 (42.6%)	1275.8 (42.4%)

3.3 Detection of Possible Occluded Enzyme Activities

3.3.1. Effect of Osmotic Shock on the Release of PAP from the Particulate fraction

Following an initial 0.5M salt-washing step, the particulate fractions were subjected to an osmotic shock by resuspending the salt-washed pellets in distilled water with gentle rehomogenisation. This osmotic shock step was employed to determine whether the PAP activity was actually membrane-bound or was in fact occluded in vesicles as has been reported for many 'membrane-bound' enzymes (Van Amsterdam et al., 1983; Dalmaz et al., 1986). Half of the pellets were resuspended in potassium phosphate buffer as a control. After vigorous washing of the pellets with either the buffer or the distilled water the samples were centrifuged as described before (Section 2.8.2.). As can be seen from the results in Table 3.3, no extra PAP activity was released from the membrane fractions following washing with either buffer or distilled water. These results strongly suggest that the PAP activity is a membrane- bound enzyme and is not just a soluble activity that has become occluded in the vesicles that are formed by the homogenisation process.

3.4.2. Effect of Osmotic Shock on the Release of PE from the Particulate Fraction

As described above, the membrane fraction was subjected to an osmotic shock in order to release any PE activity that may be occluded in vesicles formed by the membranes. The results are shown in Table 3.4. Following the osmotic shock step, another 3.46nmol/min/mL PE activity were released which only represents 0.4% of the total salt-washed particulate PE activity and is due to any occluded PE activity that was trapped in vesicles by the membranes. This figure is smaller than the 0.6% PE activity released by the buffer wash. Therefore the effect of the osmotic shock on the release of PE activity from the membranes is minimal. Any PE activity that remains in the particulate fraction following the initial salt-washing and an osmotic shock step should either be a membrane-associated or membrane-bound protein.

Table 3.3 Effect of Osmotic Shock on Particulate PAP Activity

Fraction	PAP Activity (nmol/min/mL)	% PAP Activity (as a % of the salt-washed pellet)
0.5M Salt-Washed Membrane Fractions	16.40	100.00
Control (Buffer-Washed Pellet)	15.60	93.90
Control (Buffer-Wash Supernatant)	0.00	0.00
Osmotically-Shocked Membranes	15.73	95.90
Osmotically-Released Material	0.00	0.00

Table 3.4 Effect of Osmotic Shock on Particulate PE Activity

Fraction	PE Activity (nmol/min/mL)	% PE Activity
0.5M Salt-Washed Membrane Fractions	806.00	100
Control (Buffer-Washed Pellet)	600.64	74.4
Control (Buffer-Wash Supernatant)	5.31	0.6
Osmotically-Shocked Membranes	599.80	74.4
Osmotically-Shocked Material	3.46	0.4

3.4 Salt-Washing of the Particulate Fractions

3.4.1. Effect of Repeated Salt-Washing on the Particulate PAP Activity

Following the identification of a particulate PAP activity, a series of repeated salt-washing steps were performed on the particulate fraction to determine whether the enzymes were membrane-bound or just non-specifically associated with the membrane fraction. The pellet from the osmotic shock step was repeatedly resuspended in a 4M NaCl solution. Each time the supernatant and a sample of the pellet were assayed for PAP activity. Fig. 3.12 shows how the level of PAP activity in the washes drops very quickly following sequential salt-washing, while the PAP activity in the particulate fraction remains at a constant level (12.5nmol/min/mL), which represents 74% of the activity of the post-osmotic shock pellet, and is approximately 26% of the original crude particulate pellet.

3.3.2. Effect of Repeated Salt-Washing on the Particulate PE Activity

Fig. 3.13 shows how PE activity remains associated with the particulate fraction, even after vigorous salt-washing. The level of PE activity in the final wash is negligible, while that in the particulate fraction is at approximately 35% of that in the original post-osmotic shock pellet. The figure represents 19.6% of the original crude particulate pellet. Such activity remaining associated with the particulate fraction after a series of salt washes suggests that the PE is more than just loosely associated with the membrane, as repeated salt washing should dislodge any non-specific loosely-bound proteins.

Fig. 3.12 The Effect of Repeated 4M Salt Washes on Synaptosomal PAP Activity

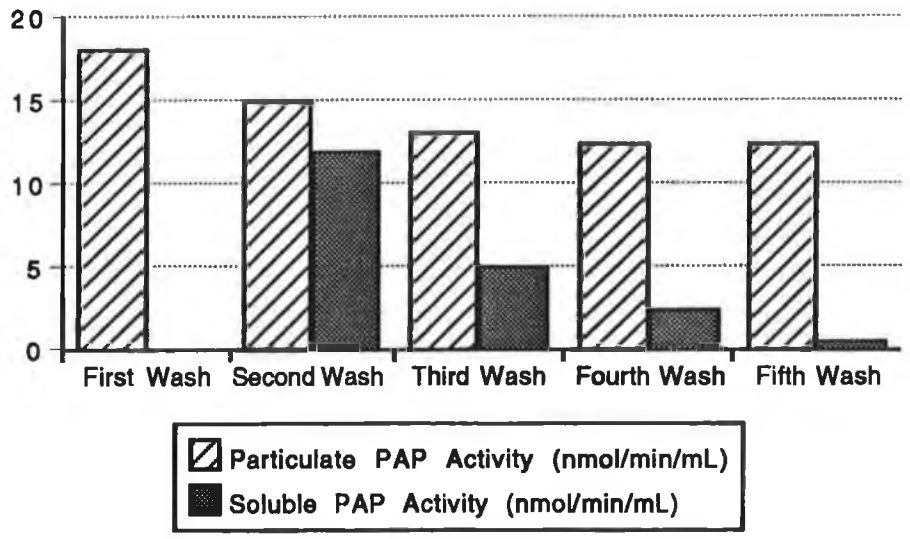
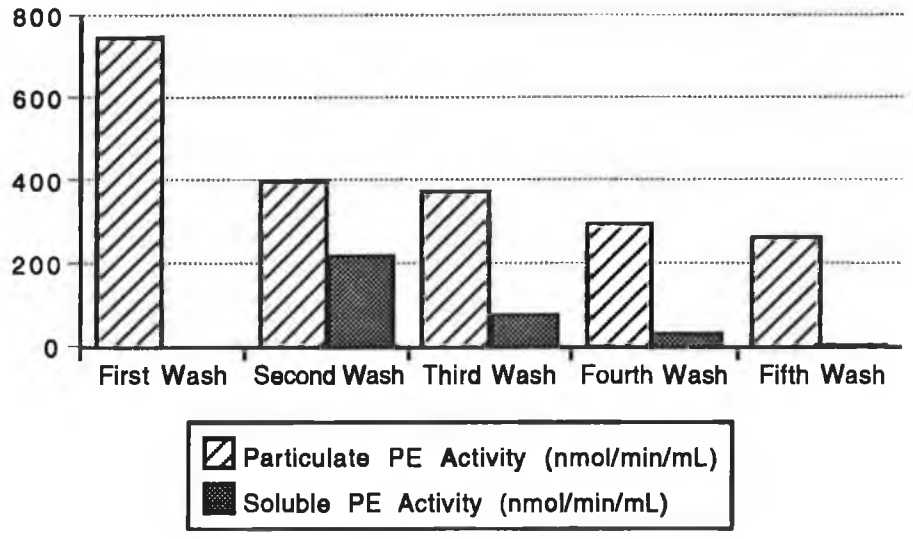


Fig. 3.13 The Effect of Repeated 4M Salt Washes on Synaptosomal PE Activity



3.5 Subcellular Localisation of the Particulate Thyroliberin-Degrading Activity

3.5.1. Subcellular Localisation of the Particulate PAP Activity

Using a combination of differential centrifugation and isopynic ultracentrafugation (with sucrose

gradients), the salt-washed particulate fractions of bovine brain were divided into three fractions. These fractions were subsequently identified as the myelin, synaptosomal membrane and mitochondrial fractions by use of specific enzyme marker assays as described in section 2.6. Following their identification, each fraction was assayed for PAP activity (Table 3.5). Following subfractionation of the salt-washed particulate fraction there was a three-fold enrichment of PAP specific activity in the synaptosomal fraction. This represented 75% of the total PAP activity. The PAP specific activity actually decreased in the myelin and mitochondrial fractions. The localisation of PAP activity with the synaptosomal membranes is in agreement with previous reports of PAP localisation (O'Connor and O'Cuinn, 1984).

Table 3.5 Subcellular Localisation of the Particulate PAP Activity

Fraction	SDH (nmol/min/ mg)	LDH (nmol/min/ mg)	5'-Ribonucleo-tidase (nmol/min/ mg)	PAP Specific Activity (nmol/min/ mg)	PAP in each fraction as a % of that in Crude Homogenate
Crude Homogenate	3.61	1.00e+5	0.57	18.50	100.00
Supernatant	2.43	3.07e+5	0.10	13.13	71.16
Salt-Washed Particulate Fraction	0.62	2.89e+4	0.76	4.92	26.70
Salt-Washed Pellet Fractionated Into:					PAP as a % of the Salt-Washed Particulate Fraction
Myelin	0.11	1.59e+4	0.32	3.41	18.45
Synaptosomal membranes	0.33	6.15e+4	1.96	13.98	75.80
Mitochondria	1.36	8.88e+4	0.44	3.66	19.80

3.5.2. Subcellular Localisation of the Particulate PE Activity

Using similar subcellular localisation methods as those employed for the localisation of PAP activity, PE activity was also found to be associated with the synaptosomal membranes (Table 3.6). The apparent low level of PE activity was the result of soluble PE being removed during osmotic shock and loosely, non-specifically-bound activity being removed by repeated salt-washing. The remaining PE activity was all membrane-associated or membrane-bound. There was a 2-fold enrichment in PE specific activity from the salt-washed fraction to the synaptosomal membranes. There was only a slight increase in specific activity in the mitochondrial fraction and a decrease in specific activity in the myelin fraction. This high figure of synaptosomal membrane-associated PE activity is in contrast to previous reports of PE activity being primarily found in the cytosolic fraction of brain. All previous reports of a particulate PE refer to its low activities, its probable presence in the membranes solely as an occluded activity, and of its presence in the crude mitochondrial fraction (Dresdner et al., 1982; Dalmaz et al., 1986). No report to date has investigated the actual location of the PE activity within the crude particulate fraction which would contain mitochondrial, microsomal and synaptosomal membranes.

The ability of the PE activity to remain in the synaptosomal membrane fraction following repeated salt-washes and hypo-osmotic shock indicates that the activity is more than just membrane-associated enzyme.

Table 3.6 Subcellular Localisation of the Particulate PE Activity

Fraction	SDH (nmol/min/ mg)	LDH (nmol/min/ mg)	5'-Ribonucleo-tidase (nmol/min/ mg)	Specific PE Activity (nmol/min/ mg)	PE in each fraction as a % of that in Crude Homogenate
Crude Homogenate	3.61	1.00e+5	0.57	833.40	100.00
Supernatant	2.43	3.07e+5	0.10	573.70	48.80
Salt-Washed Particulate Fraction	6.18	2.89e+4	0.76	159.07	19.08
Salt-Washed Pellet Fractionated Into:					PE as a % of the Salt-Washed Pellet
Myelin	0.11	1.59e+4	0.32	60.25	7.22
Synaptosomal membranes	0.33	6.15e+4	1.96	252.90	30.30
Mitochondria	1.36	8.88e+4	0.44	156.60	18.80

3.6 Phase-Partitioning Studies on the Particulate Thyroliberin-Degrading Activities

3.6.1. Phase-Partitioning Study on the Particulate PAP Activity

The phase separation of membrane proteins was first described by Bordier (1981). This technique is used to differentiate between integral and peripheral membrane proteins. A solution of the nonionic detergent Triton X-114 is homogeneous at 0°C but separates in to an aqueous phase and a detergent phase above 20°C. The Triton X-114 is used to solubilise the membrane's enzymes and the solublised material is subjected to phase separation. Hydrophilic (peripheral) enzymes are found exclusively in the aqueous phase, and integral membrane

enzymes with an amphiphilic nature are recovered in the detergent phase.
Table 3.7 shows the results of the phase-partitioning of the PAP activity from the synaptosomal membranes. 60% of the PAP activity was found in the Triton X-114-enriched hydrophobic layer indicating that it exists for the most part as an integral membrane protein, with the majority of its surface embedded in the membrane leading to its hydrophobic nature.

Table 3.7 Phase-Partitioning Study on the Synaptosomal PAP Activity with Triton X-114

Fraction	PAP activity (nmol/min/mL)	PAP Activity as a % of that in Synaptosomal Membranes
Synaptosomal Membrane	0.84	100.00
Triton X-114 Depleted Layer	0.03	3.60
Triton X-114 Enriched Layer	0.51	60.00

3.6.2. Phase-Partitioning Study on the Particulate PE Activity

Using the same procedure as described in section 3.6.1., the majority of the PE activity was found to be associated with the hydrophilic upper layer (Table 3.8). 87% of the PE activity was found in the Triton X-114 soluble layer, thereby indicating that the particulate PE is a peripheral membrane protein i.e. that the majority of the enzyme, containing its active sites, could be on the surface of the membrane and be exposed to the hydrophilic surroundings.

Table 3.8 Phase Partitioning Study on the Synaptosomal PE Activity using Triton X-114

Fraction	PE Activity (nmol/min/mL)	PE Activity as a % of that in Synaptosomal membranes
Synaptosomal Membranes	10.4	100
Triton X-114 Soluble Layer	9.02	86.7
Triton X-114 Insoluble Layer	0.84	8.7

3.7 Release of the Particulate Thyroliberin-Degrading Activities from the Synaptosomal Membranes

3.7.1. Release of Particulate PAP Activity from the Synaptosomal Membranes.

Following localisation of the PAP activity on the synaptosomal membranes, the next step was to find an efficient method to release it from the membranes. Table 3.9 shows how a number of methods were used, including the detergent Triton X-100, the proteolytic enzymes trypsin and papain. Appropriate control washes were also included. Sonication was also tried as a method of releasing the enzyme from the membranes, but it was found to completely destroy the enzyme activity. It can be seen that while some of the procedures eg trypsin and papain, are very effective in removing protein from the sample, the amount of PAP activity released is quite small, and so the specific activity of the resulting sample is very low. The most efficient means of releasing PAP activity from the synaptosomal membranes was by solubilising the enzyme from the membrane with the detergent Triton X-100. This step gives a yield of 75% PAP activity from the initial salt-washed particulate fraction. Further optimisation with Triton X-100 showed that a concentration of 0.4% (v/v) was optimal (Fig. 3.14). Triton X-114, as was used for the phase-partitioning step, was not used for enzyme solubilisation as it was too severe on the enzyme activity.

3.7.2. Release of the Particulate PE Activity from the Synaptosomal Membranes.

In order to release the PE activity from the synaptosomal membranes, an approach similar to that used for PAP was employed. As with particulate PAP activity, Triton X-100 proved to be the most efficient means of releasing PE activity from the membranes (Table 3.10). Sonication was found to almost completely inactivate the enzyme activity, yielding only 5% recovery of PE activity. Even though papain reduced the protein concentration of the sample more than any other method (90% loss of protein), it caused a very low release of PE activity (22%), resulting in a low specific activity. Fig. 3.15 shows how a concentration of 0.3-0.4% Triton X-100 was required to solubilise the maximum amount of PE activity from the synaptosomal membranes.

Table 3.9 Release of Particulate PAP from the Salt-Washed Synaptosomal Membranes

Control @ 4°C=particulate fraction incubated at 4°C for 60min
Control @30°C=particulate fraction incubated at 30°C for 60min
Sonication= 2 5-second pulses on ice
Trypsin solubilised=particulate fraction incubated at 30°C for 60 min
Papain solubilised= particulate fraction incubated at 30°C for 60 min
Triton X-100 solubilised=particulate fraction incubated on ice for 60 min

Fraction	Total Activity (nmol/min)	Total Protein (mg)	Specific Activity (nmol/min/mg)	Purification Factor	% Recovery
Salt-Washed Particulate Fraction	18.200	2800.000	0.007	1.000	100.00
Control (@ 4 C)	2.090	171.730	0.012	1.910	11.50
Control (@ 30 C)	2.750	194.130	0.014	2.200	15.10
Sonication	0.010	399.200	0.000	0.000	0.00
Trypsin Solubilised (20,800 units of activity)	5.220	446.250	0.012	1.900	28.70
Papain Solubilised (30.4 units of activity)	5.770	268.800	0.021	3.230	31.70
Triton X-100 Solubilised	13.600	329.760	0.040	6.150	74.90

Table 3.10 Release of the Particulate PE from the Salt-Washed Synaptosomal Membranes

Control @ 4°C=particulate fraction incubated at 4°C for 60min
Control @30°C=particulate fraction incubated at 30°C for 60min
Sonication= 2 5-second pulses on ice
Trypsin solubilised=particulate fraction incubated at 30°C for 60 min
Papain solubilised= particulate fraction incubated at 30°C for 60 min
Triton X-100 solubilised=particulate fraction incubated on ice for 60 min

Fraction	Total Activity (nmol/min)	Total Protein (mg)	Specific Activity (nmol/min/mg)	Purification Factor	% Recovery
Salt-Washed Particulate Fraction	820.10	2800.00	0.29	1.00	100.00
Control (@4 C)	168.10	297.07	0.57	1.97	20.50
Control (@30 C)	159.10	315.50	0.19	0.70	19.40
Sonication	46.20	400.50	0.11	0.39	5.50
Trypsin Solubilised (20,800 units of activity)	216.50	446.25	0.26	0.90	26.40
Papain Solubilised (30.4 units of activity)	181.20	268.80	0.22	0.76	22.10
Triton X-100 Solubilised	676.60	329.76	2.05	7.10	82.50

Fig. 3.14 Effects of a Range of Triton X-100 Concentrations on the Release of PAP Activity from the Synaptosomal Membranes

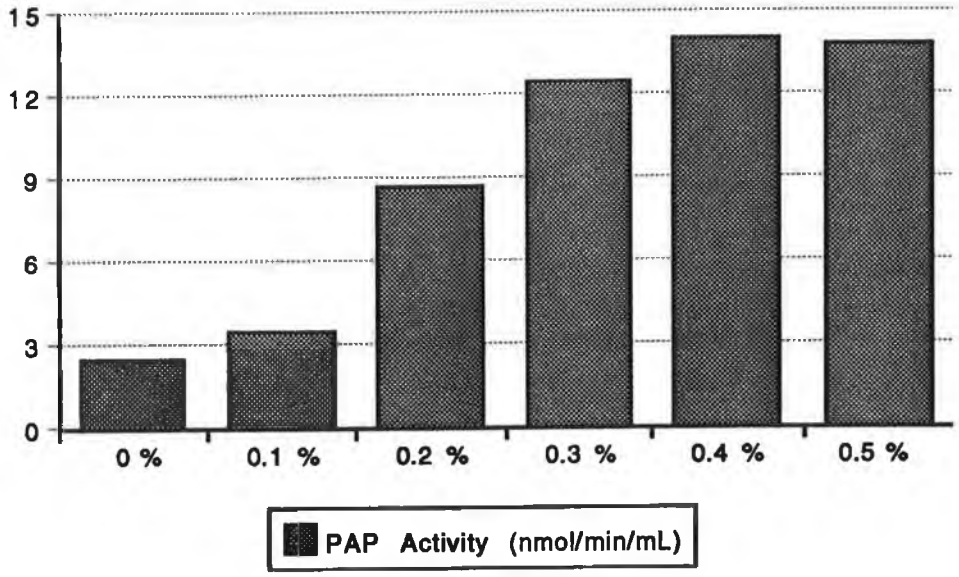
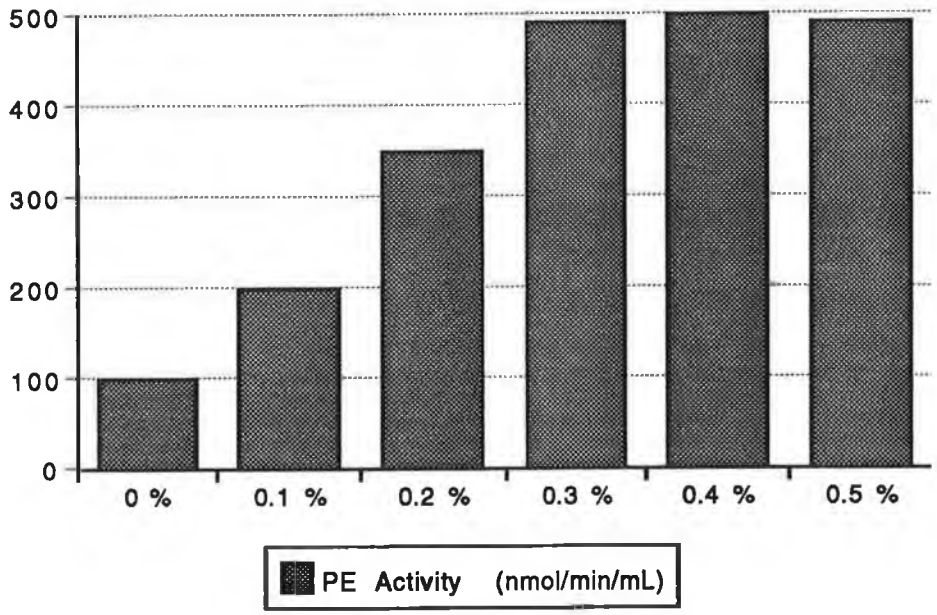


Fig. 3.15 Effects of a Range of Triton X-100 Concentrations on the Release of PE from the Synaptosomal Membranes



3.8 Purification of the Particulate PAP Activity from the Synaptosomal Membranes of Bovine Brain

3.8.1. Gel Filtration Chromatography on Sephacryl S-200 HR

PAP activity was released from the synaptosomal membranes by treatment with Triton X-100, and separated from the particulate matter by centrifugation (as outlined in section 2.10.2.1.). A 5mL sample of Triton X-100-solubilised PAP was applied to a Sephacryl S-200 column. The elution profile is shown in Fig. 3.16. The enzyme activity eluted close to the void volume and was clear from the main protein peaks. The fractions with the highest activities were pooled and were stored at 4°C.

3.8.2. Ion-Exchange Chromatography on DEAE-Sepharose

A 4mL sample of Triton X-100-solubilised PAP was applied to a DEAE- Sepharose anion exchange column. The column was equilibrated with a 50mM potassium phosphate buffer, pH 7.4. The sample was eluted using a linear salt gradient (0.2 - 0.5M NaCl). The PAP activity separated clearly from the bulk of the protein (Fig 3.17). The most active fractions were pooled and stored at 4°C.
Ion exchange chromatography gave a consistently higher yield of PAP activity than gel filtration and so it was regularly used as the first step in the purification scheme.

3.8.3. Concentration of the Post-Ion Exchange PAP Activity

Following ion-exchange the PAP activity was concentrated by reverse dialysis on a bed of PEG 6000. This step was incorporated into the purification scheme as the volume of the pooled fractions following ion exchange was too large to apply to a 2mL phenyl sepharose column. A 5mL post-ion exchange PAP sample was concentrated down to 2 mLs.

3.8.4. Hydrophobic Interaction Chromatography on Phenyl Sepharose

The concentrated synaptosomal PAP sample was applied to a phenyl sepharose column under high salt conditions (2M $(NH_4)_2SO_4$). The PAP activity was eluted using a positive linear glycerol gradient (0-25%) and a negative salt gradient (2M-0). The protein content of the samples was at a minimum following ion exchange and so there was very little protein to be separated (Fig 3.18). The most active synaptosomal PAP fractions were pooled and were stored at 4°C.

Table 3.11 summarises the purification scheme for synaptosomal PAP. There was a 20.7% yield of activity from the original synaptosomal fraction and the enzyme was purified 644-fold.

Fig. 3.16. Elution Profile of Synaptosomal PAP from Sephacryl S-200 HR

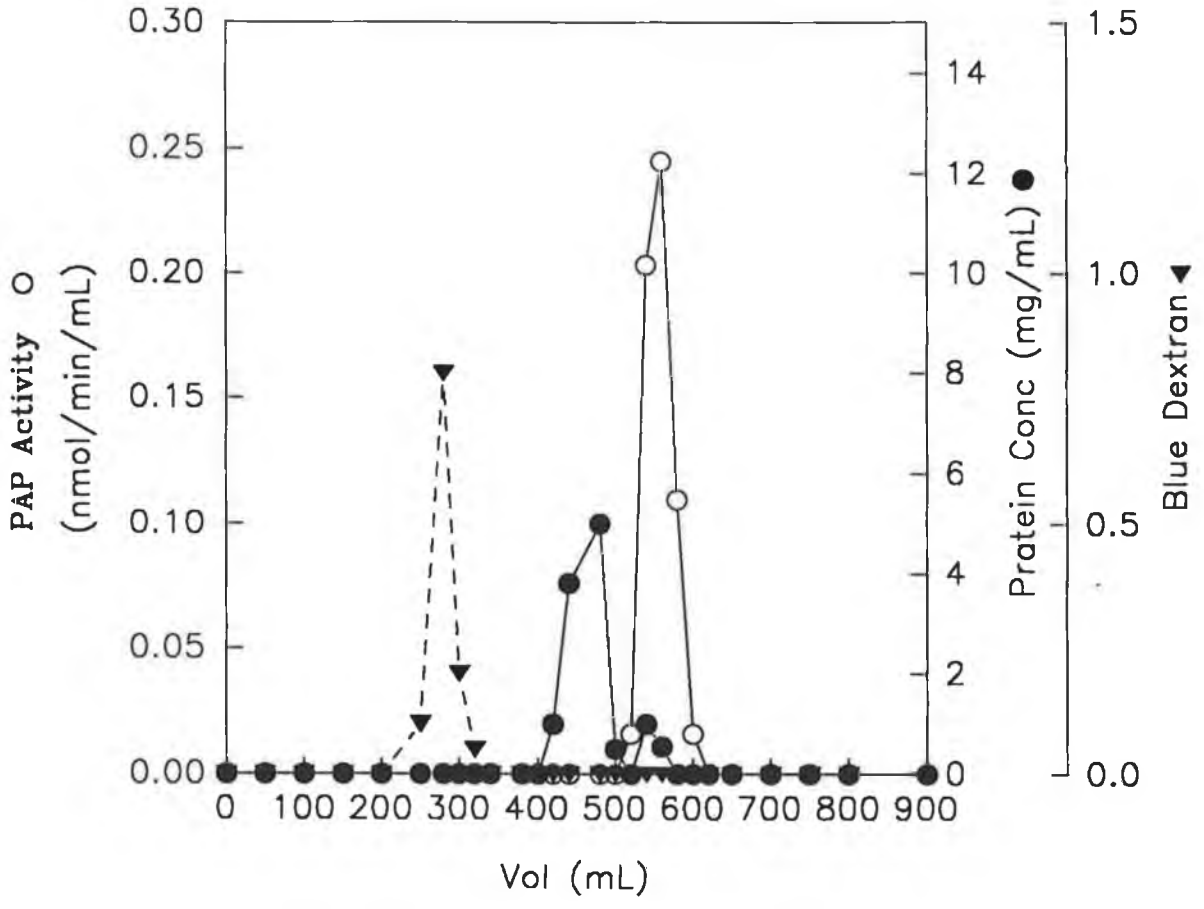
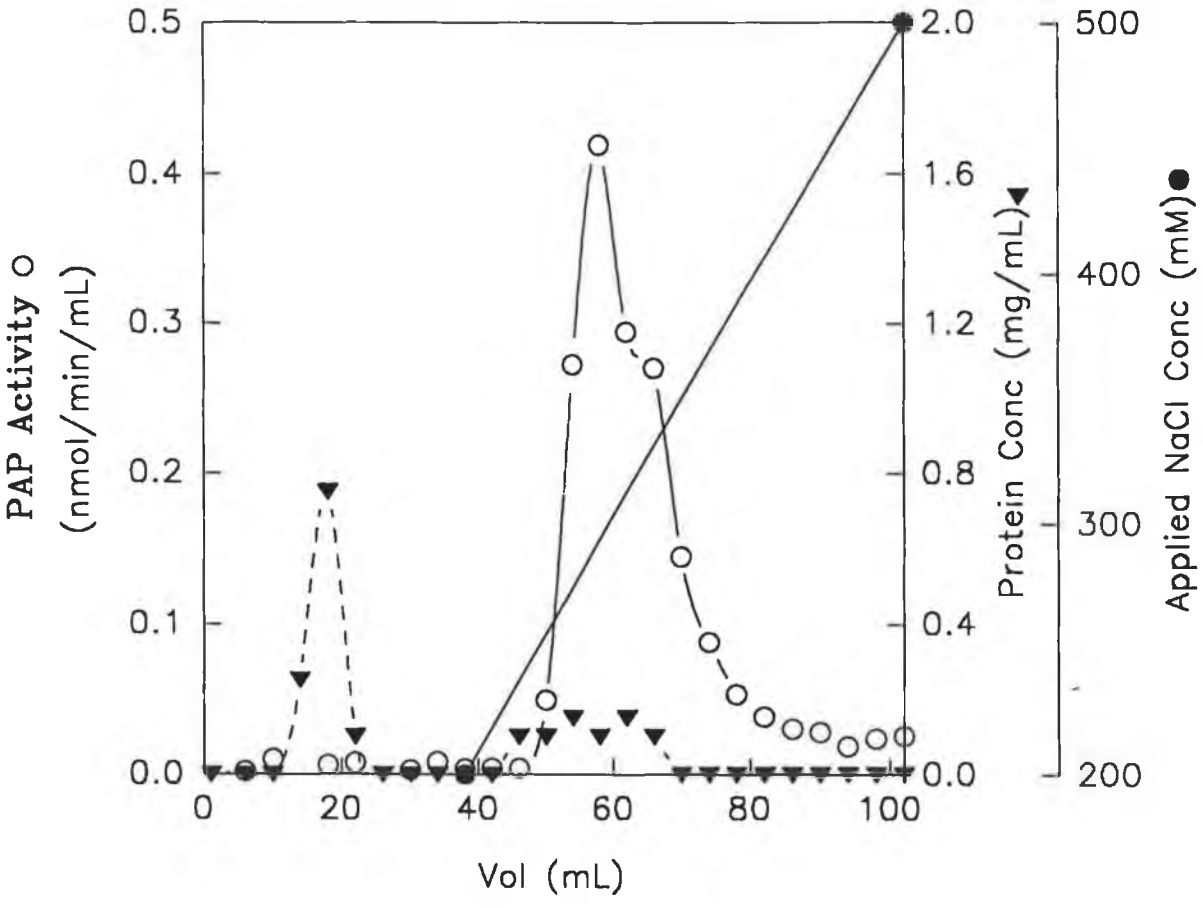


Fig. 3.17. Elution Profile of Synaptosomal PAP from DEAE Sepharose



Vol (mL)

Fig. 3.18. Elution Profile of Synaptosomal PAP from Phenyl-Sepharose

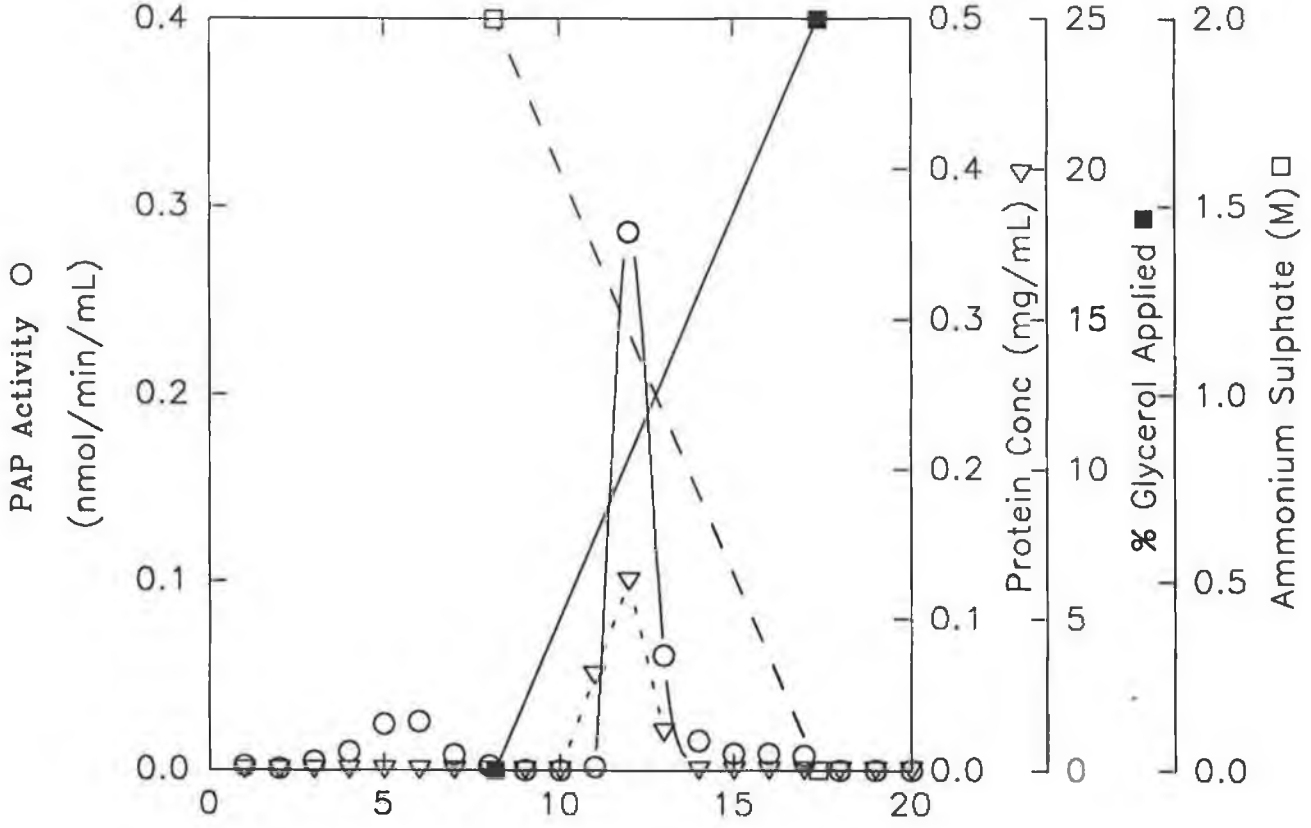


Table 3.11 Purification Table for Purified Synaptosomal PAP

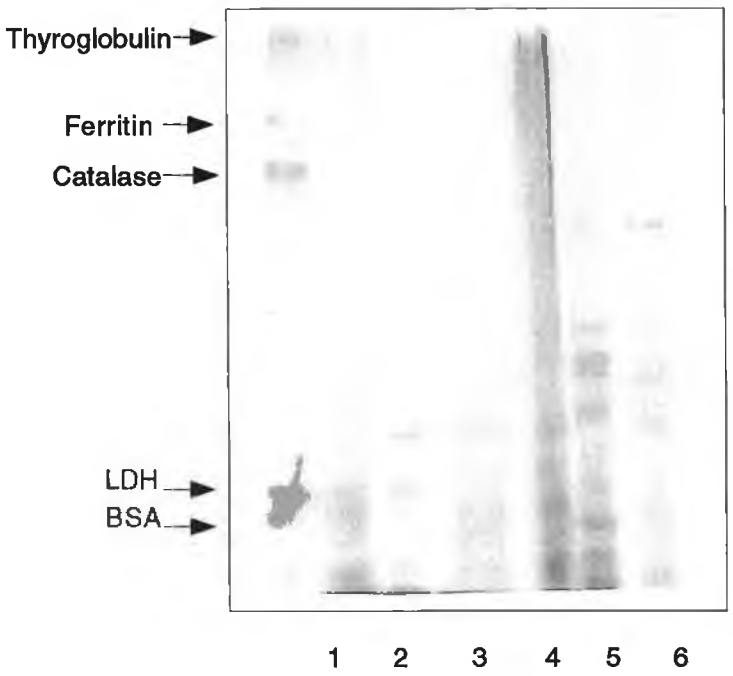
Fraction	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	% Recovery	Purification Factor
Crude Homogenate	6857.500	190.00	0.02	*	*
Synaptosomal Fraction	2838.00	69.00	0.02	100.00	1.00
Salt-Washed Synaptosomal Fraction	2010.00	20.20	0.01	29.30	0.05
Triton X-100 Solubilised	300.50	14.80	0.05	21.40	2.50
Post Ion-Exchange	23.50	16.70	0.63	24.20	35.50
Post Phenyl Sepharose	1.11	14.30	12.88	20.70	644.00

3.8.5. Assessment of the Synaptosomal PAP Purification Scheme by Electrophoresis

3.8.5.1. Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on a sample from each of the purification stages in order to monitor the actual purification and to estimate the molecular weight of the purified enzyme. Fig. 3.19 shows how the molecular weight of the purified synaptosomal PAP corresponds to a molecular weight of 230kDa.

Fig. 3.19 A 7.5% SDS-PAGE of the Bovine Synaptosomal PAP



1. Crude Homogenate
2. Synaptosomal Fraction
3. Salt-Washed Synaptosomal Fraction
4. Triton X-100 Solubilised Fraction
5. Post Ion Exchange Fraction
6. Post Phenyl Sepharose Fraction

3.9 Purification of the Particulate Prolyl Endopeptidase Activity from the Synaptosomal Membranes of Bovine Brain

3.9.1. Gel Filtration Chromatography on Sephacryl S-200HR

A 5mL Triton X-100-solubilised sample (as described in section 2.8.4.2.) was applied to a 500mL Sephacryl S200HR column on the Pharmacia Biopilot FPLC system. As can be seen in Fig. 3.20, the PE activity eluted much later than the synaptosomal PAP activity and was well separated from any major protein peaks. The most active fractions were pooled and stored at -20°C.

3.9.2. Hydroxylapatite Chromatography

Following gel filtration, a 10mL PE sample was dialysed for 3 hours into 20mM potassium phosphate buffer, pH 7.4. This sample was then applied to a calcium-phosphate (hydroxylapatite) column and the prolyl endopeptidase activity was eluted with a linear phosphate gradient (20-500mM) (Fig. 3.21). The PE eluted away from most of the protein peaks, which were already quite low (less then 0.1mg/mL). The most active fractions were pooled and stored at -20°C.

3.9.3. Hydrophobic Interaction Chromatography

5mL of a post-hydroxylapatite sample were applied to a phenyl-sepharose column under high salt conditions ($2M(NH_4)_2SO_4$). The PE activity was eluted using a positive linear (0-50%) glycerol gradient and at this stage the protein levels were almost undetectable (0.04mg/mL). Under these conditions, the synaptosomal PE activity eluted at approximately 45% glycerol (Fig.3.22).

Table 3.12 summarises the purification scheme for the synaptosomal PE activity. There was a 23% recovery of activity from the initial particulate fraction and the prolyl endopeptidase was purified 1407-fold.

Fig. 3.20. Elution Profile of Synaptosomal PE from Sephacryl S-200 HR

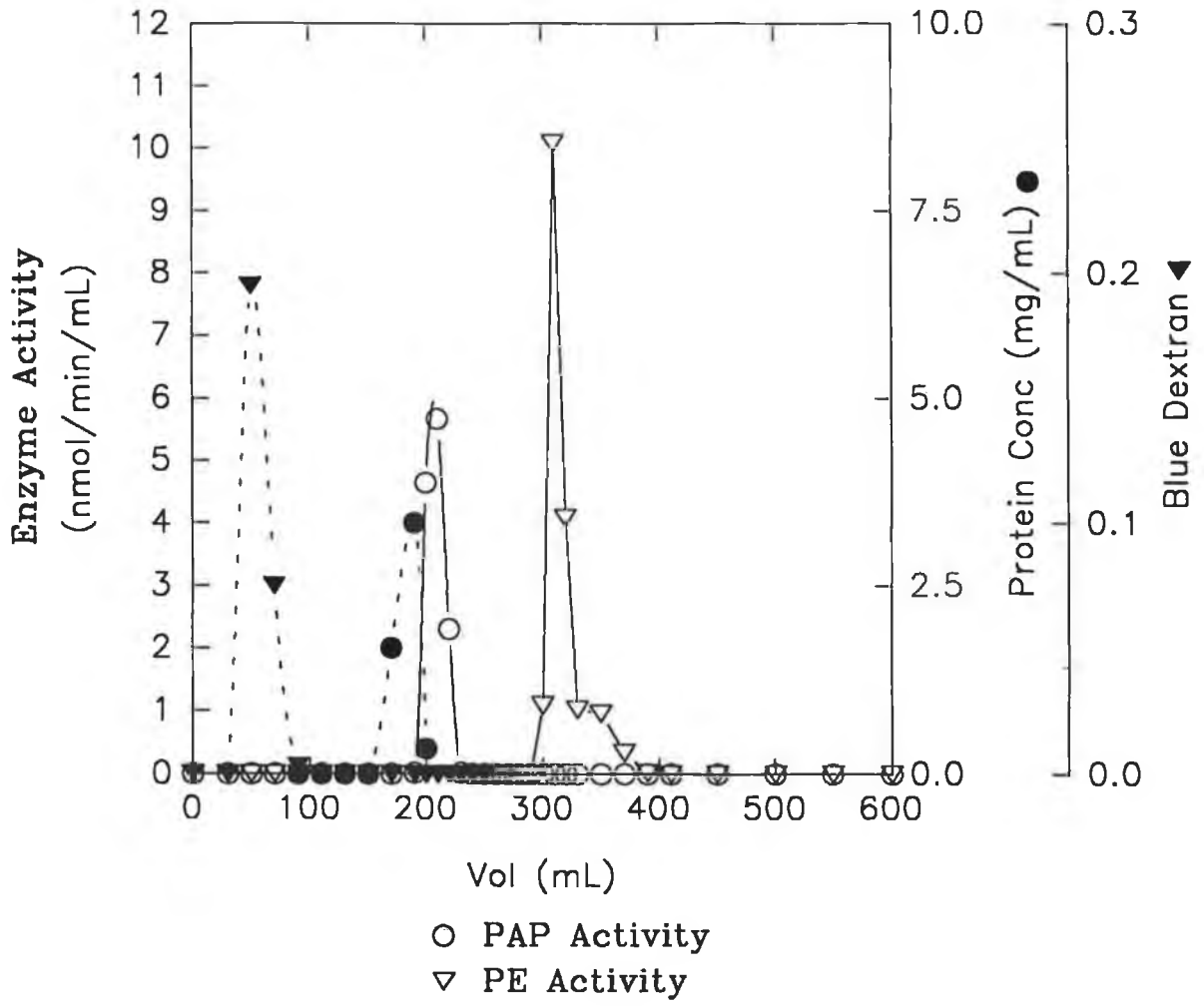


Fig. 3.21. Elution Profile of Synaptosomal PE from Hydroxylapatite

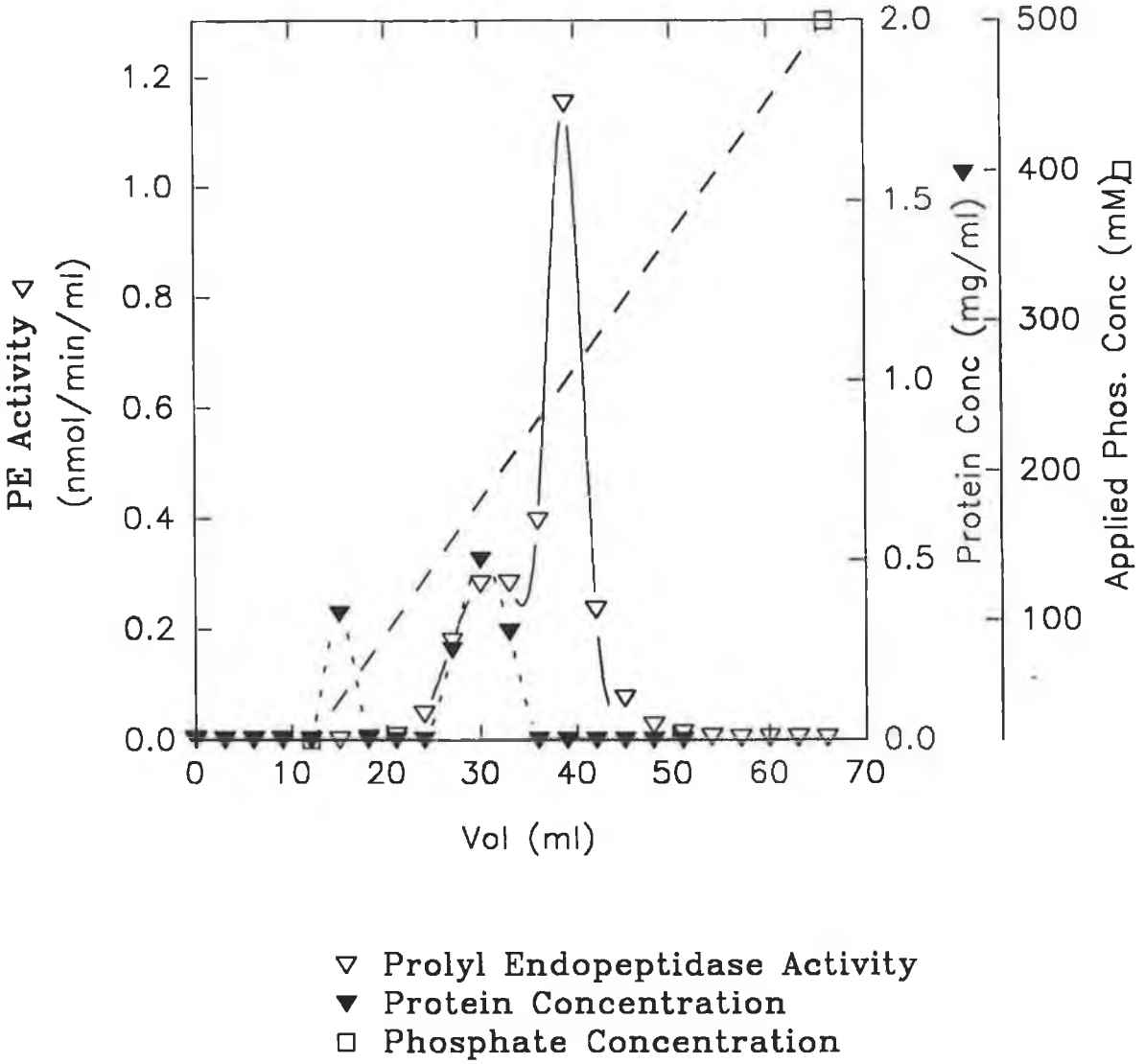


Fig. 3.22. Elution Profile of Synaptosomal PE from Phenyl-Sepharose

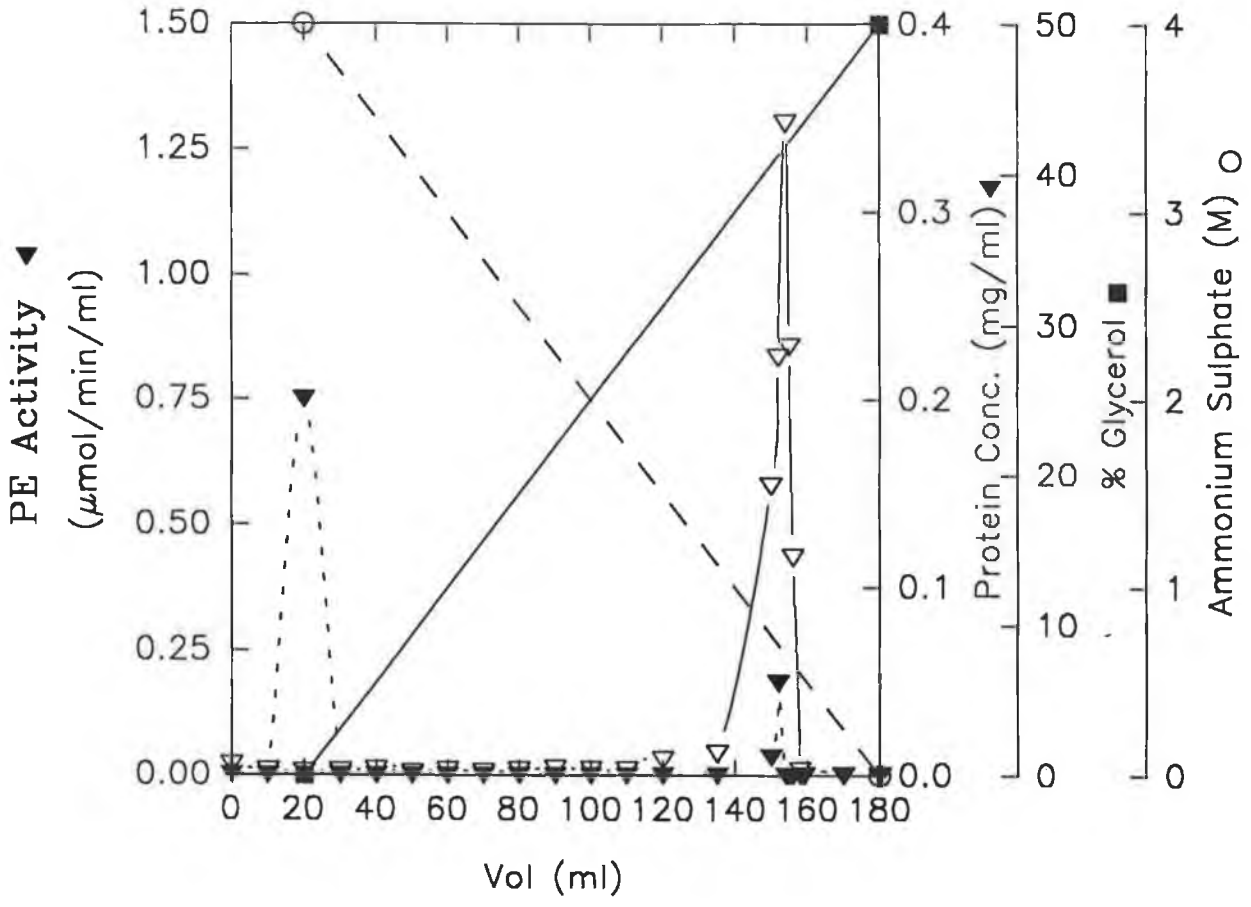


Table 3.12 Purification Table for Purified Synaptosomal PE

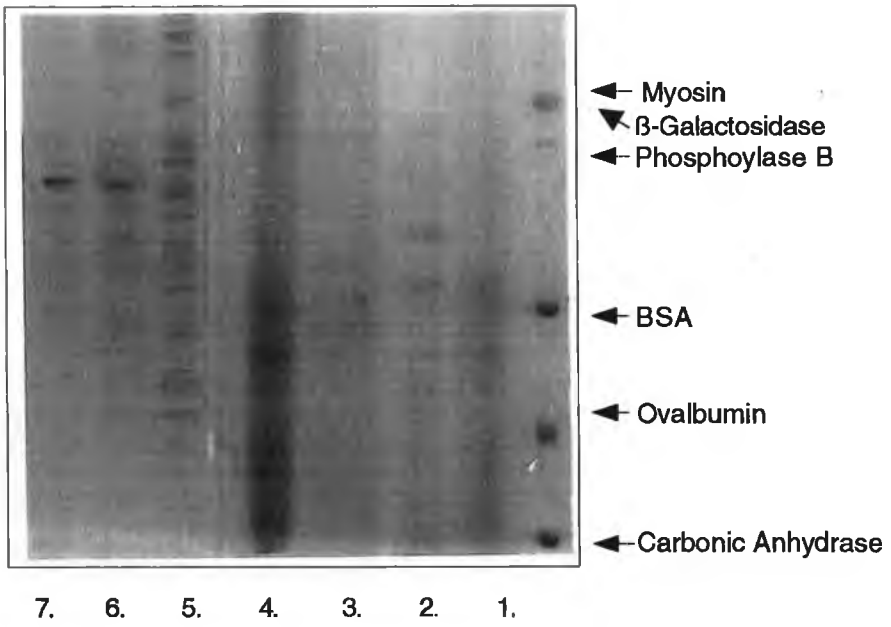
Fraction	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	% Recovery	Purification Factor
Crude Homogenate	6890.00	2907.50	0.36	*	*
Synaptosomal Fraction	2845.00	1300.00	0.46	100.00	1.00
Salt-Washed Synaptosomal Fraction	2000.00	850.90	0.43	65.50	0.93
Triton X-100 Solubilised	305.90	680.70	2.23	52.40	4.90
Post Gel Filtration	32.25	444.86	13.80	34.20	30.00
Post Hydroxyl-apatite	5.50	391.05	71.10	30.00	154.60
Post Phenyl Sepharose	0.47	304.30	647.40	23.40	1407.50

3.9.4. Assessment of the Synaptosomal PE Purification Scheme by Electrophoresis

3.9.4.2. Sodium Dodecyl-Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

In Fig. 3.23 an SDS-Gel of the purified synaptosomal PE confirms the subunit molecular weight of the enzyme as being in the region of 87kDa.

Fig. 3.23. A 10% SDS-PAGE of Bovine Synaptosomal PE



1. Crude Homogenate
2. Synaptosomal Fraction
3. Salt-Washed Synaptosomal Fraction
4. Triton X-100 Solubilised Fraction
5. Post Gel Filtration Fraction
6. Post Hydroxylapatite Fraction
7. Post Phenyl Sepharose Fraction

3.10 Characterisation of the Thyroliberin-Degrading Activities from the Synaptosomal Membranes of Bovine Brain

3.10.1. Molecular Weight Determination of the Synaptosomal PAP Activity

The molecular weight of the synaptosomal PAP activity was determined by gel filtration on a sephacryl S-200 column (as described in section 2.9.1.). A plot of Ve/Vo versus the logarithm of the molecular weight of a number of standard proteins was linear as shown in Fig. 3.24. The synaptosomal PAP solubilised by Triton X-100 was found to have a Ve/Vo value which corresponded to a molecular weight of approximately 230kDa.

3.10.2. Molecular Weight Determination of the Synaptosomal PE Activity

The molecular weight of the synapotosomal PE was estimated in the same way as described in section 3.10.1. The molecular weight was found to be 87kDa (Fig. 3.25).

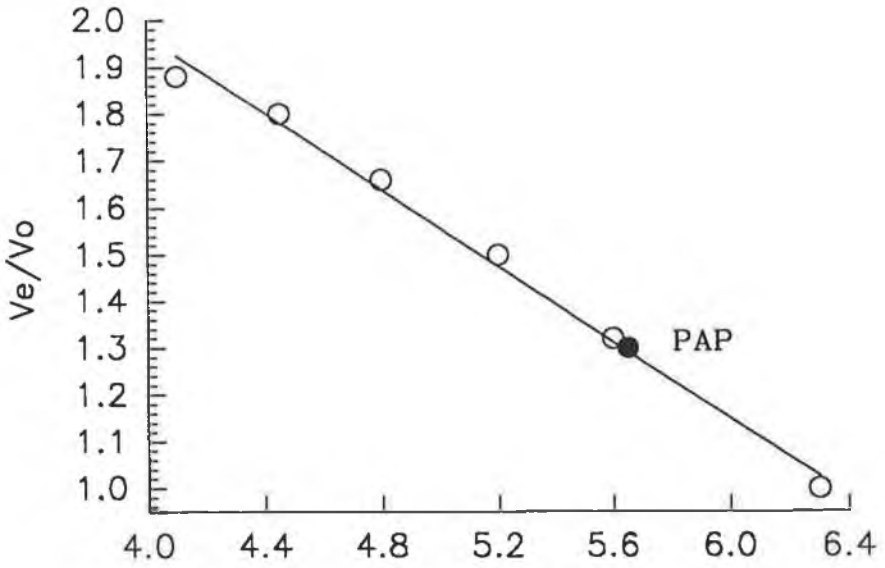
3.10.3. pH-Rate Profile of the Synaptosomal PAP Activity

The activity of the synaptosomal PAP was assayed for activity over a range of pH units from 5 to 8.5 using 100mM MES, pH 5-6.5, 100mM potassium phosphate, pH 6.5-7.5 and 100mm Tris-HCl, pH 7-8.5. The purified synaptosomal PAP had an optimum between pH 7.0 and 7.5 (Fig. 3.26). This optimum was achieved in both potassium phosphate and Tris-HCl buffers. The enzyme activity dropped dramatically in the Tris-HCl buffer once its pH went over 7.5. The activity of the PAP in MES was much lower than in either of the other two buffers, but the MES pH range was quite acidic (pH5-6.5). From this data it can be seen that the choice of physiological pH 7.4 for all assays was a suitable one.

3.10.4. pH-Rate Profile of the Synaptosomal PE Activity

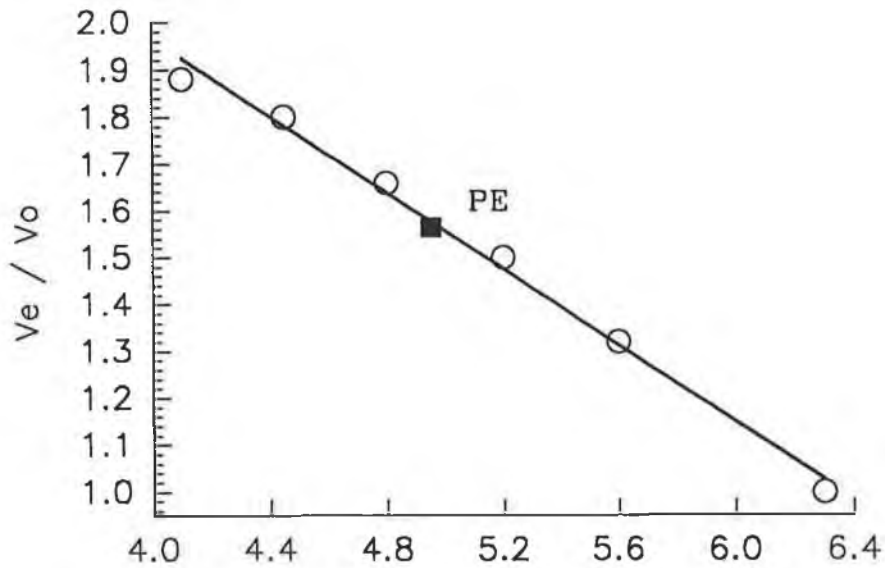
The activity of the PE purified from the synaptosomal membranes was stable over a broader range of pH units than the synaptosomal PAP activity (Fig. 3.27). The prolyl endopeptidase activity was high in all three buffers from pH 5.5 through to pH 8. There appeared to be a preference by the enzyme for potassium phosphate buffer at pH 7.0, but this increase in activity was only slight. Only at pH 8.5 did the PE activity begin to decrease.

Fig. 3.24. Molecular Weight Calibration Curve for Synaptosomal PAP



PAP M.W. = 230kDa

Fig. 3.25. Molecular Weight Calibration Curve for Synaptosomal PE



PE M.W. = 87 kDa

Fig. 3.26. pH Profile for Purified Synaptosomal PAP

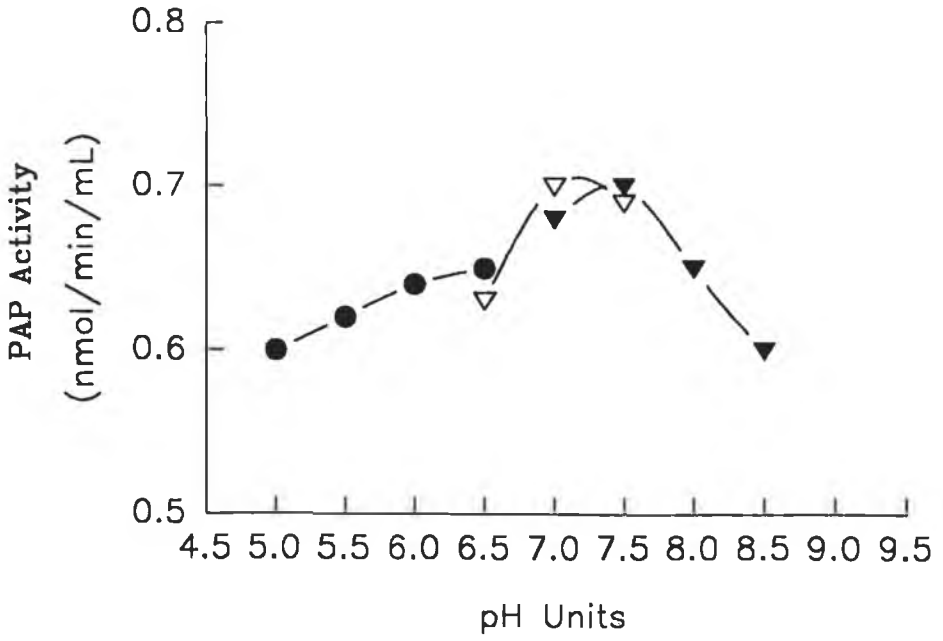
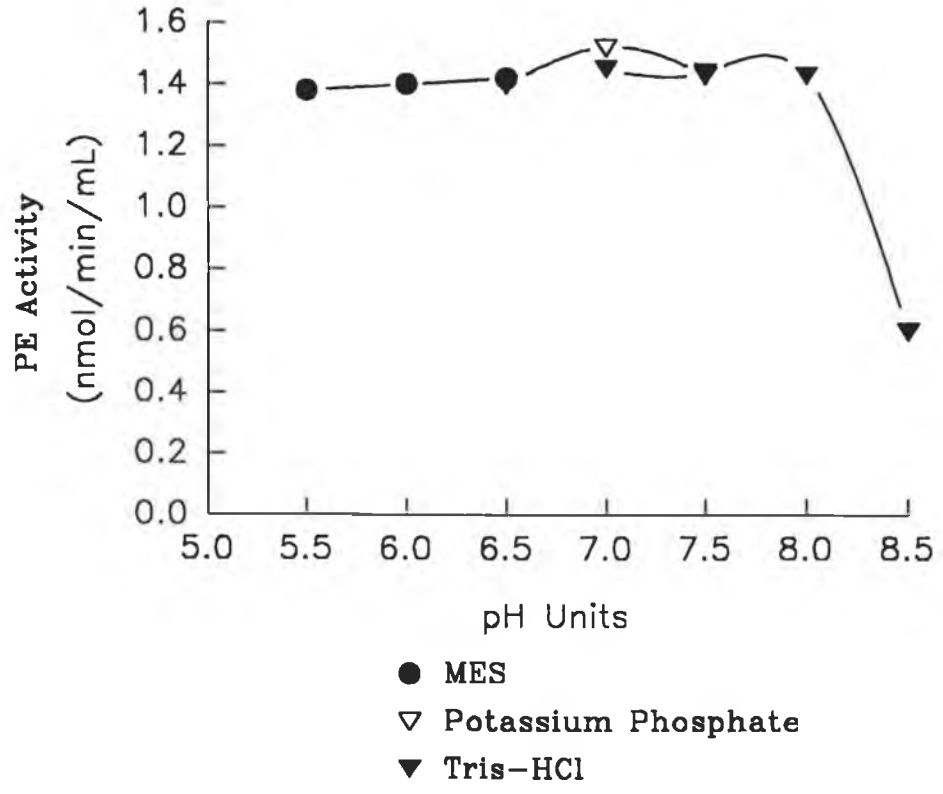


Fig. 3.27. pH Profile for Purified Synaptosomal PE

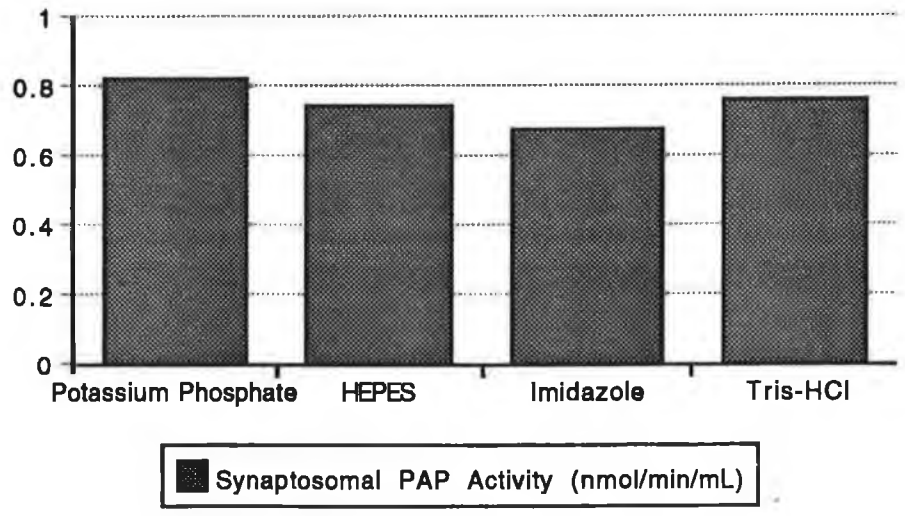


3.10.5. Effect of Different Buffers on Thyroliberin-Degrading Activities

3.10.5.1. The activity of the synaptosomal PAP in a range of buffers

The activity of the Triton X-100-solubilised PAP in a range of different buffers is shown in Fig. 3.28.
The enzyme was most active in 100mM potassium phosphate buffer, but appeared to have an equal preference for both 100mM HEPES and Tris-HCl. Its activity was lowest in 100mM Imidazole buffer (20% of that in phosphate buffer).

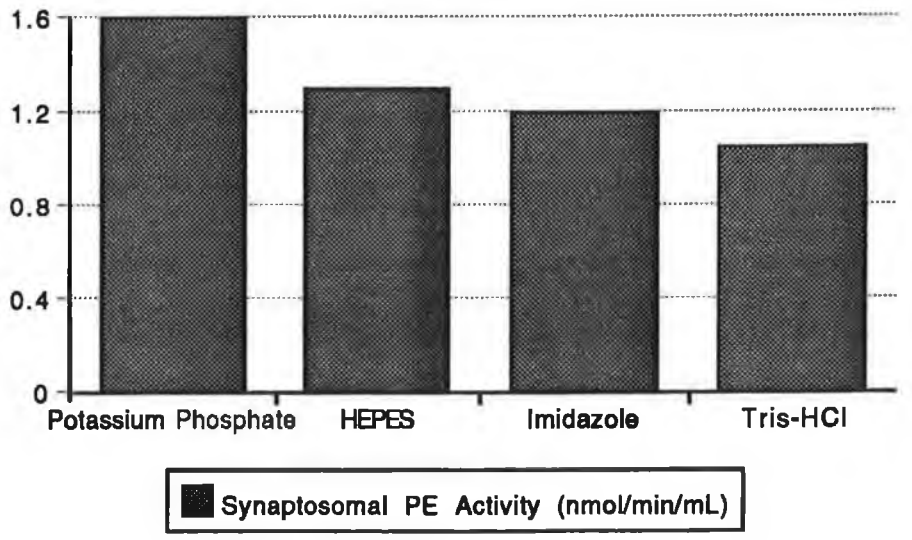
Fig. 3.28 Synaptosomal PAP Activity in a Range of Buffers at 100mM Concentration, pH 7.4



3.10.5.2. The activity of the synaptosomal prolyl endopeptidase activity in a range of buffers

PE showed a marked preference for 100mM potassium phosphate buffer, having at least 20% more activity in it than in any of the other buffers (Fig. 3.29). Its lowest activity was found in Tris-HCl, and it seemed to have an equal preference for both HEPES and Imidazole.

Fig. 3.29 Synaptosomal PE Activity in a Range of Buffers at 100mM Concentration, pH 7.4



3.10.6. Stability Studies on the Purified Synaptosomal Thyroliberin-Degrading Activities Under Different Conditions

3.10.6.1. Effect of temperature on the stability of the synaptosomal PAP activity

Both the Triton X-100-solubilised and purified synaptosomal PAP activities were quite unstable in contrast to the synaptosomal PAP purified from other sources (O'Connor and O'Cuinn, 1984; Wilk and Wilk, 1989). The Triton X-100-solubilised PAP in 100 mM potassium phosphate buffer, pH 7.4, was placed under different storage conditions in order to increase the stability of its activity. The activity of the sample was monitored over time. The first of the conditions investigated was temperature (Fig. 3.30). The activity of the sample at room temperature was most stable and the activity of the PAP increased slightly with time. At 4°C, the activity of the PAP initially decreased, but then the activity levelled out without any further decrease in activity. The synaptosomal activity PAP activity at -20°C was the most unstable, as it lost 75% of its activity in the first 24 hours which was not recovered over the time of monitoring.

The purified synaptosomal PAP was also assayed under the same conditions. Its activity was found to be the most stable at 4°C, when it only lost 15% of its activity over 100 hours (Fig. 3.31). At room temperature, the condition preferred at the Triton X-100-solubilised stage, the PAP activity dropped by 25% over time. When stored at -20°C, the PAP activity decreased by 50%.

Fig. 3.30. Triton X-100-Solubilised PAP Activity Over Time

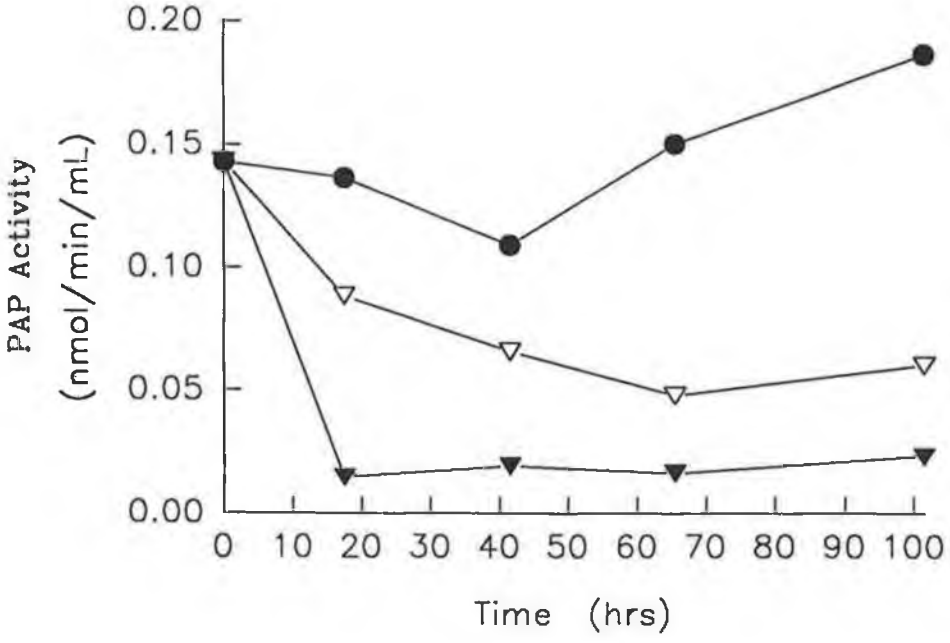
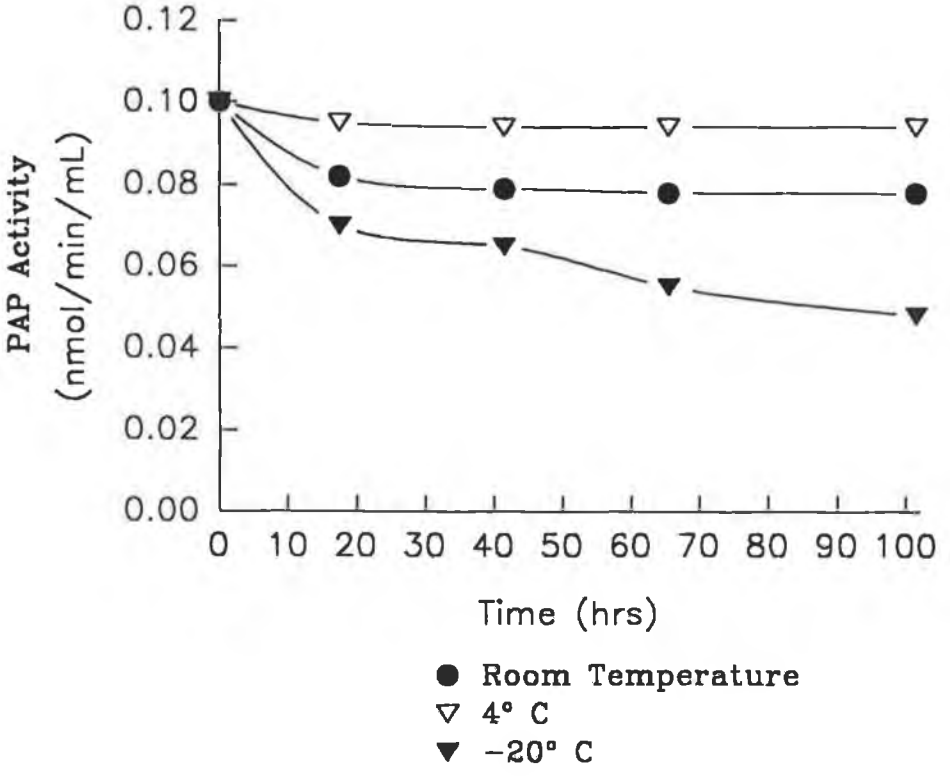


Fig. 3.31. Purified PAP Activity Over Time



3.10.6.2. Effect of glycerol on the stability of the purified synaptosomal PAP activity

The effect of glycerol as a stabilising agent was investigated with regard to purified synaptosomal PAP activity when stored at 4°C and -20°C. Fig. 3.32a shows the effect of glycerol on synaptosomal PAP activity when stored at 4°C. 20% (v/v) glycerol proved to have the most stabilising effect on the PAP activity, although 10% and 30% were also quite effective. The activity of the sample dropped by nearly 50% in the absence of glycerol, but only dropped by an average of 20% in the presence of glycerol. When samples were stored at -20°C (Fig. 3.32b), glycerol again provided a stabilising influence on the PAP activity, but it was unable to prevent a huge drop in the enzyme activity. Even with 30% (v/v) glycerol, there was a 35% drop in the synaptosomal PAP activity. Without any glycerol, the activity dropped by 80%.

3.10.6.3. Effect of BSA on the stability of the purified synaptosomal PAP activity

BSA is commonly used as a stabilising agent for enzymes at low concentration. 1 and 2% (w/v) BSA and a combination of 1% (w/v) BSA and 10% (v/v) glycerol were used in an attempt to increase the stability of the synaptosomal PAP at 4°C and -20°C. At 4°C, the combination of BSA and glycerol proved quite effective with very little loss in activity (Fig. 3.33a). 1 and 2% BSA on their own resulted in 70% and 43% loss in activity respectively. At -20°C, the combination again provided the most protection against inactivation, but the activity still dropped by 57% of the original (Fig. 3.33b).

3.10.6.4. Effect of different buffers on the stability of the purified synaptosomal PAP activity

The possibility of providing increased stability to the purified PAP activity by storage in a different buffer was investigated. The enzyme was dialysed into the different buffers and was stored at 4°C and -20°C. The results from section 3.10.5. showed that the PAP activity was relatively unaffected by different buffers (Fig. 3.28).
Fig. 3.34a shows how the different buffers have little effect on the stability of the purified PAP activity when stored at 4°C. The different buffers provided even less protection against inactivation when the PAP was stored at -20°C (Fig. 3.34b).
Following these experiments, the most effective method of storage of the purified enzyme was in 100mM potassium phosphate buffer,pH 7.4, with 10% (v/v) glycerol and 1% (w/v) BSA at 4°C. From the phenyl sepharose step, the enzyme is already in glycerol and so 1% BSA was added to the sample. Under these conditions, the purified PAP activity was stable for up to 1 week.

Fig. 3.32a. Effect of Glycerol on Purified PAP Activity Stored at 4°C

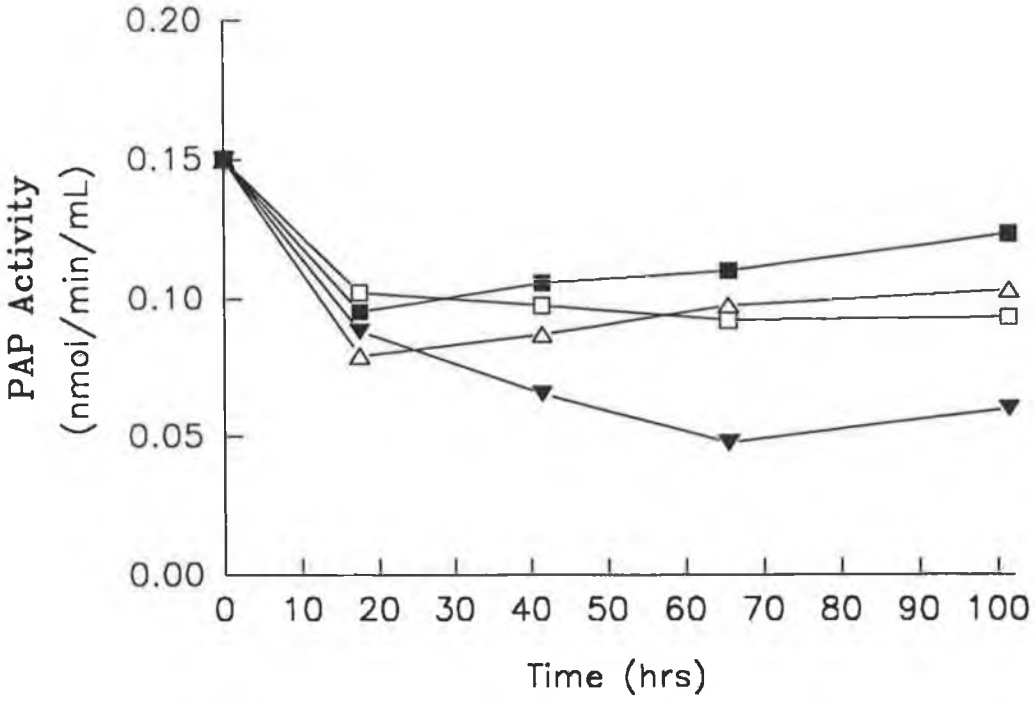


Fig. 3.32b. Effect of Glycerol on Purified PAP Activity Stored at -20°C

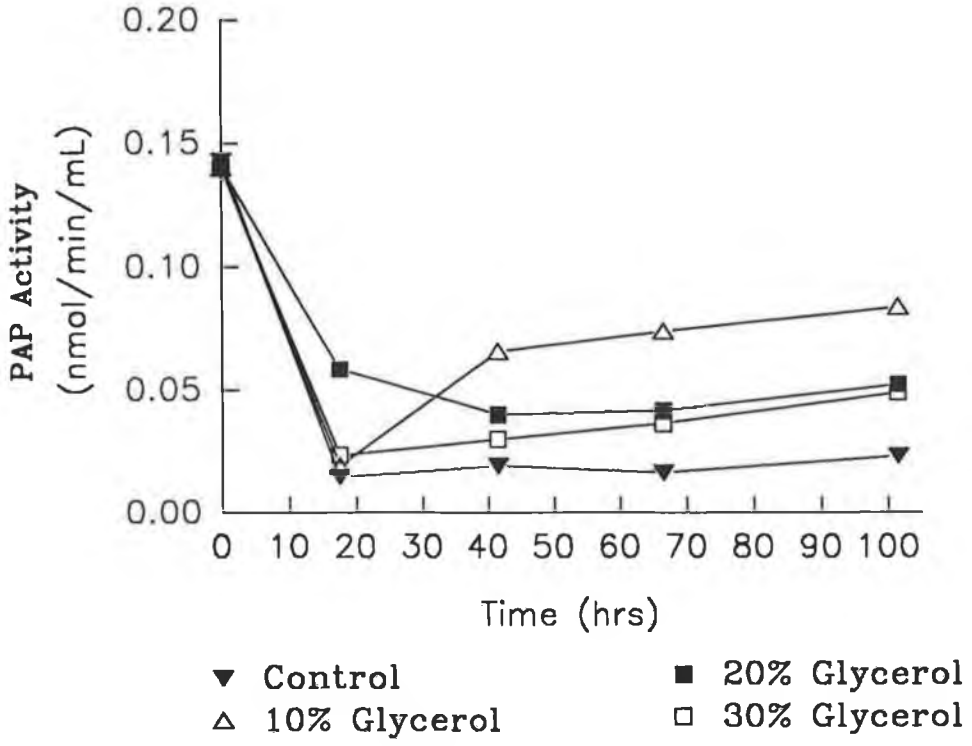


Fig. 3.33a. Effect of BSA on Purified PAP Activity Stored at 4°C

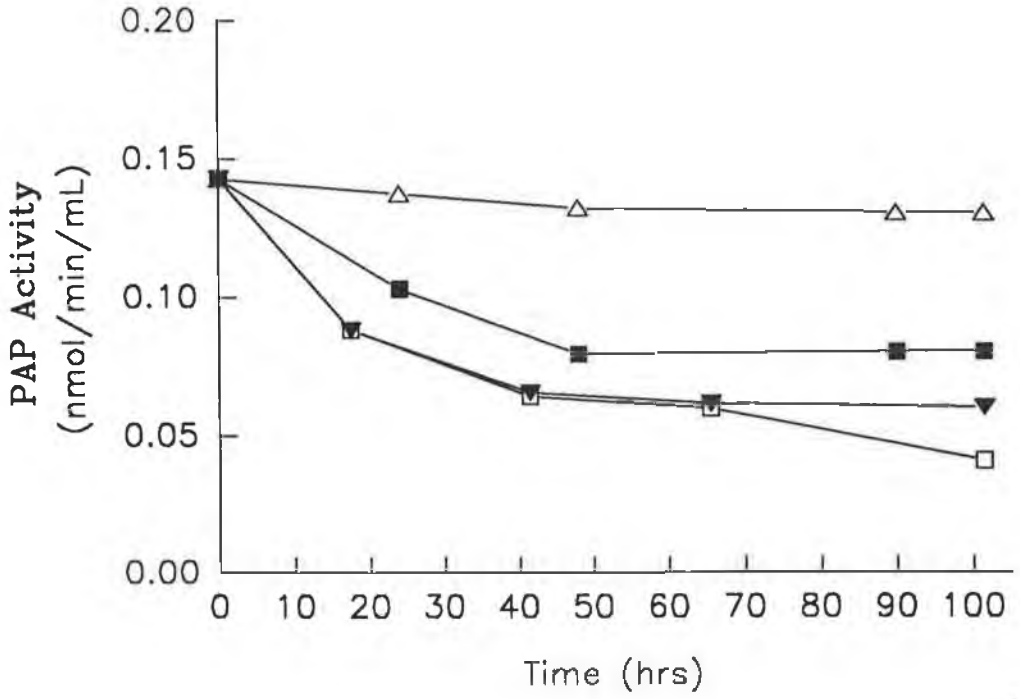


Fig. 3.33b. Effect of BSA on Purified PAP Activity Stored at -20°C

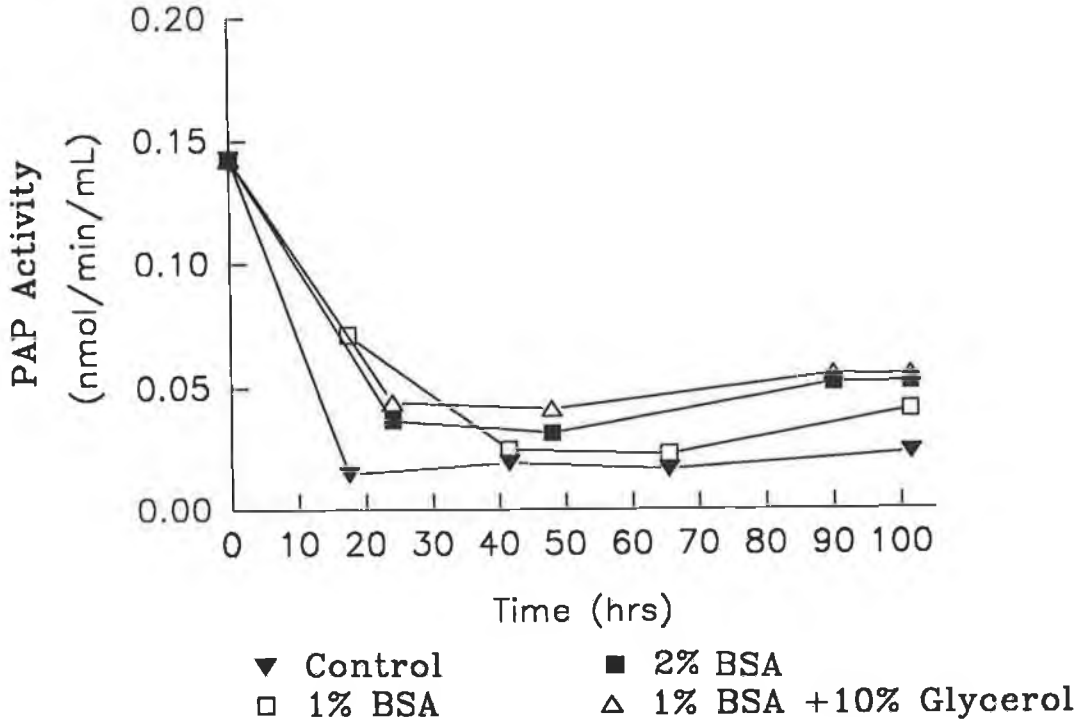


Fig. 3.34a. Effect of Different Buffers at pH 7.4 on Purified PAP Activity Stored at 4°C

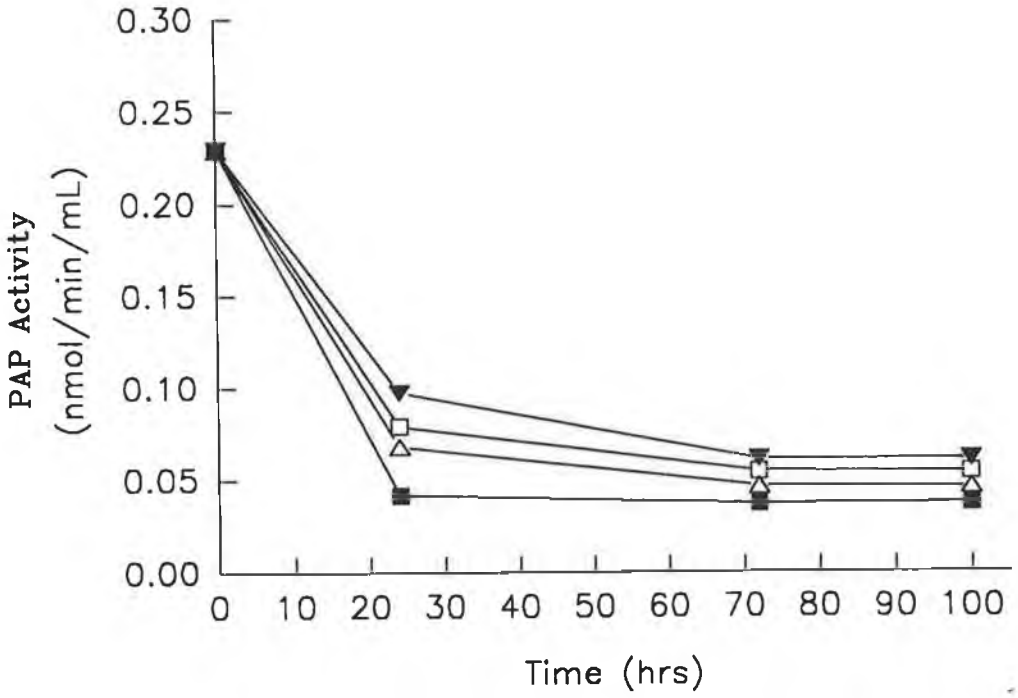
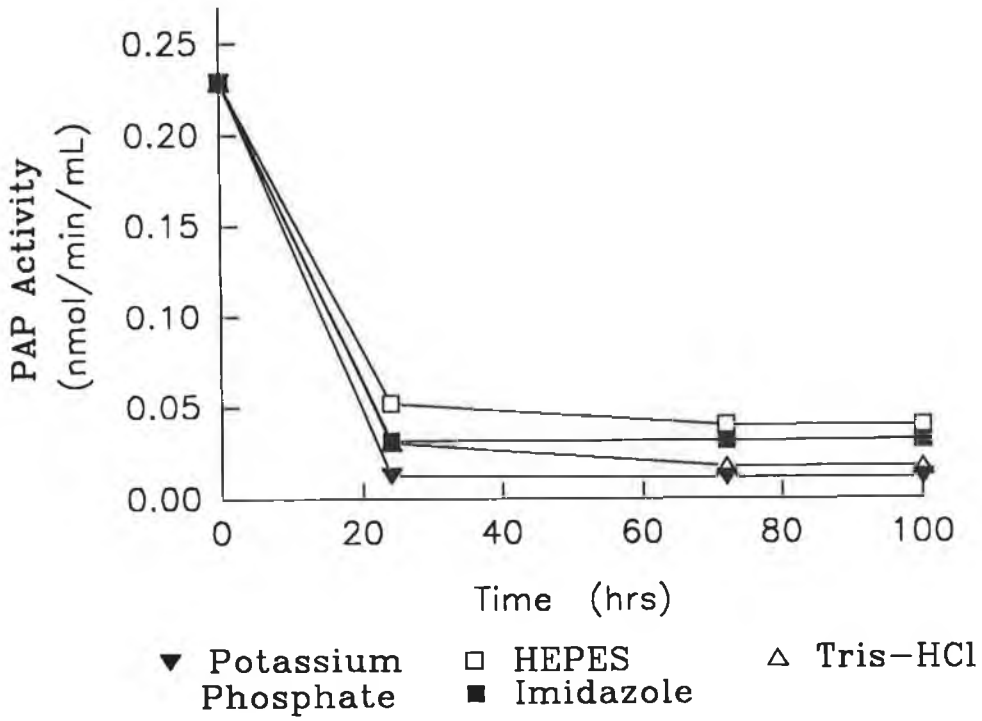


Fig.3.34b. Effect of Different Buffers at pH 7.4 on Purified PAP Activity Stored at -20°C



3.10.6.5. Effect of different storage conditions on the stability of the purified synaptosomal PE activity

Post-phenyl Sepharose PE activity proved to be quite stable under a range of different storage conditions, such as different temperatures, in the presence of glycerol and when stored in different buffers. The results of the various storage conditions on PE activity are shown.
Fig. 3.35 shows that PE activity was quite stable regardless of the temperature at which it was stored. The activity remained slightly higher when the sample was frozen, than at 4°C. At room temperature the activity decreased by approximately 50% after 100 hours storage. In Fig. 3.36, the sample was stored at -20°C in the presence of 10% and 20% (v/v) glycerol and this resulted in only a 5% loss of activity even after 100 hours. Post-phenyl sepharose PE was already in glycerol and from the graph it can be seen that this inclusion of glycerol does not have any adverse effect on the purified prolyl endopeptidase activity.

Prolyl endopeptidase was also stored in a number of different buffers, in order to assess their effects on its activity. It was shown earlier (Fig. 3.29) that purified PE activity showed a preference for potassium phosphate, pH 7.4, and was least active in Tris-HCl. This same preference was displayed when the purified PE was dialysed into three different buffers- HEPES, Imidazole and Tris-HCl, and was then frozen at -20°C in these buffers (Fig. 3.37). The PE activity in HEPES and Imidazole was only 10% less than that in 100mM potassium phosphate, but the PE activity when frozen in Tris-HCl dropped by 25% of the original.

Fig. 3.35. Effect of Storage Temperature on PE Activity Over Time

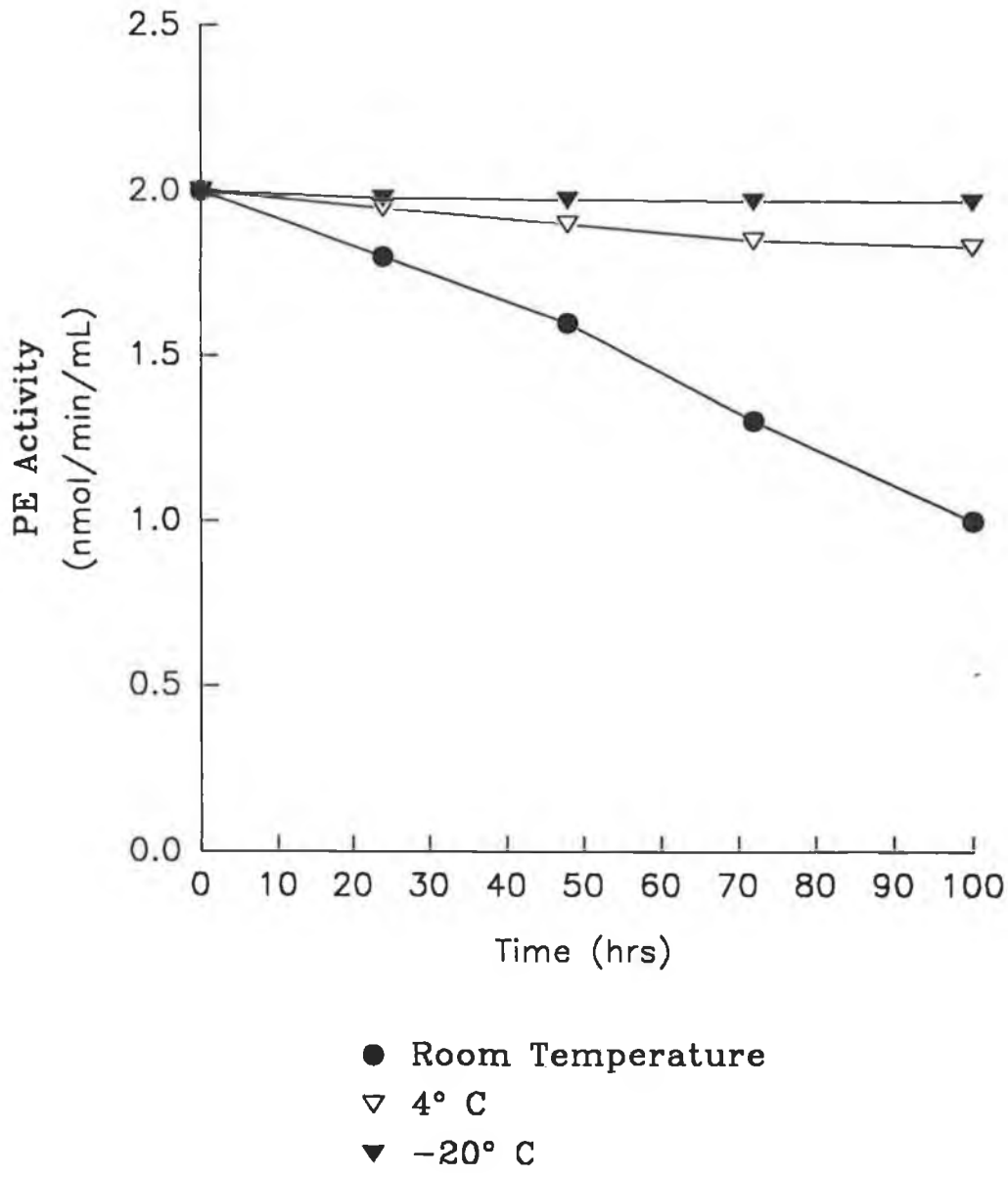


Fig. 3.36. Effect of Glycerol on PE Activity Stored at -20°C

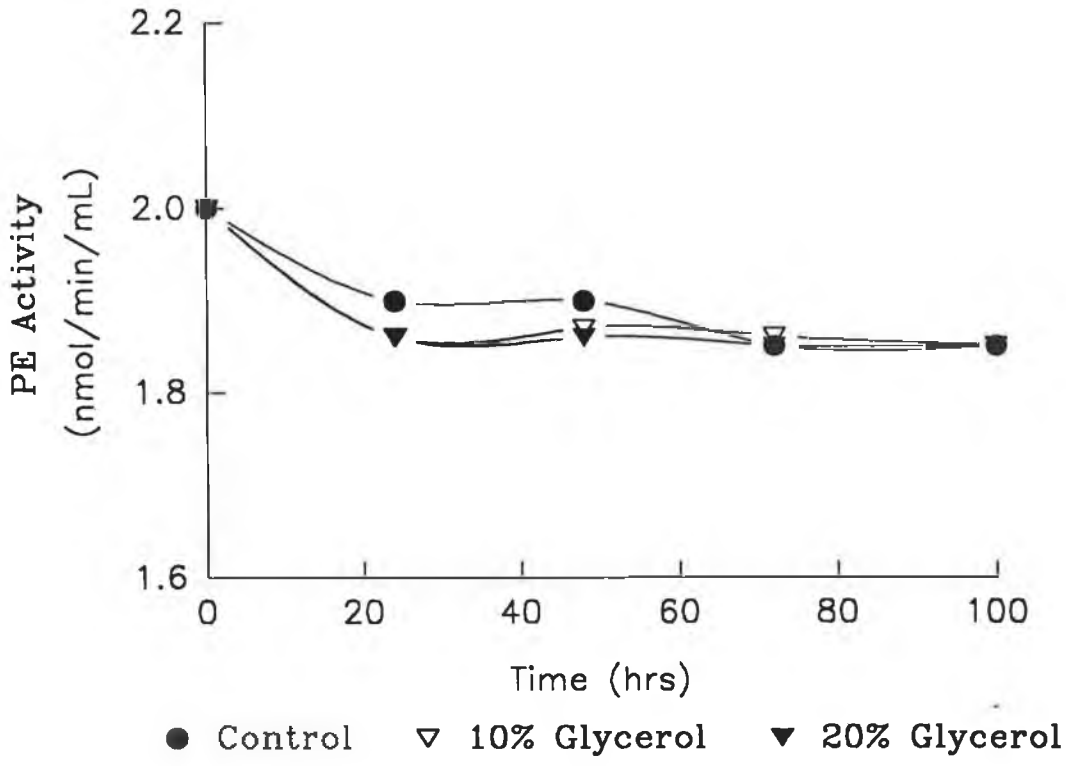
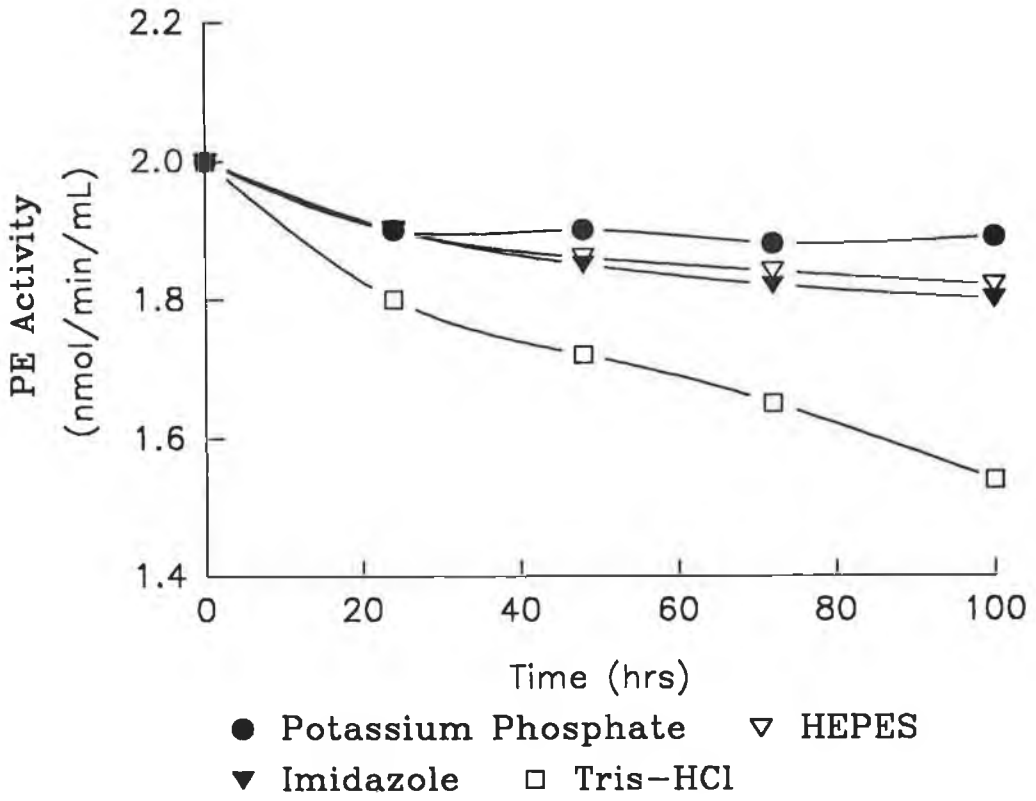


Fig. 3.37. Effect of Different Storage Buffers on PE Activity at -20°C



3.10.7. The Substrate Specificity of the Thyroliberin-Degrading Activities from the Synaptosomal Membranes of Bovine Brain.

3.10.7.1. The substrate specificity of the synaptosomal PAP

The substrate specificity of the purified synaptosomal PAP was investigated by HPLC based on a method by Martini et al. (1985) and modified by Griffiths (1993). A chromatogram showing the cleavage of TRH by the purified synaptosomal PAP is shown in Fig. 3.38. The cleavage products retention times were compared to those of standards and were identified as cyclo(His-Pro) and pyroglutamic acid. The retention time of pyroglutamic acid was only 2.5 mins and so it was easy to detect its production. The production of <Glu indicated that cleavage of the substrate occurred by PAP, as it is the only enzyme capable of cleaving the <Glu-His bond. Table 3.13 shows the complete range of substrates which were assayed for cleavage by synaptosomal PAP. As can be seen, PAP purified from the synaptosomal membranes of bovine brain, displays the same narrow substrate specificity as the PAP type II from guinea-pig brain (O'Connor and O'Cuinn,1985). Synaptosomal PAP will only cleave the tripeptide <Glu-His-ProNH_2 or very closely related peptides such as <Glu-His-Trp (LHRH 1-3), <Glu-His-ProOH (acid thyroliberin) or <Glu-(Me)His-ProNH_2 (a thyroliberin analogue). Another thyroliberin analogue <Glu-ProNH_2 was not cleaved, showing the absolute requirement for a <Glu-His in the first and second position. Longer peptides such as neurotensin, bombesin and LHRH were not cleaved, despite the fact that PAP will cleave LHRH(1-3). Even the short dipeptide <Glu-His was not cleaved, showing how PAP has a specificity for tripeptides. <Glu-His-Gly (the anorexogenic peptide) was not cleaved either, even though it appears to fulfil the requirements for cleavage by PAP i.e. it is a tripeptide with <Glu-His in the first two positions. The glycine in the third position instead of the cyclic proline (or the cyclic tryptophan in LHRH 1-3) may be the reason for this lack of cleavage by the synaptosomal PAP.

3.10.7.2. The substrate specificity of the synaptosomal prolyl endopeptidase

Prolyl endopeptidase was shown to have a broad specificity when it was purified from the cytosolic fraction of rat (Andrews et al., 1982) and bovine (Yoshimoto et al., 1983) brains. Since a particulate PE has not previously been characterized, it was of interest to investigate whether the particulate PE from bovine brain would display a similar broad substrate specificity to its cytosolic counterpart. Fig. 3.39 shows how the cleavage products of thyroliberin incubated with prolyl endopeptidase are resolved by HPLC. The production of acid thyroliberin is clear and separates well from thyroliberin. The cleavage of a number of proline-containing peptides was also investigated by TLC and the cleavage products were visualised by Pauly's reagent and Ninhydrin.

Table 3.13 Substrate Specificity of the Purified Synaptosomal PAP Activity

+ Indicates that cleavage of the peptide occurred

- Indicates that cleavage of the peptide did not occur

Substrate	Number of Amino Acids	Cleavage of the Substrate
<Glu-His	2	-
Thyroliberin Analogue <Glu-ProNH2	2	-
Thyroliberin <Glu-His-ProNH2	3	+
Acid Thyroliberin <Glu-His-ProOH	3	+
LHRH(1-3) <Glu-His-Trp	3	+
Thyroliberin Analogue <Glu-(Me)His-ProNH2	3	+
Anorexogenic peptide <Glu-His-Gly	3	-
LHRH <Glu-His-Trp-Ser-	10	-
Neurotensin (1-4) <Glu-Leu-Tyr-Glu-	13	-
Bombesin (1-4) <Glu-Gln-Arg-Leu-	14	-

Table 3.14 shows a range of substrates which were cleaved by the synaptosomal PE. The substrate specificity of the PE was examined before and after its release from the membrane, to ensure that the solubilisation of the enzyme did not affect its physiological activity. PE has a very broad specificity and cleaved all of the proline-containing peptides shown. Bombesin, a neuropeptide without a proline moiety was included to demonstrate the requirement for a

proline for cleavage by PE. It is of interest to note the cleavage points of the substrates with 2 or more proline moieties. In the case of bradykinin, PE cleaves the Pro^3-Gly^4 and Pro^7-Phe^8 bonds and not the Pro^2-Pro^3 bond. For substance P, PE cleaves the Pro^4-Gln^5 bond and not the Pro^2-Lys^3 bond. This demonstrates a certain level of specificity which was previously reported by Camargo et al. (1979) for the cytosolic PE. They described the PE as an endo-oligopeptidase with a specificity toward small peptides. This specificity appears to be solely dependent on peptide size and there is no evidence to suggest that the bovine synaptosomal PE has a requirement for a basic amino acid prior to the proline moiety as described by Tate (1981) for bovine cytosolic PE. While some of the peptides do have a basic amino acid prior to the proline (eg thyroliberin, LHRH, neurotensin, angiotensin and substance P), others do not including bradykinin and the specific PE substrate Z-Gly-ProMCA. Vasopressin and oxytocin which have also been reported to be cleaved by the cytosolic PE (Welches et al., 1993) do not have a basic amino acid prior to the proline either.

Table 3.14 Substrate Specificity of the Purified Synaptosomal PE Activity

+ Indicates that cleavage of the peptide occurred

- Indicates that cleavage of the peptide did not occur

Peptide	Position of Cleavage	Synaptosomal Membrane-Bound PE	Purified PE
Thyroliberin <Glu-His-ProNH2	Pro3-NH2	+	+
LHRH <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2	Pro9-Gln10	+	+
Neurotensin <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-LeuNH2	Pro7-Arg8 Pro10-Tyr11	+	+
Angiotensin II Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	Pro7-Phe8	+	+
Bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Pro3-Gly4 Pro7-Phe8	+	+
Bradykinin (1-7) Arg-Pro-Pro-Gly-Phe-Ser-Pro	Pro3-Gly4	+	+
Substance P Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-	Pro4-Gln5	+	+
Bombesin <Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-MetNH2	No Proline	-	-

Fig. 3.38. HPLC Profile of Synaptosomal PAP Activity on Thyroliberin

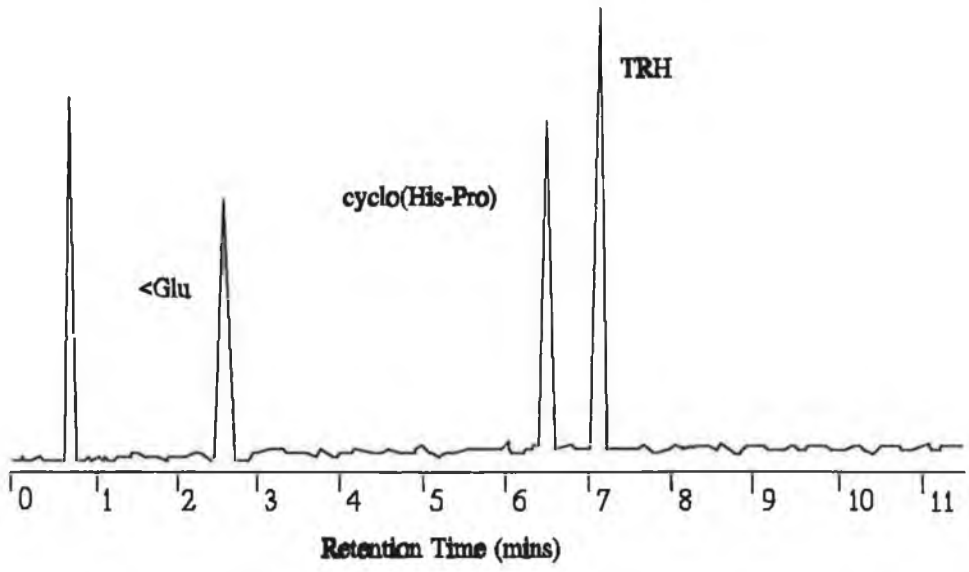
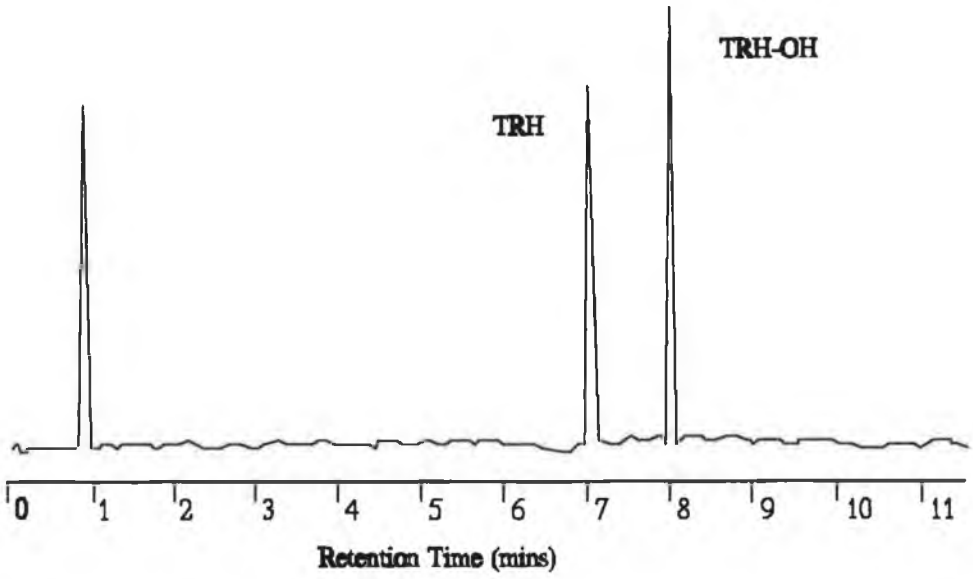


Fig. 3.39. HPLC Profile of Synaptosomal PE Activity on Thyroliberin



3.10.8. Preliminary Kinetic Studies on the Synaptosomal Thyroliberin-Degrading Activities

3.10.8.1. Preliminary kinetic studies on the purified synaptosomal PAP

The kinetics of hydrolysis of <Glu-His-ProMCA by the purified synaptosomal PAP were examined as outlined in section 2.14. As shown in Fig. 3.40a, the hydrolysis of the synthetic substrate, <Glu-His-ProMCA was found to obey normal Michaelis-Menten kinetics and Km and Vmax values of 100μM and 0.85nmol/min/mg respectively were calculated. The Lineweaver-Burk plot had a correlation coefficient of 0.99. The effect of the presence of a variety of peptides containing N-terminal pyroglutamyl residues, on the hydrolysis of <Glu-His-ProMCA by the purified synaptosomal PAP were examined.

The data resulting from this study was fitted to three types of plots, the Lineweaver-Burk, the Eadie-Hofstee and the Hanes-Woolf plot to compare the results (Figs. 3.40a-c). The use of the Lineweaver-Burk, although the most common, has been widely criticised (Henderson, 1992) as it groups the data points close to the origin and ignores the weighting of points. Both the Eadie-Hofstee and the Hanes-Woolf plots are preferable as both involve only one recipricol value, and are used in this case to confirm the findings of the Lineweaver-Burk plots. Table 3.15a shows the results when the data was fitted to the Lineweaver-Burk plot. The most unusual feature was the presence of several non-competitive inhibitors, especially acid thyroliberin and the thyroliberin analogue, <Glu-(Me)His-ProNH$_2$. Due to their similarity in structure to thyroliberin, a competitive inhibition may have been expected. Their high Ki values indicate that they are not as effective inhibitors as may have been anticipated either. When compared to the synthetic peptides <Glu-His-Gly and <Glu-His-Trp, whose Kis are 10 fold lower than those of acid thyroliberin and <Glu-(Me)His-ProNH$_2$, they appear to be quite ineffective. However, the Ki value for acid thyroliberin is still in the μM range and so must be considered to be an effective inhibitor.

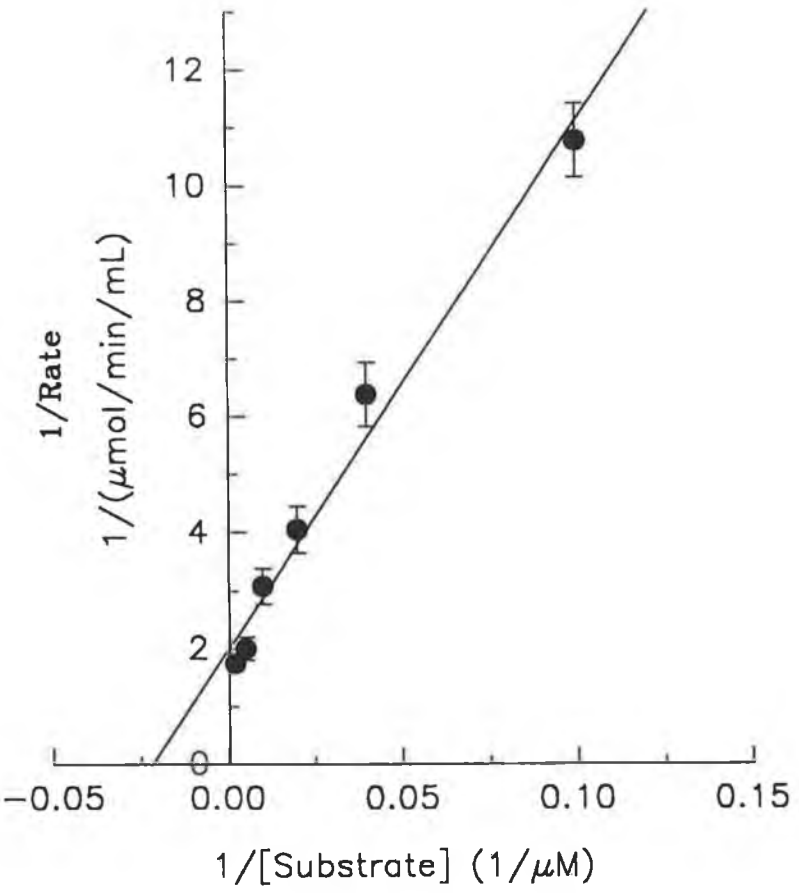
Previously characterised synaptosomal PAP activities (O'Connor and O'Cuinn, 1985) have demonstrated a very high affinity for LHRH and thyroliberin (20μM and 42μM respectively), but the Ki values obtained from the synaptosomal PAP activity in bovine brain are at least an order of magnitude lower for the same two peptides (6.6 and 0.72μM respectively).

The dipeptide <Glu-His was the only non-competitive inhibitor found for the synaptosomal PAP from guinea-pig brain (O'Connor and O'Cuinn, 1985), and again for the bovine brain synaptosomal PAP it was found to be an effective non-competitive inhibitor with a Ki of 1.2μM.

Looking at the results from the Eadie-Hofstee plots (Fig. 3.15b), the first notable difference was the reduced number of non-competitive inhibitors. Only LHRH and <Glu-His were found to be non-competitive inhibitors and this finding was later confirmed by the Hanes-Woolf plots (Fig. 3.15c). The synthetic peptides <Glu-His-Gly and <Glu-His-Trp again were seen to be very effective competitive inhibitors with Kis of 0.38μM and 0.53μM respectively. These Kis were even lower than those obtained by the Lineweaver-Burk plots and seem to indicate that synaptosomal PAP has an even higher affinity for these peptides than originally thought.

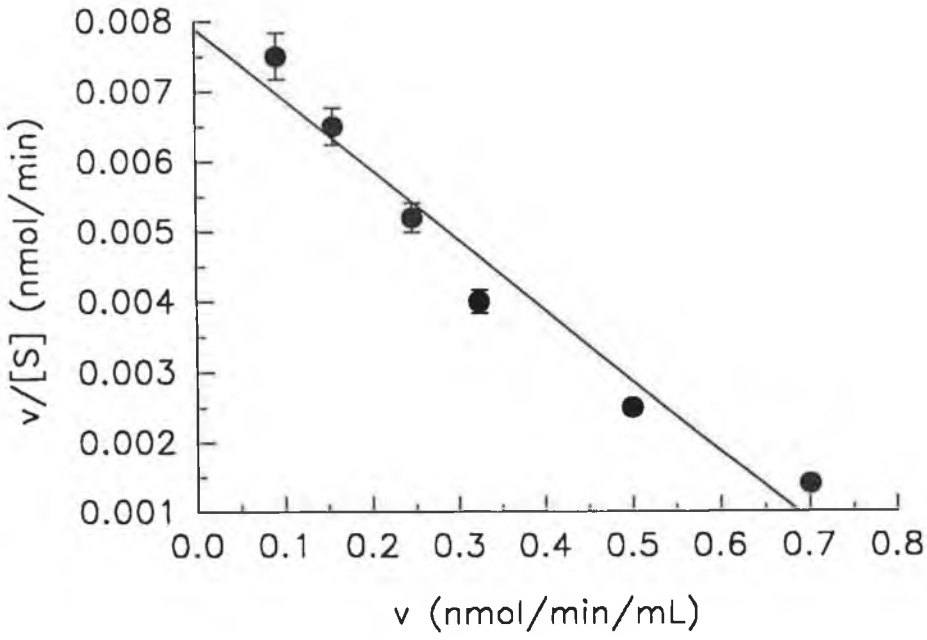
Fig. 3.40a. Lineweaver-Burk Plot for Synaptosomal PAP with <Glu-His-ProMCA

(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)



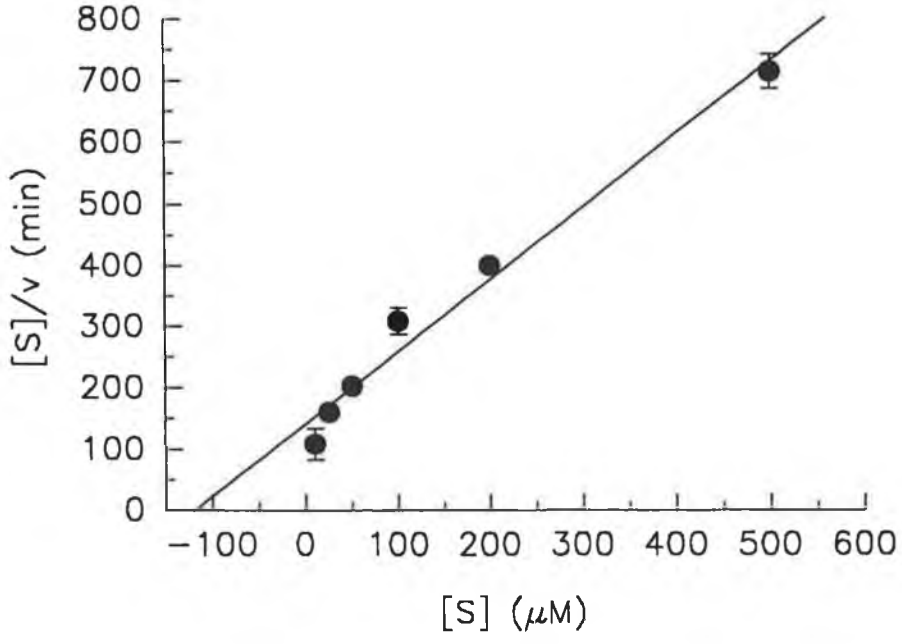
Regression Coefficient = 0.99

Fig. 3.40b. Eadie-Hofstee Plot for Synaptosomal PAP with <Glu-His-ProMCA
(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)



Regression Coefficient = 0.977

Fig. 3.40c. Hanes-Woolf Plot for Synaptosomal PAP with <Glu-His-ProMCA



Regression Coefficient = 0.99

Thyroliberin also was shown to be an effective competitive inhibitor of the synaptosomal PAP activity with a Ki of 0.45μM, again lower than was estimated with the Lineweaver-Burk plot (0.72μM). The acid thyroliberin and the thyroliberin analogue once more had higher Ki values than some of the other synthetic peptides (4.2 and 2.6μM respectively), but these figures were also lower than those estimated with the Lineweaver-Burk plots. A final comparison was made by fitting the data to the Hanes-Woolf plot, believed by some to be the most accurate of the three (Endrenyi and Kwong, 1972). A similar order was found using this method as had been seen with the other two methods. This method confirmed the findings of the Eadie-Hofstee that only two of the peptides were in fact non-competitive inhibitors, LHRH and <Glu-His. The fact that the Lineweaver-Burk plot seemed to indicate that there were more non-competitive inhibitors than those two, can be explained by the errors involved in using that method of presenting data. The synthetic peptides, <Glu-His-Gly and <Glu-His-Trp were again found to be the most competitive inhibitors with Ki values of 0.99 and 1.126μM respectively. Also the thyroliberin analogue, <Glu-(Me)His-ProNH$_2$ was found to be the least effective inhibitor, although it had a Ki value of 7.9μM. In Figs 3.41, 3.42 and 3.43, there is an example of a competitive (thyroliberin) and a non-competitive (LHRH) inhibitor, as plotted by each of the three methods.

The overall result seems to indicate the presence of two non-competitive inhibitors, LHRH and <Glu-His for the synaptosomal PAP activity and all the others appear to be competitive inhibitors. One unusual feature of these preliminary studies is the fact that thyroliberin analogue, <Glu-(Me)His-ProNH$_2$ does not compete as effectively as some of the synthetic peptides for the synaptosomal PAP activity. The most remarkable feature of these results is the fact that the Ki values are so low, indicating a highly specific enzyme.

Table 3.15 Activity of Synaptosomal PAP Towards Thyroliberin and Other Peptides

C = Competitive NC = Non competitive

Table 3.15 (a) Inhibition constants using Lineweaver-Burk plots

Peptide	Whether Hydrolysed	Inhibition	Ki (μM)
<Glu-His-Gly	-	C	0.45
<Glu-His-Trp	+	C	0.60
Thyroliberin	+	C	0.72
<Glu-His	-	NC	1.20
Bombesin	-	C	3.00
Neurotensin	-	C	5.93
LHRH	-	NC	6.60
<Glu-ProNH2	-	NC	7.40
Acid Thyroliberin	+	NC	7.50
<Glu-(Me)His-ProNH2	+	NC	8.40

Table 3.15 (b) Inhibition constants using Eadie-Hofstee plots

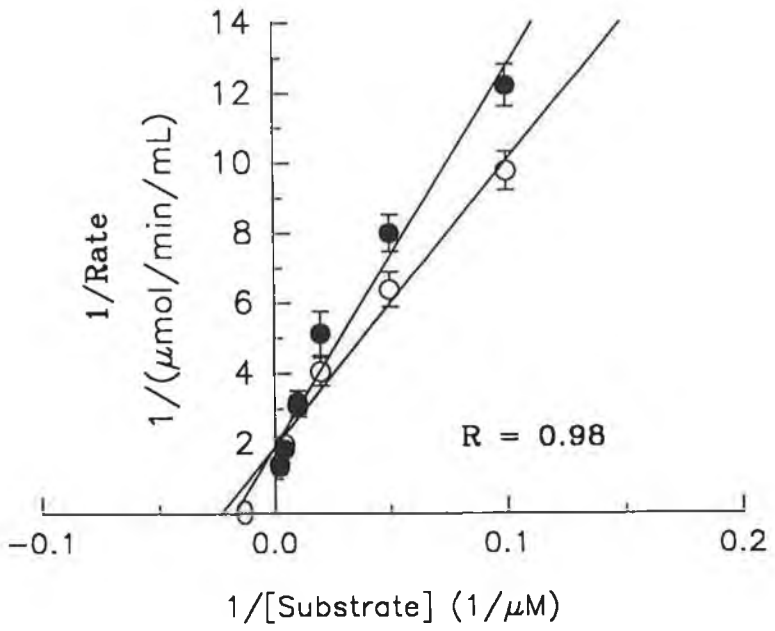
Peptide	Whether Hydrolysed	Inhibition	Ki (μM)
<Glu-His-Gly	-	C	0.38
Thyroliberin	+	C	0.45
<Glu-His	-	NC	0.45
<Glu-His-Trp	+	C	0.53
LHRH	-	NC	1.01
<Glu-(Me)His-ProNH2	+	C	2.60
Bombesin	-	C	3.50
Neurotensin	-	C	3.80
Acid Thyroliberin	+	C	4.20
<Glu-ProNH2	-	C	4.75

Table 3.15 (c) Inhibition constants using Hanes-Woolf plots

Peptide	Whether Hydrolysed	Inhibition	Ki (μM)
<Glu-His	-	NC	0.82
<Glu-His-Gly	-	C	1.00
<Glu-His-Trp	+	C	1.13
Bombesin	-	C	1.95
Thyroliberin	+	C	1.98
LHRH	-	NC	4.03
Acid Thyroliberin	+	C	6.20
Neurotensin	-	C	6.73
<Glu-ProNH2	-	C	6.76
<Glu-(Me)His-ProNH2	+	C	7.90

Fig. 3.41a. Competitive Inhibition of Synaptosomal PAP by Thyroliberin (Lineweaver-Burk Plot)

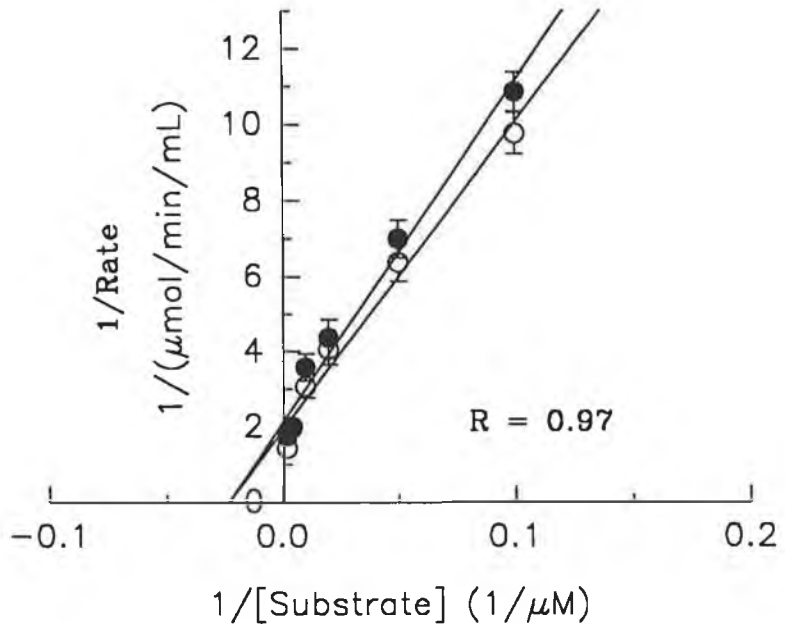
(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)



○ Lineweaver Burk Plot with 1mM <Glu−His−ProMCA

● Lineweaver Burk Plot with 1mM Thyroliberin

Fig. 3.41b. Non-Competitive Inhibition of Synaptosomal PAP by LHRH (Lineweaver-Burk Plot)



○ Lineweaver Burk Plot with 1mM <Glu−His−ProMCA

● Lineweaver Burk Plot with 1mM LHRH

Fig. 3.42a. Competitive Inhibition of Synaptosomal PAP by Thyroliberin (Eadie-Hofstee Plot)

(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)

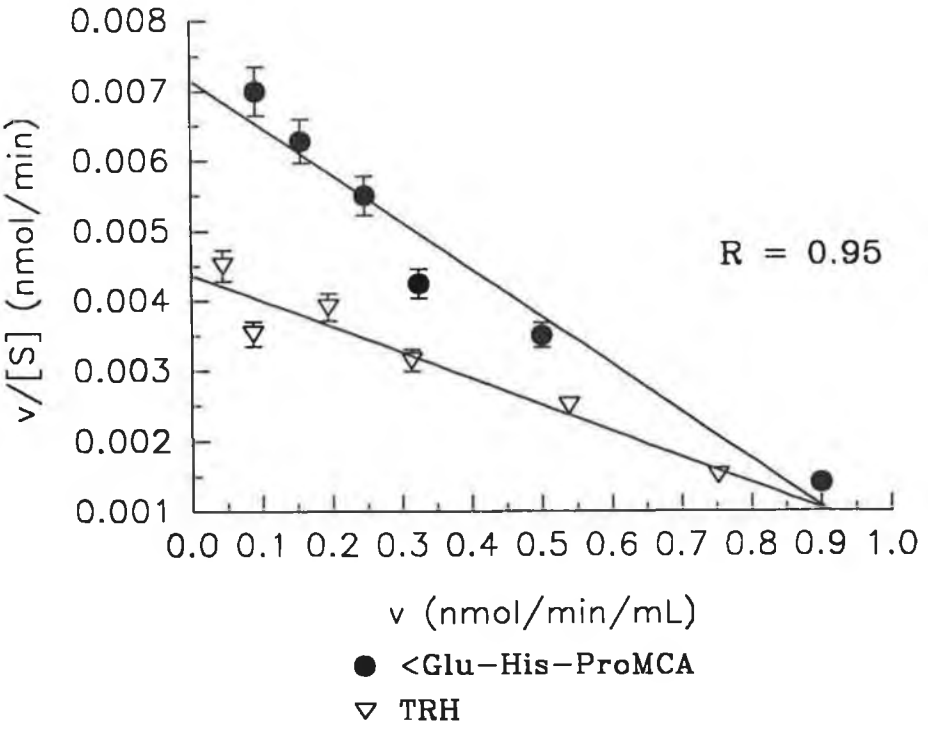


Fig. 3.42b. Non-Competitive Inhibition of Synaptosomal PAP by LHRH (Eadie-Hofstee Plot)

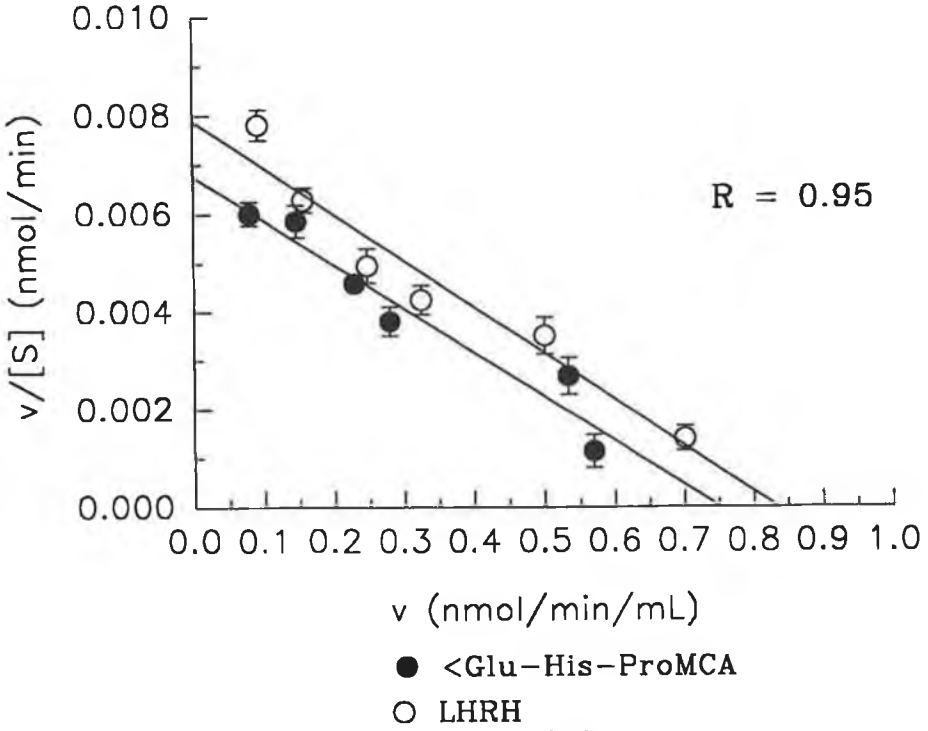
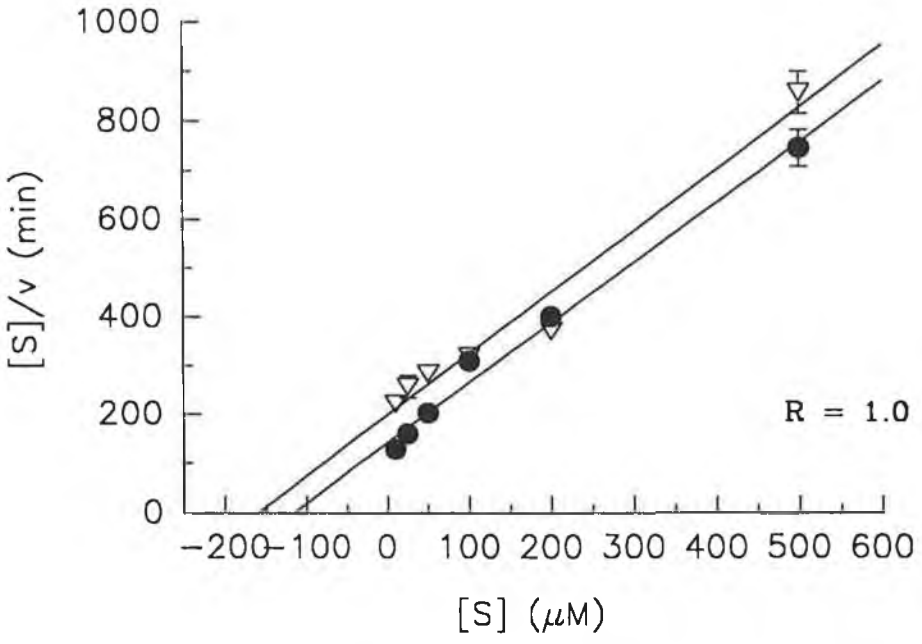


Fig. 3.43a. Competitive Inhibition of Synaptosomal PAP by Thyroliberin (Hanes-Woolf Plot)

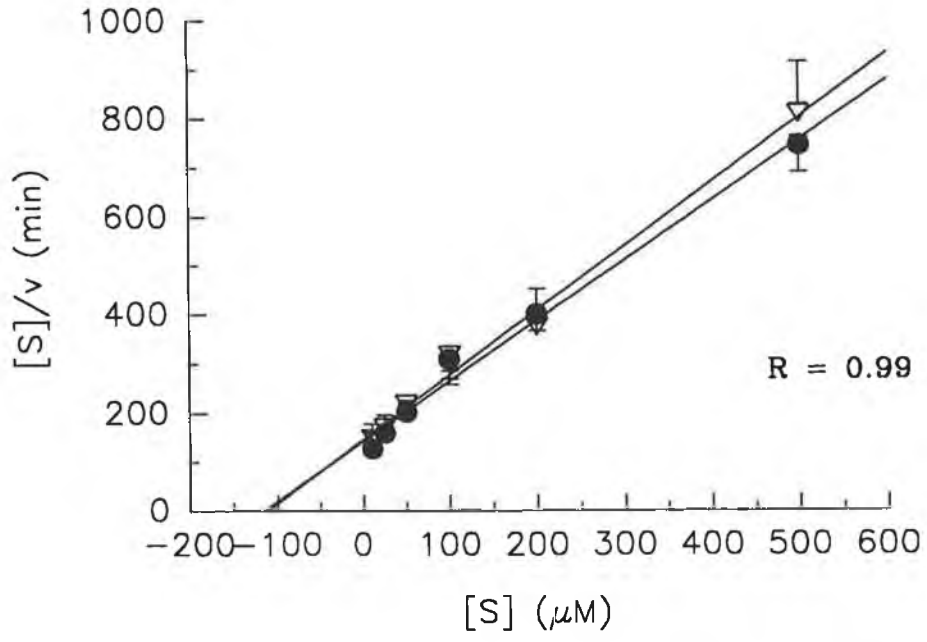
(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)



● Hanes–Woolf Plot with <Glu–His–ProMCA

▽ Hanes–Woolf Plot with 1mM Thyroliberin

Fig. 3.43b. Non-Competitive Inhibition of Synaptosomal PAP by LHRH (Hanes-Woolf Plot)



● Hanes–Woolf Plot with <Glu–His–ProMCA

▽ Hanes–Woolf Plot with 1mM LHRH

3.10.8.2. Preliminary kinetic studies on the purified synaptosomal PE

As with the synaptosomal PAP, the data resulting from these preliminary kinetic studies on the purified synaptosomal PE was fitted to the three different plots, the Lineweaver-Burk, the Eadie-Hofstee and the Hanes-Woolf plot (Fig. 3.44a-c) Synaptosomal PE had a Km of 60μM for the substrate Z-Gly-ProMCA and a Vmax of 747.3nmol/min/mg. The Ki values resulting from the three plots correlated very well (Tables 3.16a-c). The non-competitive inhibitors, bradykinin, angiotensin II and substance P had the lowest Kis in all cases. The most effective competitive inhibitor was LHRH which had Ki values between 0.51 and 0.74μM depending on the plot.
Thyroliberin and acid thyroliberin were the two substrates with the highest Ki values regardless of the plot used to present the data. The values obtained with the Eadie-Hofstee and Lineweaver-Burk plots are quite close to the Km values which can be seen from Tables 3.16a-c, when the two plots became almost indistinguishable (Fig. 3.46a). Even though a synaptosomal PE activity has never been characterised to date, the cytosolic PE has been studied in detail from several sources. It appears to have a high affinity for Angiotensin II, Bradykinin, Substance P and Neurotensin, with less affinity for TRH and LHRH (Welches et al., 1993). The actual Ki values for the natural peptides with the cytosolic PE are all approximately 10-fold higher than those estimated for the synaptosomal PE (Table 3.16a), thus making the synaptosomal membrane-bound PE a far more specific enzyme, which may be as a result of its association with the membrane. A comparison of the three types of plots, each showing a competitive and a non-competitive inhibitor are shown in Fig. 3.45-3.47.
Fig. 3.48 shows the Lineweaver-Burk plot for PE with the thyroliberin substrate, <Glu-His-ProMCA. For <Glu-His-ProMCA, PE had a Km of 72.6μM and a Vmax of 418.4nmol/min/mL.

Table 3.16 Activity of Synaptosomal PE Towards Thyroliberin and Other Peptides

Table 3.16 (a) Inhibition constants using Lineweaver-Burk plots

Peptide	Whether Hydrolysed	Inhibition	Ki (μM)
Bradykinin	+	NC	0.15
Angiotensin II	+	NC	0.18
Neurotensin	+	NC	0.25
Substance P	+	NC	0.64
LHRH	+	C	0.74
Acid Thyroliberin	-	C	22.85
Thyroliberin	+	C	44.93

Fig. 3.16 (b) Inhibition constants using Eadie-Hofstee plots

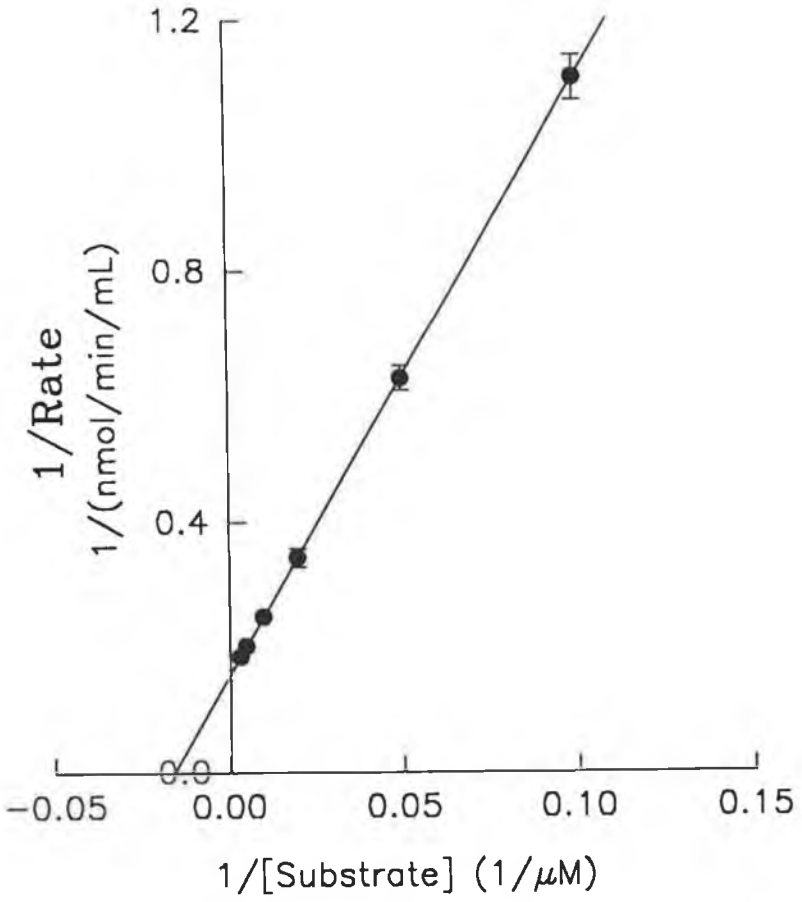
Peptide	Whether Hydrolysed	Inhibition	Ki (μM)
Bradykinin	+	NC	0.10
Angiotensin II	+	NC	0.11
Neurotensin	+	NC	0.14
LHRH	+	C	0.60
Substance P	+	NC	0.79
Acid Thyroliberin	-	C	9.80
Thyroliberin	+	C	53.07

Fig. 3.16 (c) Inhibition constants using Hanes-Woolf plots

Peptide	Whether Hydrolysed	Inhibition	Ki (μM)
Bradykinin	+	NC	0.17
Angiotensin II	+	NC	0.18
Neurotensin	+	NC	0.33
LHRH	+	C	0.51
Substance P	+	NC	0.52
Acid Thyroliberin	-	C	7.40
Thyroliberin	+	C	11.70

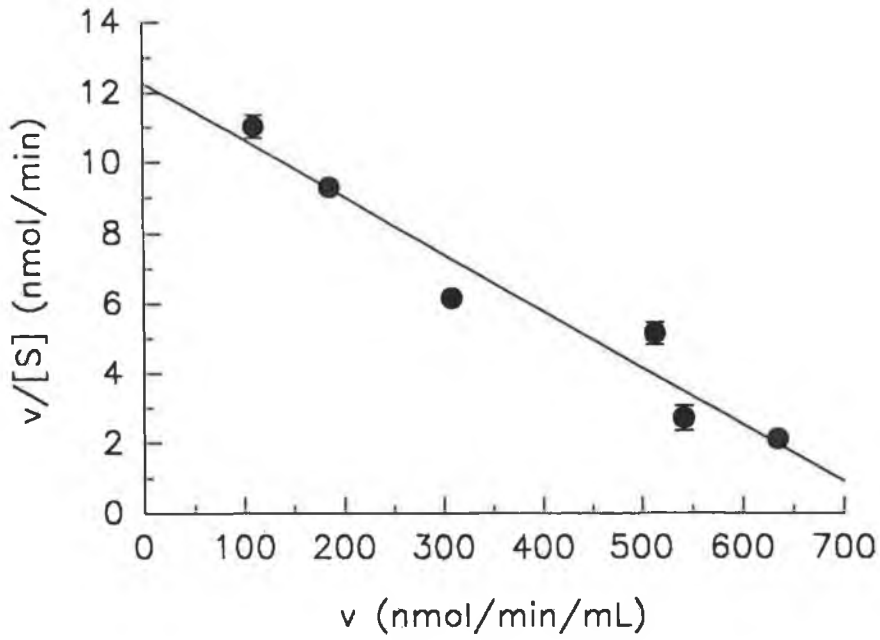
Fig. 3.44a. Lineweaver-Burk Plot for Synaptosomal PE with Z-Gly-ProMCA

(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)



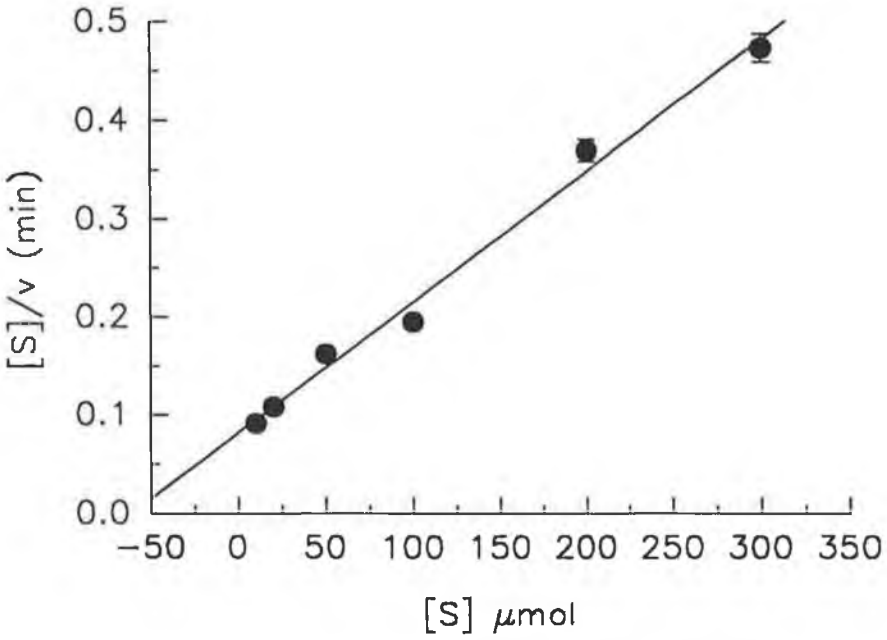
Regression Coefficient = 1.0

Fig. 3.44b. Eadie-Hofstee Plot for Synaptosomal PE with Z-Gly-ProMCA



Regression Coefficient = 0.97

Fig. 3.44c. Hanes-Woolf Plot for Synaptosomal PE with Z-Gly-ProMCA



Regression Coefficient = 1.0

Fig. 3.45a. Competitive Inhibition of Synaptosomal PE by Acid Thyroliberin (Lineweaver-Burk Plot)

(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)

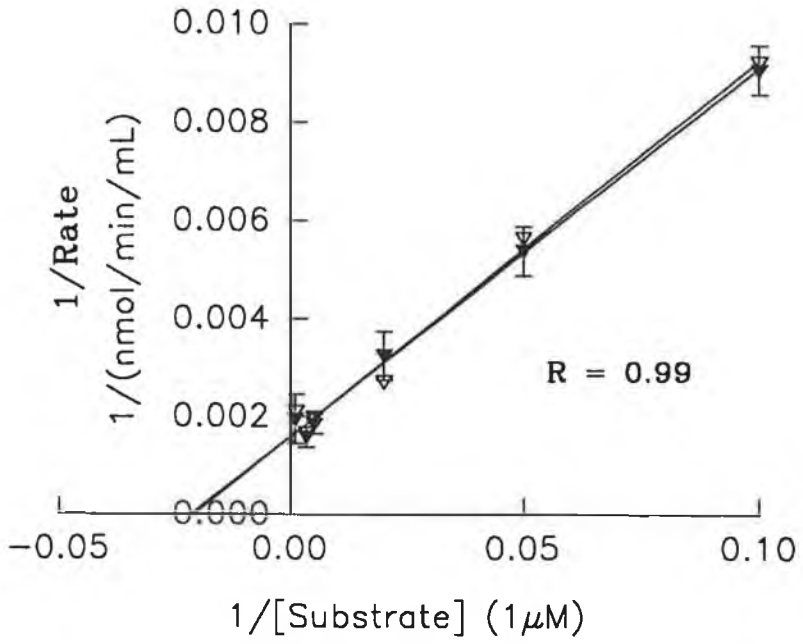


Fig. 3.45b. Non-Competitive Inhibition of Synaptosomal PE by Bradykinin (Lineweaver-Burk Plot)

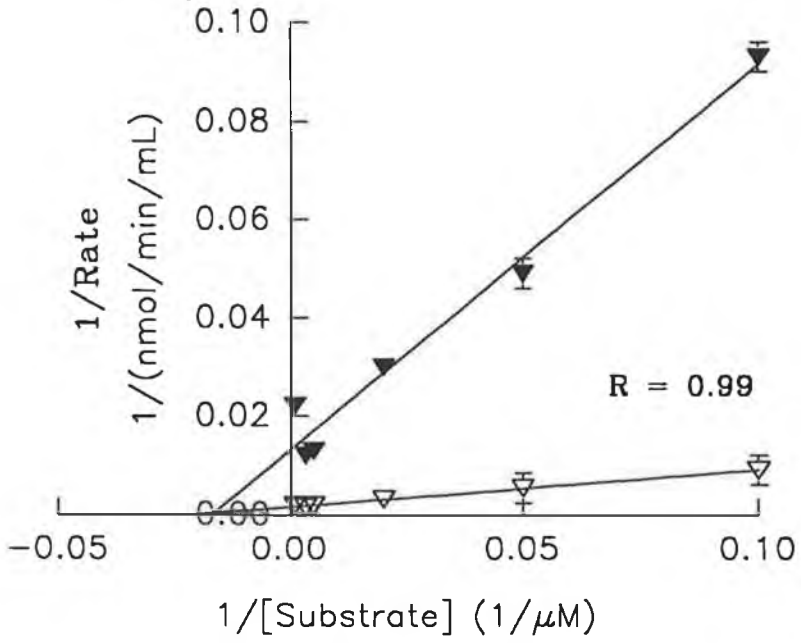


Fig. 3.46a. Competitive Inhibition of Synaptosomal PE by Acid Thyroliberin (Eadie-Hofstee Plot)

(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)

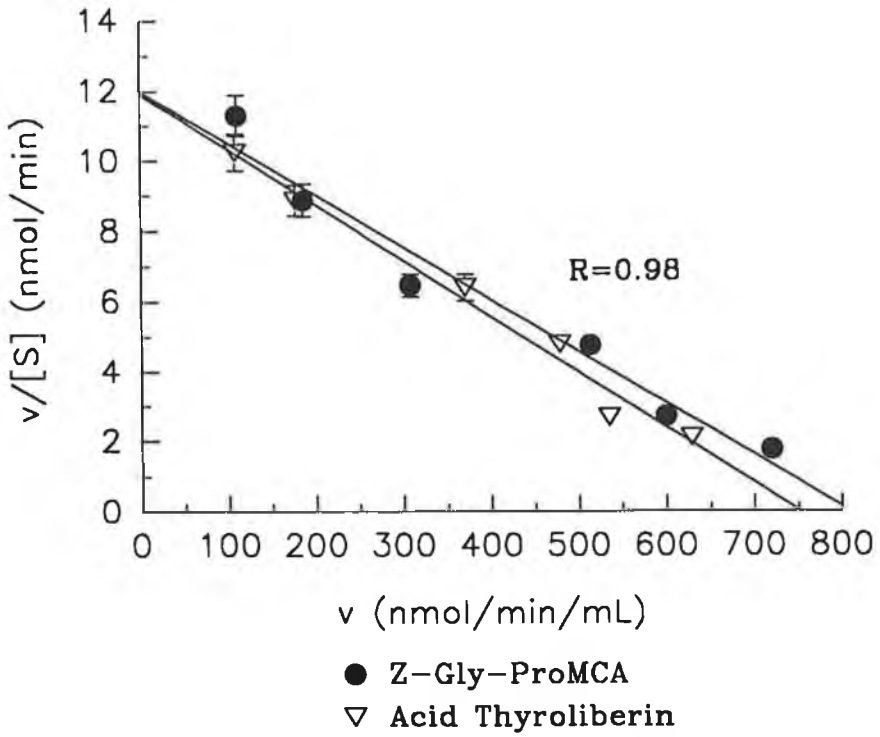


Fig. 3.46b. Non-Competitive Inhibition of Synaptosomal PE by Bradykinin (Eadie-Hofstee Plot)

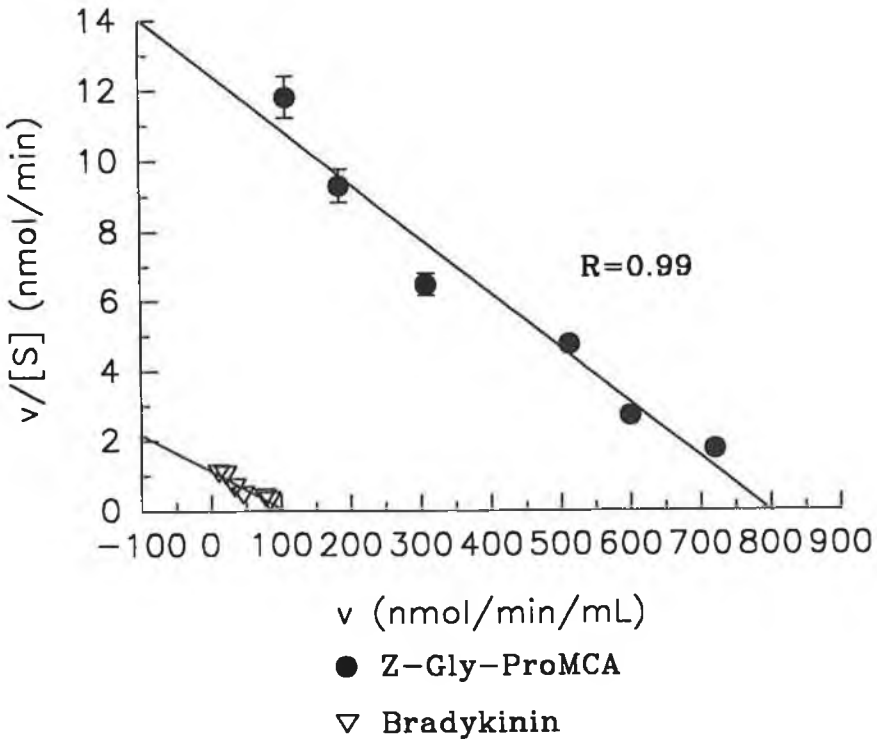


Fig. 3.47a. Competitive Inhibition of Synaptosomal PE by Acid Thyroliberin (Hanes-Woolf Plot)

(Error bars represent S.E.M. = ∂/ √ n , where ∂ = standard deviation and n = number of samples)

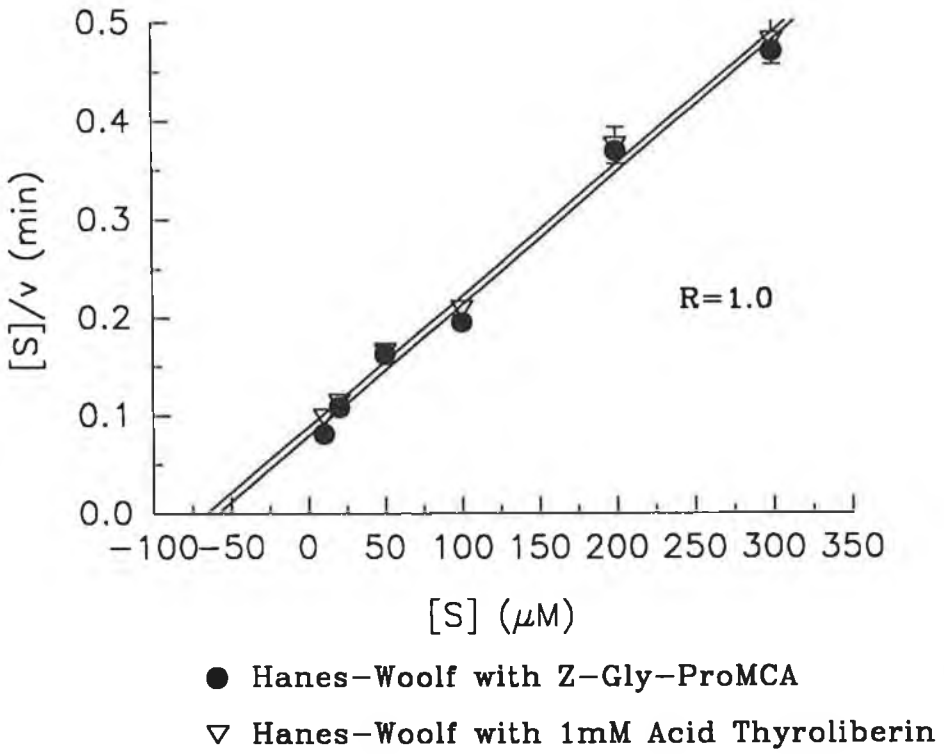


Fig. 3.47b. Non-Competitive Inhibition of Synaptosomal PE by Bradykinin (Hanes-Woolf Plot)

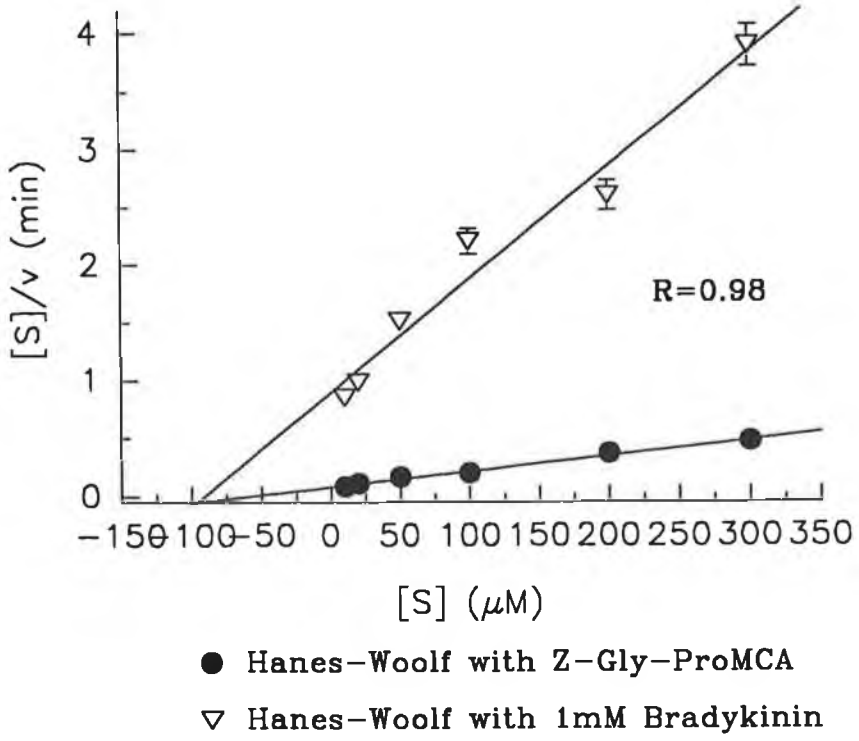
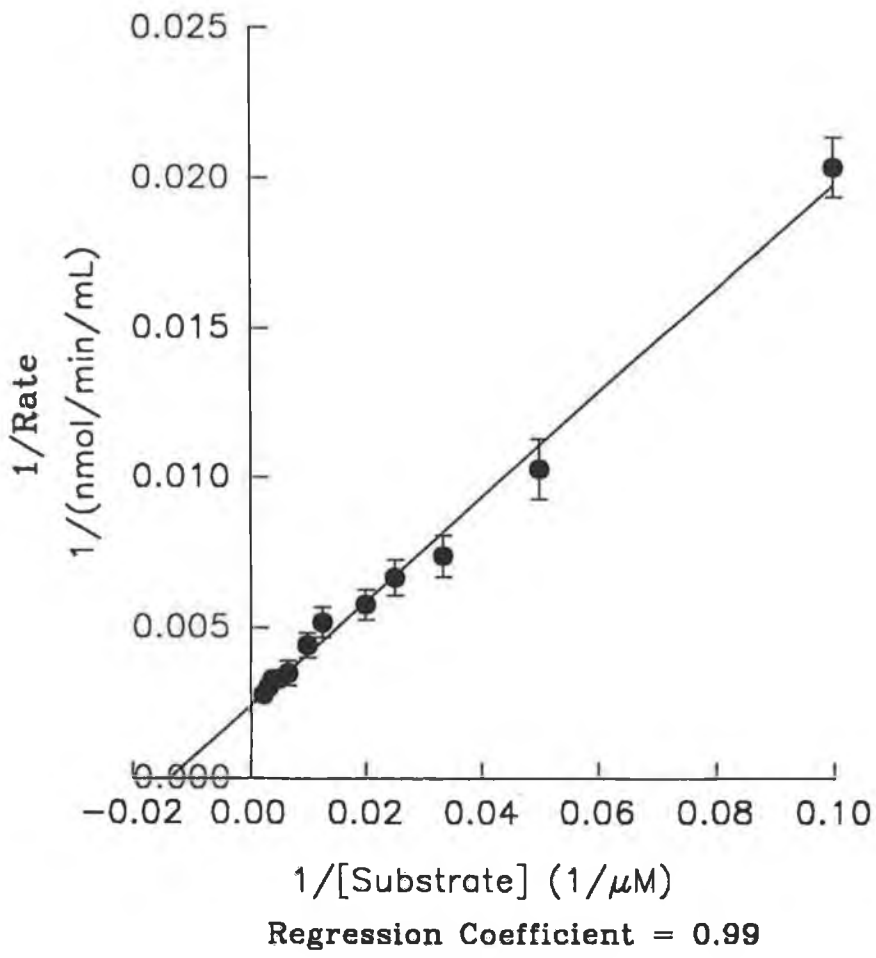


Fig. 3.48. Lineweaver-Burk Plot for Synaptosomal PE with <Glu-His-ProMCA
(Error bars represent S.E.M. = $\partial/\sqrt{n}$, where ∂ = standard deviation and n = number of samples)



3.10.9. Effect of Functional Reagents on the Thyroliberin-Degrading Activities of the Synaptosomal Membranes of Bovine Brain

3.10.9.1. Effect of inhibitors on the synaptosomal PAP activity

The synaptosomal membrane-bound, Triton X-100-solubilised and the purified synaptosomal PAP activities were incubated with a range of functional reagents as described in section 2.16. The effects of these reagents on PAP activity are shown in Table 3.17. There was no significant difference in the effect of the functional reagents on the profile between the synaptosomal membrane-bound PAP and the solubilised activity.

1. The Sulphydryl Reagents: 2- Iodoacetamide, iodoacetate, N-ethylmaleimide and PCMB were all found to be inhibitors to some extent of the synaptosomal PAP activity. At both 1 and 10mM concentrations, iodoacetamide and iodoacetate inhibited the PAP activity by over 90% for the purified enzyme. N-ethylmaleimide was the least inhibitory of the sulphydryl reagents, yet even at 1mM it inhibited 50% of the PAP activity. PCMB at 1mM concentration, completely inhibited the enzyme whether membrane-bound or purified. It was less effective at 0.1mM concentration,causing only 30% inhibition of the purified enzyme. The inhibitory effects of these sulphydryl reagents on the synaptosomal PAP activity indicates that this PAP contains a thiol group essential for its activity.

2. The Metal Chelators: 1,10-phenanthroline proved to be an effective PAP inhibitor. At 1mM it inhibited the PAP activity (whether membrane-bound, Triton X-100-solubilised or purified) by over 90%. At 0.1mM , it was not quite as effective. The other two metal chelators-8-hydroxyquinoline and EDTA, were not found to be inhibitors of the enzyme activity. They both inhibited up to 35% of the PAP activity, but were not as potent as 1,10-phenanthroline at either of the two concentrations examined.

3. The Serine Protease Inhibitors: Benzamidine and PMSF were not particularly potent inhibitors of the synaptosomal PAP. At best 10mM benzamidine inhibited only 35% of the Triton X-100-solubilised PAP activity. Because these inhibitors have little or no effect on the PAP activity, it can be concluded that PAP must not have an essential serine group in its structure.

N-acetylimidazole is a functional reagent with a preference for phenolic groups, but it will also react with His, Tyr and Cys residues. It is not a strong inhibtior of the synaptosomal PAP and only inhibits the purified enzyme by 30% even at 10mM concentration.

4. Puromycin and Bacitracin: Two protease inhibitors of microbial origin, puromycin and bacitracin, were also incubated with the synaptosomal PAP to assess their effects on the enzyme's activity. At 1 and 0.1mM concentration the puromycin inhibited the Triton X-100-solubilised PAP by up to 25%. However the membrane-bound PAP was inhibited by over 50% by 1mM puromycin. The reason for this inhibition is not clear, but the solubilizing action of the Triton X-100 of the PAP from the membrane affords it some protection against the inhibitory actions of puromycin.

The specific prolyl endopeptidase inhibitor, Z-Pro-prolinal, had no effect on the synaptosomal PAP activity.

Table 3.17 Inhibitor Profile of Particulate Pyroglutamate Aminopeptidase Activity

* Concentration of Bacitracin was 500 units of activity

** S.D. Calculated for triplicates using $\sqrt{(\bar{x^2}) - (\bar{x})^2}$ where X = reading

Inhibitor	Conc (mM)	Purified Particulate PAP (as a % of that in control) **(+/-S.D.)
Iodoacetamide	10	**4.91 (+/- 0.25)**
	1	**7.85 (+/-0.31)**
Iodoacetate	10	**4.00 (+/-0.15)**
	1	**5.10 (+/-0.20)**
N-Ethylmaleimide	10	**14.26 (+/-3.60)**
	1	52.71 (+/-9.40)
PCMB	1	**0.000**
	0.1	68.50 (+/-4.80)
Benzamidine	10	77.75 (+/-13.1)
	1	75.35 (+/-7.90)
PMSF	1	72.95 (+/-1.05)
	0.1	71.9 (+/-6.80)
N-Acetylimidazole	10	70.3 (+/-5.13)
	1	81.35 (+/-2.35)
1,10-Phenanthroline	10	**7.22 (+/-6.10)**
	1	71.9 (+/-0.87)
8-Hydroxyquinoline	1	65.0 (+/-3.05)
EDTA	10	**78.0 (+/-9.00)**
	1	**88.0 (+/-2.20)**
Puromycin	1	81.3 (+/-4.20)
	0.1	83.9 (+/-2.50)
Bacitracin	*	27.5 (+/-2.50)
Z-Pro-prolinal	0.1	100.000

3.10.9.2. Effect of inhibitors on the synaptosomal PE activity

As for the synaptosomal PAP, the synaptosomal PE was assayed in the presence of a range of functional reagents at three different stages, membrane-bound, Triton X-100-solubilised and purified PE. The results of these tests on the purified enzyme are shown in Table 3.18.

1. The Thiol Protease Inhibitors: 2-Iodoacetamide, iodoacetate, PCMB and N-ethylmaleimide were the first group to be investigated for their effects on PE. Neither 2-iodoacetamide or iodoacetate were strong inhibitors of the PE activity. 2-Iodoacetamide inhibited the purified enzyme up to 40% at a concentration of 10mM, while at 1mM it had no effect. Iodoacetate was less effectve- at 10mM it only inhibited 30% of the purified PE activity and caused no inhibition of the membrane-bound or Triton X-100-solubilised enzyme's activity at 10mM or 1mM concentrations (results not shown). PCMB and N-ethylmaleimide were very effective inhibitors of PE both at the membrane-bound and solubilised stages. PCMB completely inactivated the PE activity, with only 5and 10% activity remaining for the purified enzyme at 10 and 1mM concentrations respectively. N-ethylmaleimide was less effective at the lower concentration (1mM), but still inhibited 35% of the purified PE's activity.

2. The Metal Chelators: 1,10-phenanthroline completely inactivated the purified enzyme at 10mM concentration and only 12% activity remained after 1mM 1,10-phenanthroline. At the higher concentration, it inhibited 65% of the membrane-bound and Triton X-100-solubilised PE activities, but was less effective at 0.1mM for these two stages (results not shown). EDTA did not cause any significant inhibition of the PE at any concentration. 8-hydroxyquinoline however proved to be quite a strong inhibitor of the purified enzyme at the 10mM concentration, inhibiting its activity by over 60%. It was far less effective at lower concentrations or when the enzyme was still membrane-bound.

3. The Serine Protease Inhibitors: Benzamidine and PMSF did not cause any significant inhibition of the PE activity at any concentration.

4. The Microbial Protease Inhibitors: Puromycin and bacitracin, were also assessed for their efficacy as PE inhibitors. At 1mM concentration puromycin inhibited the purified PE activity by 43%, but was quite ineffective at 0.1mM. Bacitracin, at 500 units of enzyme activity, was quite an effective inhibitor as it inhibited both the membrane-bound and the Triton X-100-solubilised PE activities by 60%. N-acetylimidazole was not a strong inhibitor, as it only inhibited the purified enzyme by 24% even at 10mM concentration. Z-Pro-prolinal, the specific prolyl endopeptidase inhibitor, completely inactivated the PE activity, whether membrane-bound or solubilised at 10^{-4}M.

While the thiol protease inhibitors proved to be the most effective inhibitors of the synaptosomal PE activity, suggesting that the PE has an essential thiol group at its active site, the metal chelator 1,10-phenanthroline, when present in even low concentrations is very effective in inactivating the PE activity. This inactivation by a metal chelator may suggest the requirement for a metal for PE activity. This idea is strengthened by the 60% inactivation of the purified PE's activity by 10mM 8-hydroxyquinoline.

Table 3.18 Inhibitor Profile of Particulate Prolyl Endopeptidase Activity

* The concentration of Bacitracin used was 500 units of activity

** S.D. Calculated for triplicates using $\sqrt{(\overline{x^2}) - (\overline{x})^2}$ where X = reading

Inhibitor	Conc (mM)	Purified Synaptosomal PE (Activity as a % of that in Control) **(+/-S.D.)
2-Iodoacetamide	10	59.70 (+/-4.93)
	1	101.4 (+/-13.77)
Iodoacetate	10	70.44 (+/-1.03)
	1	79.60 (+/-3.50)
PCMB	10	**5.40 (+/-0.60)**
	1	**10.8 (+/-0.90)**
N-Ethylmaleimide	10	**0.79 (+/-0.03)**
	1	65.5 (+/-0.80)
Benzamidine	10	81.8 (+/-8.57)
	1	87.7 (+/-11.3)
PMSF	1	94.17 (+/-0.90)
	0.1	87.17 (+/-7.90)
1,10-Phenanthroline	10	**0.00**
	1	**12.25 (+/-1.46)**
EDTA	10	86.40 (+/-2.83)
	1	101.5 (+/-0.10)
8-Hydroxyquinoline	10	**39.02 (+/-3.42)**
	1	95.12 (+/-8.50)
Puromycin	1	57.34 (+/-2.02)
	0.1	100.5 (+/-1.90)
Bacitracin	*	**37.9 (+/-2.00)**
N-Acetylimidazole	10	76.72 (+/-10.5)
	1	98.32 (+/-8.90)
Z-Pro-prolinal	0.1	**0.00**

3.10.10. Effect of EDTA and DTT on the Thyroliberin-Degrading Synaptosomal Activities

3.10.10.1. Effect of EDTA and DTT on the synaptosomal PAP activity

Due to the previously reported synaptosomal PAP activities being strongly inhibited by EDTA and DTT, even at 1mM concentrations (O'Connor and O'Cuinn, 1984; Friedman and Wilk, 1986; Wilk and Wilk, 1989), the bovine synaptosomal PAP was assayed in the presence of a range of concentrations of EDTA and DTT to see if the enzyme's activity was effected at any concentration. Fig. 3.49 shows how the synaptosomal PAP activity remains constant regardless of EDTA concentration. The inclusion of DTT however had a dramatic effect on PAP activity. The addition of 1mM DTT increased PAP activity by 40%. This rise in activity did not continue with increasing DTT concentration, the maximum enzyme activity was achieved in the presence of 1mM DTT.

3.10.10.2. Effect of EDTA and DTT on synaptosomal PE activity

Prolyl endopeptidase from the synaptosomal membranes of bovine brain was incubated with a range of EDTA and DTT concentrations. The enzyme's activity was hardly effected across the range of EDTA, it maintained its activity between 1.6 and 1.65nmol/min/mL regardless of EDTA concentration (Fig. 3.50). There is only a slight increase in PE activity following the inclusion of DTT - the PE increases from 1.5 to 1.6nmol/min/mL with 1mM DTT, but does not increase with increasing DTT concentration.

3.10.11. Effect of Metal Ions on the Synaptosomal Thyroliberin-Degrading Activities

3.10.11.1. Effect of metal ions on synaptosomal PAP activity

Even though the synaptosomal PAP from bovine brain was not behaving as a classical metalloenzyme (see Table 3.18), it was previously reported that the synaptosomal PAP from other sources did have an essential metal in its active site (Czekay and Bauer, 1993). For this reason, the effect of a series of 1mM concentration metal ions was investigated (Fig.3.51). Zn^{2+}, Co^{2+}, Fe^{2+} and Ag^{2+} were very inhibitory of the PAP activity, but none of the other metals tested had strong inhibitory action. In fact, Li^{+}, Fe^{3+}, Ca^{2+} and K^{+} all sightly increased the PAP's activity.

Fig. 3.49. Effect of EDTA and DTT on Purified Synaptosomal PAP Activity

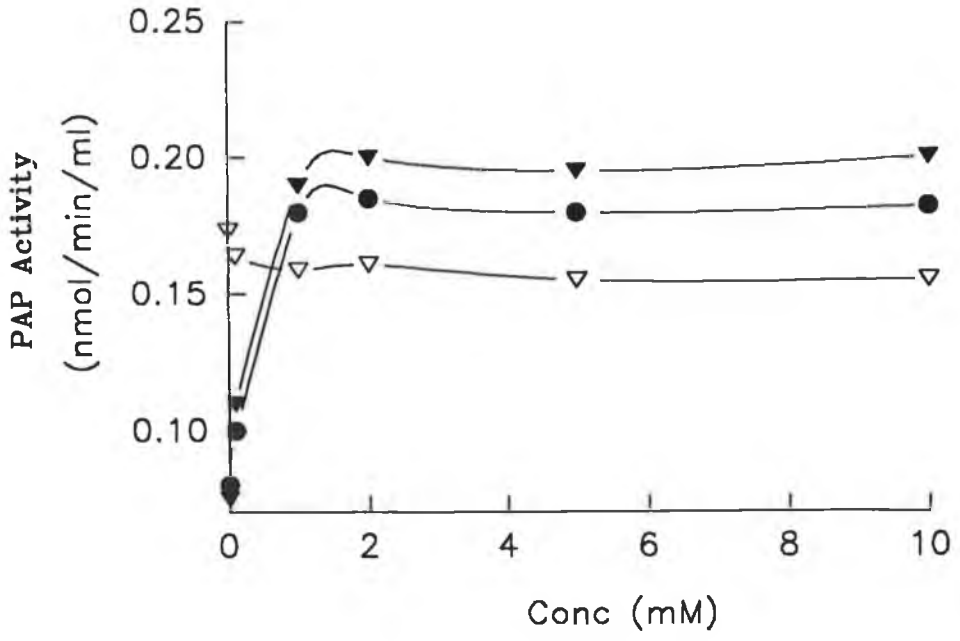


Fig. 3.50. Effect of EDTA and DTT on Purified Synaptosomal PE Activity

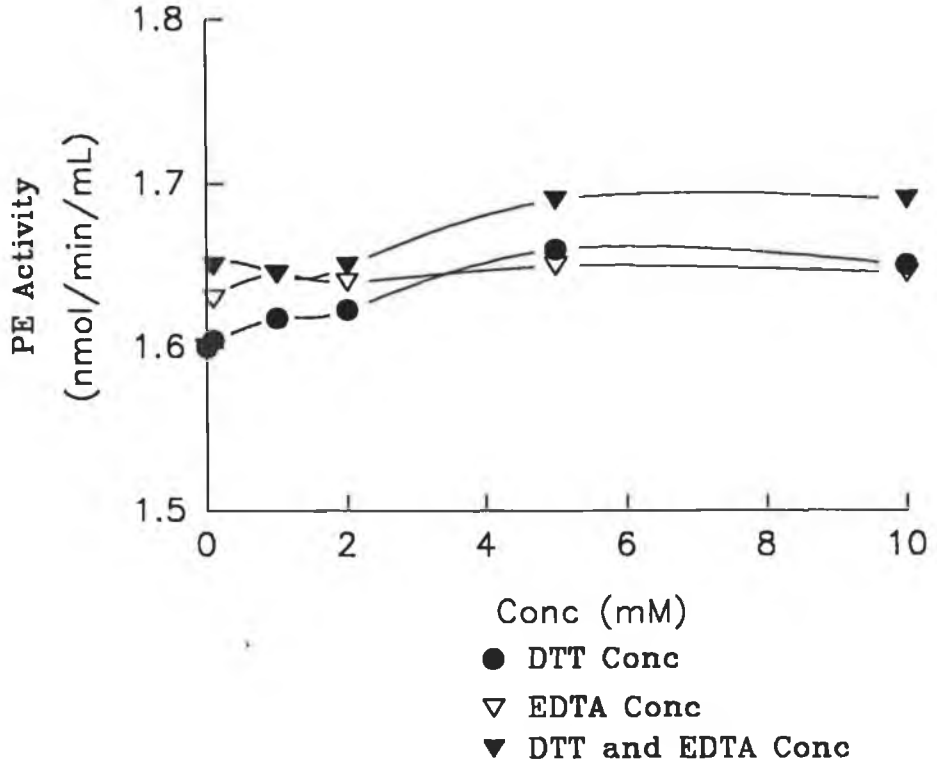
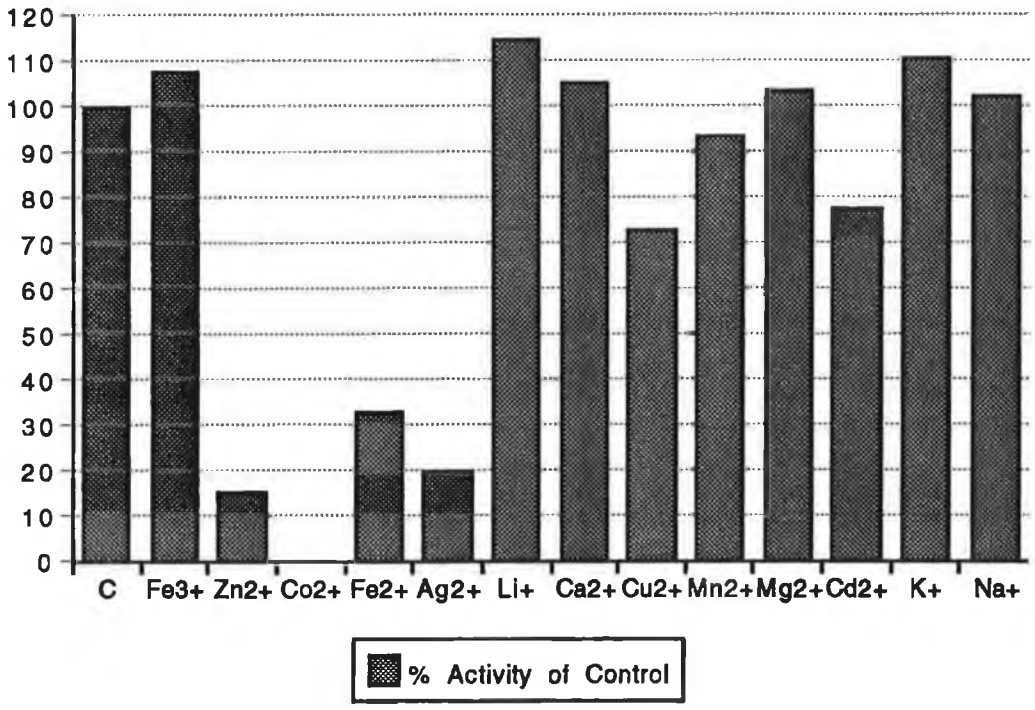


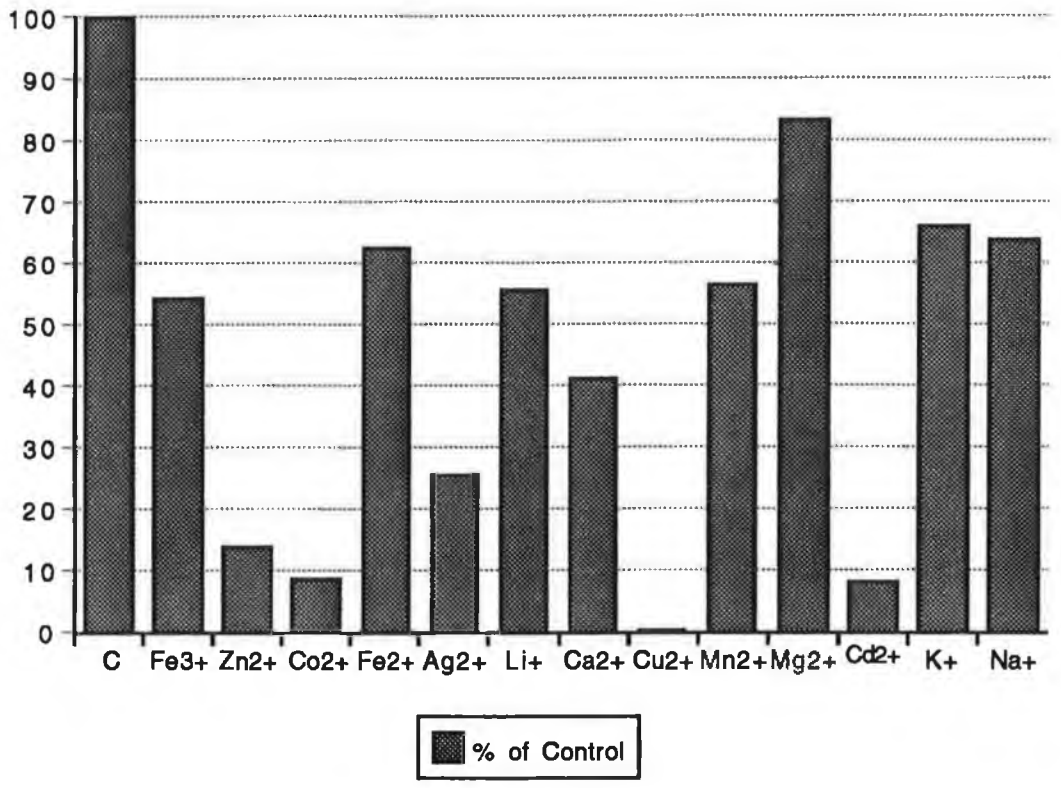
Fig. 3.51 Effect of 1mM Metal Ions on Purified Synaptosomal PAP Activity



3.10.11.2. Effect of metal ions on synaptosomal PE

Purified synaptosomal PE was found to be inhibited by 2 metal chelators, 1,10-phenanthroline and 8-hydroxyquinoline at high concentrations, suggesting that it had a requirement for a metal in its active site (Fig. 3.52). Zn^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+} were all strong inhibitors of the PE activity at 1mM concentration, while none of the metals tested increased the PE activity. The metal ions had a far greater effect on the prolyl endopeptidase activity than they had on the synaptosomal PAP.

Fig. 3.52 Effect of 1mM Metal Ions on Purified Synaptosomal PE Activity



4. Discussion

4. Discussion

4.1. Development of a new fluorimetric assay for the detection of particulate PAP activity

Since its discovery in 1968 (Doolittle and Armentrout, 1968), cytosolic pyroglutamate aminopeptidase (E.C. 3.4.19.3) has been known for its cleavage of the N-terminal pyroglutamyl from a wide range of peptides. For this reason the substrate routinely used for the detection of soluble PAP activity has been <Glu-MCA (Fujiwara and Tsuru, 1978). In 1984, when the first particulate pyroglutamate aminopeptidase activity (E.C. 3.4.9.-) was discovered in guinea pig brain (O'Connor and O'Cuinn, 1984), it was only detectable with thyroliberin as a substrate, as it did not cleave <Glu-MCA. In the early 1980's Bauer and Kleinkauf (1980) had developed an assay capable of detecting PAP activity, which was based on its ability to degrade thyroliberin. Using radiolabelled thyroliberin as a substrate, they developed an assay that was sensitive and accurate for the quantitation of particulate PAP activity. However it was also time-consuming, complex and expensive. It was not until 1986 when Friedman and Wilk began using a chromogenic substrate for particulate PAP activity that the investigation began into new assay systems. They used the substrate <Glu-His-Pro-2naphthyl amide (NA) to detect particulate PAP activity. Unfortunately the cleavage of the <Glu-His bond solely will not allow the release of the chromophore, the Pro-2NA bond also needs to be cleaved. Friedman and Wilk counteracted this problem by the introduction of DAP IV into the reaction sequence, thereby developing the assay as a coupled-enzyme assay. However the Pro-2NA bond is susceptible to cleavage by a prolyl endopeptidase, and therefore a specific PE inhibitor, Z-Pro-prolinal also had to be included in the reaction mixture. While this assay system held distinct advantages over the radiolabelled thyroliberin assay, in that it was faster, more economical and did not involve the use of radioactivity, it also had some disadvantages. The use of the second enzyme, DAP IV was a problem as this enzyme was not available commercially and so had to be purified to homogeneity in the laboratory prior to its use in the assay system. In addition, the use of naphthylamides has been restricted due to their identification as carcinogens. For these reasons, the development of a new fluorimetric substrate by Bachem in 1989 opened the pathway for new assay systems specifically designed for the detection of particulate PAP activity. This new substrate was <Glu-His-ProMCA which had a 7-amino-4-methyl coumarin label attached. The MCA is highly fluorescent, leading to a far more sensitive reading than any chromophore could yield. It is also more economical to use as only picomolar quantities are required for detection. However this substrate also requires the use of DAP IV for the cleavage of the Pro-MCA. DAP IV was purified in the laboratory from rat liver (section 2.4) and Z-Pro-prolinal, the specific PE inhibitor was kindly donated by Sherwin Wilk.

The initial experiments involved the establishment of the assay parameters. By varying the concentrations of substrate, PAP and DAP IV, the optimum conditions for the detection of PAP activity were established. PAP activity from rat liver cytosol (purified in the laboratory) was used as a positive control for all the initial assays as the commercially available PAP activity was found to be inconsistent and unreliable in its activity levels and stability. In order to yield valid results a

number of criteria had to be ensured:

1. The substrate and coupling-enzyme (DAP IV) concentration should never become rate-limiting.
2. There should never be inhibitors of the enzyme (PAP) present in the reaction mixture.
3. All the environmental factors such as pH, temperature and ionic strength should be controlled.

4.1.1. The fluorimetric coupled enzyme assay

The assay used 100µL of sample with 400µL of 0.1mM <Glu-His-ProMCA. These quantities ensured that neither the substrate nor the enzyme were limiting the reaction. 20µL of 10^{-5} M Z-Pro-prolinal was included in the reaction to ensure that any PE activity present would be inhibited. This inhibition was shown to be comprehensive (Fig. 3.3) and not time dependent (Fig. 3.4).

The coupling enzyme, DAP IV, was the final ingredient in the reaction mixture to be investigated. If assays of this type are to yield valid results it is essential that the coupling enzyme never becomes rate-limiting so that the measured rate is always determined by the activity of the enzyme under investigation. Fig. 3.5 shows how the amount of DAP IV was varied to assess its effects on MCA release. 40µL of DAP IV was found to be sufficient to ensure that it was present in excess and that it would not limit the rate of reaction.

The spontaneous and non-enzymatic cyclisation of His-ProNH_2 to yield His-Pro and a free amide group has been well documented (Prasad and Peterofsky, 1976; Prasad et al.,1987). Bungaard and Moss (1990) studied the kinetics of this cyclisation and found that the cyclisation proceeded at maximum rate in phosphate buffer between pH 6.9 and 7.4 at 37°C. The cyclisation of His-ProMCA was investigated in Fig. 3.6. In the absence of DAP IV, MCA was also released but the actual release of MCA was far lower, yielding only 30% of the MCA released when DAP IV is present in the reaction mixture. In order to encourage the spontaneous cyclisation mechanism and to ensure that the presence of DAP IV was not interfering with the measurement of any kinetic constants, the reaction was allowed to proceed for 60 mins, prior to the addition of the coupling enzyme DAP IV. Fig. 3.6 shows that after a further 60 mins the reaction has gone to completion, and the possibility of DAP IV interfering with the measurement of any kinetic parameters was eliminated.

The metal chelator, 1,10-phenanthroline, was found to be an effective synaptosomal PAP inhibitor, withot having any effect on the DAP IV activity. It was added to the reaction mixture at the same time as the DAP IV to ensure that there was no further PAP activity, and to allow the DAP IV to cleave the His-ProMCA which had been produced by the first enzymic reaction. The inclusion of the 1,10-phenanthroline guaranteed that the reaction went to completion. By taking all these parameters into account, it was ensured that neither the substrate nor the coupling enzyme were ever limiting the reaction, there could be no interference in the measurement of kinetic parameters by the coupling enzyme and the entire reaction went to completion within the assay time.

The coupled-enzyme fluorimetric assay was found to be an improvement on the coupled-enzyme spectrophotometric assay developed by Friedman and Wilk (1986), as it is more sensitive, more economical and does not involve the use of any known toxic or carcinogenic substances. Its major disadvantage is the requirement for DAP IV, the coupling enzyme , as this enzyme is not available commercially and has to be purified in the lab prior to performing the assay.

4.1.2 Spontaneous cyclisation assay

If His-ProNH_2 non-enzymatically cyclises to yield His-Pro + NH_2 *in vivo*, was it not possible to utilise this mechanism in an assay? This would eliminate the use of a coupling enzyme and the need for purifying DAP IV for the reaction. Bungaard and Moss (1990) showed that phosphate buffer pH 6-7.4 catalysed the cyclisation ($t_{1/2}$ of 140min at pH 6-7 at 37°C). By omitting DAP IV from the reaction mixture, it was shown that MCA was indeed released over time, however the quantities were quite low (Fig. 3.8). The possibility of stopping the reaction with a basic species to encourage the cyclisation reaction instead of an acid was considered, but at high pH fluoresence is quenched. The temperature after the reaction was stopped was varied in order to encourage the cyclisation of His-ProMCA to His-Pro and free MCA. Fig. 3.9 showed that although the levels of MCA released following 30 mins at 80°C increased greatly, there was also a considerable amount of MCA released due to degradation of the substrate at such a high temperature. In order for the spontaneous cyclisation reaction assay to yield valid results, it is important to monitor the degradation of the substrate in the 80°C waterbath. Taking this disadvantage into account, the spontaneous cyclisation assay is an efficient, sensitive and economical assay, and has the advantage over the coupled-enzyme assay of not requiring a second enzyme, DAP IV.

4.2. Pyroglutamate aminopeptidase activity in bovine brain

4.2.1. Demonstration of two PAP activities in bovine brain

Cytosolic and particulate PAP activity has been studied in several species including guinea-pig (O'Connor and O'Cuinn, 1984), rabbit (Wilk et al., 1988) and rat (Garat et al., 1985). In this study bovine brain was chosen as a source of the PAP enzymes due to its large size and its ready availability. It proved to be a source rich in enzyme activity, activity higher than in any other species previously studied. Initially the cytosolic and particulate fractions were examined for PAP activity using the <Glu-His-ProMCA substrate (in order to detect both types of PAP activity) in the absence and in the presence of DTT and EDTA. Cytosolic PAP characteristically was found to have a requirement for DTT for activity (Browne and O'Cuinn, 1983), and previously studied particulate PAP from rat, rabbit and guinea-pig showed that the particulate PAP activity was strongly inhibited in the presence of EDTA and showed no requirement for DTT (Garat et

al., 1985; Wilk and Wilk, 1989; O'Connor and O'Cuinn, 1984). These differing requirements for the sulphydryl reagent and the metal chelator were typically used as a means of distinguishing between cytosolic and particulate PAP activity. It was interesting therefore to note that the first indication of the bovine PAP displaying a species difference lay in the fact that while the soluble PAP behaved characteristically by increasing its activity in the presence of DTT, the particulate PAP activity behaved totally out of character by actually increasing the level of its total activity from 26.1µmol/min to 66.26µmol/min in the presence of DTT and EDTA. This figure represented 35% of the total PAP activity in the crude homogenate. While these results were unusual it was too soon to comment, without first ensuring that all the increased activity was due to the particulate fraction, and was not due to some soluble PAP activity entrapped by the membranes. This possibility was examined in section 2.8.

4.2.2. Effect of osmotic shock

In order to release any soluble enzyme activity which may have become entrapped in vesicles formed by the membranes during the homogenisation procedure, an osmotic shock step was performed on the membranes to release any such activity following the initial salt wash. Van Amsterdam et al., (1983) claimed that many loosely and non-specifically membrane-associated proteins were mistaken for membrane-bound activities, due to insufficient washing of the membranes. They also suggested the use of osmotic shock to release any entrapped vesicles formed by the membranes during homogenisation which may be harbouring occluded enzyme activity. The results of such a step are shown in Tables 3.3 and 3.4. The particulate fraction was subjected to an osmotic shock step, when the pellet was resuspended with hand homogenisation in distilled water. One pellet was washed in buffer as a control. As can be seen in Table 3.3, there was no extra PAP activity released following washing with distilled H_2O or with buffer, 95% of the activity remained in the particulate fraction. At this stage, following the initial salt-wash and the osmotic shock step, 23.7% of the original crude particulate PAP activity is still remaining in the particulate fraction, indicating that the activity is at least membrane-associated and not just loosely and non-specifically attached to the membranes.

4.2.3 Salt-washing of the membranes

Following osmotic shock, the membranes were subjected to a series of vigorous salt-washing steps with 4M NaCl. The purpose of the salt was to remove any loosely-bound proteins from the surface of the membranes, which were now fully exposed following the osmotic shock step, and to ensure that any remaining activity was definitely membrane-associated if not membrane-bound. Following the four salt washes 74% of the PAP activty remained in the particulate fraction. This figure represents 19% of the original crude particulate PAP activity which is still associated with the membranes.

4.2.4. Subcellular localisation of the particulate PAP activity of bovine brain

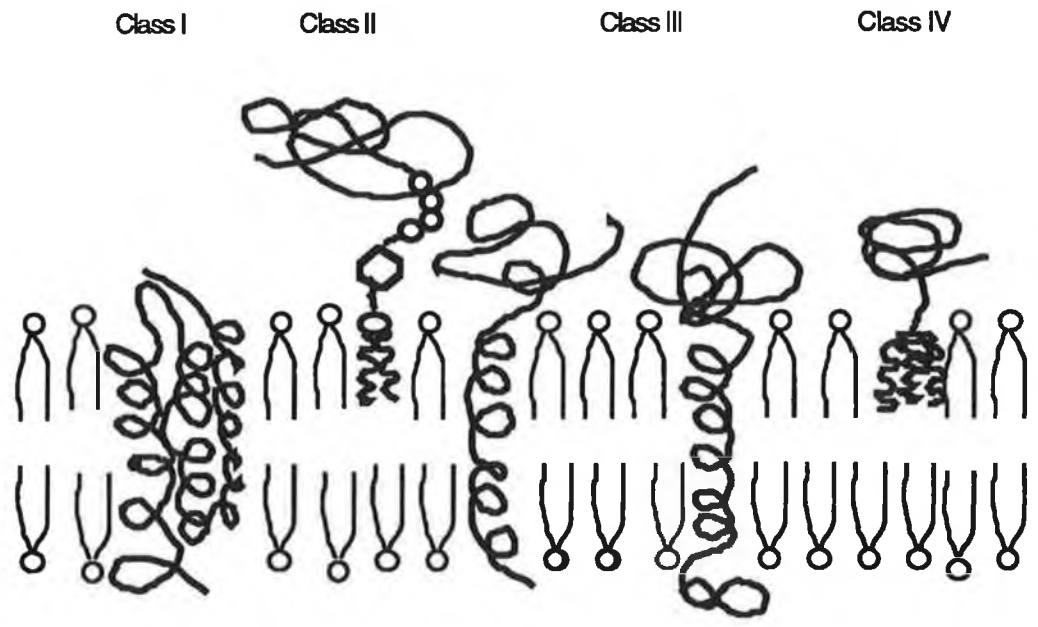
PAP activity had previously been localised in the synaptosomal membranes of guinea-pig (O'Connor and O'Cuinn, 1984), rabbit (Wilk and Wilk, 1989) and rat (Torres et al., 1986)brains. In order to investigate whether the bovine particulate PAP activity shared the same location or not, a combination of differential centrifugation and isopynic ultracentrifugation steps were employed. The salt-washed particulate fraction was divided into three fractions which were identified as the myelin, synaptosomal membranes and mitochondria by specific enzyme marker assays (section 3.5). The majority of the particulate PAP specific activity was enriched in the fraction identified as the synaptosomal membranes, with 75.8% of the salt-washed particulate PAP activity being associated with this fraction. Small levels of activity were also found in the myelin and mitochondria, which was probably due to the mixing of the three layers during their removal. Following salt-washing, osmotic shock and subcellular localisation, approximately 17% of the original crude particulate PAP activity is still remaining associated with the synaptosomal membranes.

4.2.5. Phase partitioning studies on the synaptosomal thyroliberin-degrading PAP activity

The single-most important feature to ascertain about a membrane protein at the outset is its mode of association with the bilayer. Integral (or intrinsic) membrane proteins are integrated into the hydrophobic phase and require disruption of the phospholipid bilayer or cleavage of the polypeptide from its membrane anchor for its release. Integral membrane proteins are classified into four groups depending on the proportion of their structure that it is in contact with the hydrophobic phase of the bilayers (Fig.4.1) (Findlay, 1990).

Peripheral (or extrinsic) membrane proteins on the other hand, are associated with the membrane surface through interactions either with other proteins or with the exposed regions of phospholipid. An assay developed by Bordier (1981) was used to determine the exact nature of the synaptosomal PAP and PE activities. A non-ionic detergent, Triton X-114, was used to solubilise the membrane enzymes. During solubilisation the non-ionic detergent displaces most lipid molecules in contact with the hydrophobic domain of the integral membrane protein and leads to the formation of a soluble protein-detergent mixed miscelle. Above certain temperatures, referred to as cloud points, phase separation occurs until two clear phases are formed,detergent-depleted and detergent-enriched respectively . The hydrophobic integral membrane proteins are located primarily in the detergent-enriched phase and the peripheral membrane proteins are in the detergent-depleted phase. Using this method 60% of the synaptosomal PAP activity was located in the detergent-enriched phase, identifying it as an integral membrane-protein.

Fig. 4.1. Structural Types of Integral Membrane Proteins



Class I: Proteins in which a substantial proportion of their mass is embedded in the bilayer
Class II: Proteins bound to the bilayer via a phosphotidyl inositol-carbohydrate moiety attached to the C-terminus of the polypeptide chain
Class III: Proteins anchored in the bilayer by a single transmembrane segment
Class IV: Proteins associated with the bilayers via fatty acid acyl and/or diacylglycerol moieties covalently attached to the N-terminus of the polypeptide chain .
The latter categories of membrane proteins contain most or all of their mass in the aqueous phase.

4.2.6. Release of the particulate thyroliberin-degrading PAP activity from the synaptosomal membranes

When the exact location of the thyroliberin-degrading PAP activity was established, it was necessary to find an efficient method to effect the release of the activity from the membranes. The most commonly used methods- detergents, proteolytic enzymes and sonication were all tried for their efficacy in causing the release of the membrane-bound activity. It was found that for the synaptosomal PAP activity, the detergent Triton X-100 at a concentration of 0.4% (v/v) was the most efficient, releasing nearly 75% of the PAP activity from the salt-washed

synaptosomal fraction. The requirement for such a high concentration of Triton X-100 (0.4% v/v) in order to release the PAP activity from the membranes lent further evidence to the fact that the enzyme activity is membrane-bound. A requirement for such high detergent concentrations for effecting the release of the enzyme from the synaptosomal membranes, added to the repeated salt-washing and the osmotic shock step, further confirms its status as a membrane-bound activity. The bovine synaptosomal PAP activity may therefore be classed as an integral synaptosomal membrane-bound enzyme. This specific location is in agreement with previously characterised particulate PAP activities from rat (Charli et al., 1988) and rabbit brain (Wilk and Wilk, 1989).

4.2.7. Purification of the synaptosomal PAP by column chromatography

A series of column chromatography steps was used to finalise the purification scheme for the synaptosomal PAP activity. These steps, including gel filtration, ion exchange and hydrophobic interaction chromatography, resulted in a 20.7% yield of synaptosomal PAP activity from the particulate fraction with a purification factor of 644. This apparent low level of enzyme activity is due primarily to its instability (Section 4.2.8.), as it was found to be very difficult to maintain its activity after 24 hours. This instability is in marked contrast to the extremely stable particulate PAP activity isolated from guinea-pig brain (O'Connor and O'Cuinn, 1984) and rabbit brain (Wilk et al., 1988).

The gel shown in Fig 3.19 show that the enzyme was purified to homogeniety. The SDS gel shows that the purified enzyme corresponds closely to the molecular weight marker catalase (230kDa). This size for the synaptosomal PAP was confirmed by running another set of molecular weight markers over the Sephacryl S-200 column, and is the same as the MW for the guinea-pig and rabbit particulate PAP II.

4.2.8. Characterisation of the synaptosomal PAP activity from bovine brain

4.2.8.1. Stability Studies on the Synaptosomal PAP Activity from Bovine Brain

The characterisation of the bovine brain synaptosomal PAP involved a series of experiments designed to yield a fuller understanding of the behaviour of this enzyme, which already appeared to be different in many ways to its guinea-pig and rabbit brain counterparts. The first of these experiments was a series of stability studies on the synaptosomal PAP activity.

The bovine synaptosomal PAP displayed a narrow pH optimum in the neutral range (pH 7.0-7.5) in contrast to the broad pH range of the guinea-pig enzyme (O'Connor and O'Cuinn, 1984) and the rat enzyme (Charli et al., 1984). Its activity in different buffers was examined at both the Triton X-100-solubilised stage and for the purified enzyme for two reasons. The first was to see if the enzyme displayed a marked preference for any particular buffer and secondly to see if any of the buffers could increase the stability of the enzyme. At the Triton X-100-solubilised stage (Fig. 3.28), the enzyme did not appear to show any great preference for any of the buffers, although its activity was highest in potassium phosphate buffer. This slight preference was also

displayed once the enzyme was purified. However, regardless of the buffer system, the activity of the purified enzyme was unstable when stored at 4°C or at -20°C (Figs. 3.36 a and b). Its stability was also examined by trying a range of commonly used stabilisation techniques. The addition of glycerol and BSA to the purified enzyme was examined when stored at both 4°C and -20°C. The most effective stabilisation technique was found to be the inclusion of 10% (v/v) glycerol and 1% BSA (w/v). As the synaptosomal PAP is eluted from the phenyl sepharose column with a glycerol gradient, it was not necessary to add any extra glycerol, and 1% (w/v) BSA was added routinely to the purified enzyme for added stability at 4°C. The activity continued to decrease at -20°C regardless of the amount of BSA, and was found to be the most stable with the addition of 10% glycerol. However this still resulted in a 30% loss of activity. This instability is in marked contrast to the reported long-term stability of the guinea-pig and rat brain synaptosomal PAP enzymes (O'Connor and O'Cuinn, 1984; Garat et al., 1985).

4.2.8.2. Substrate Specificity of the Synaptosomal PAP Activity

The narrow substrate specificity of the synaptosomal PAP from guinea-pig brain and other sources has been well documented (O'Connor and O'Cuinn, 1985: Elmore et al., 1990), where the enzyme has been shown to only cleave the N-terminal pyroglutamate from thyroliberin or very closely related peptides. It was also found that any substitution of pyroglutamyl at the N-terminus completely abolished the enzyme's ability to hydrolyse the peptide. There has been some tolerance shown to substitutions of the $ProNH_2$ residue resulting in cleavage of acid thyroliberin, LHRH (1-3) (<Glu-His-Trp), and the anorexogenic peptide, <Glu-His-Gly. This narrow substrate specificity was examined for the bovine brain synaptosomal PAP by HPLC. Like its guinea-pig counterpart, the bovine synaptosomal PAP displayed a narrow substrate specificity, cleaving only thyroliberin or its most closely related peptides i.e. acid thyroliberin, LHRH (1-3) and <Glu-(Me)His-$ProNH_2$, a thyroliberin analogue (Table 3.15). The anorexogenic peptide <Glu-His-Gly, which was cleaved by the guinea-pig synaptosomal PAP was not hydrolysed by the bovine enzyme. Longer or shorter peptides were not cleaved nor were peptides without <Glu-His at the N-terminus of the sequence.

4.2.8.3. Preliminary Kinetic Studies on the Synaptosomal PAP Activity

The synaptosomal PAP was found to obey Michealis-Menten kinetics with a Km of 100μM and a Vmax of 0.85nmol/min/mL for the substrate <Glu-His-ProMCA. This Km is only slightly higher than that shown for the synaptosomal guinea-pig PAP II (40μM) using the radiolabelled thyroliberin as substrate, and the Vmax is also quite similar (1.1nmol/min/mL) (O'Connor and O'Cuinn, 1985). This Km value suggests a similarly high affinity for the substrate as has been reported for other synaptosomal PAP activities. The difference in Km values may be due to the fact that <Glu-His-ProMCA is a new synthetic substrate, as opposed to the natural substrate previously used (radiolabelled thyroliberin). Wilk and Wilk (1989), the only other reported users of a synthetic substrate for the estimation of kinetic values, estimated a Km of 44μM for the rabbit brain synaptosomal PAP using the substrate <Glu-His-ProNA .

The most interesting feature of the studies previously performed on the guinea-pig synaptosomal PAP, was that LHRH, although not hydrolysed by particulate PAP , showed a greater affinity for the enzyme (Ki=20μM), than thyroliberin itself (Ki=40μM) (O'Connor and O'Cuinn, 1985). It was of special interest then in this study to see if this phenomenon held true for the synaptosomal PAP activity from bovine brain.

As outlined in section 3.10.8, the data resulting from the kinetic studies was fitted to three plots, the Lineweaver-Burk, the Eadie-Hofstee and the Hanes-Woolf plots. The reasons for using three different types of plots are many. Firstly, each in their own way has some inherent inaccuracies, and so to reduce the errors involved in calculating the kinetic constants, all three were used. Secondly, the results from the first plot (Lineweaver-Burk) with the synaptosomal PAP yielded such unusual results, that the other two plots were used to confirm these findings. While competitive inhibition is a regular occurrance, many researchers claim that non-competitive inhibition is a phenomenon that rarely occurs in practice, and in reality should be termed 'mixed inhibition' (Cornish-Bowden, 1979). However this study found that there existed two non-competitive inhibitors for the synaptosomal PAP from bovine brain, LHRH and <Glu-His, and these findings were confirmed by all three plots. The other peptides which were seen to be non-competitive initially when plotted on Lineweaver-Burk plots, were found to actually be competitive when plotted using both of the other two methods. This apparent reversal of results by different plots must be due to the errors incurred by the use of the double recipricol plot and explains why it is the least popular of the plots for estimation of the kinetic constants, even though it remains the most popular for displaying data (Henderson, 1992). Regardless of the method used to represent the data, the Ki values obtained were all quite similar. They were all in the μM range indicating that the bovine synaptosomal PAP has a high affinity for all the peptides examined.

The high affinity of the guinea-pig particulate PAP for LHRH was mentioned earlier, where the enzyme was shown to have a higher affinity for LHRH than for thyroliberin itself (O'Connor and O'Cuinn, 1985). This was not the case for the bovine synaptosomal PAP, all three plots showed that the enzyme had a higher affinity for thyroliberin than for LHRH. However, the enzyme consistently had higher affinities for <Glu-His-Gly (the anorexogenic peptide) and for <Glu-His-Trp (LHRH(1-3)). It may have been expected that the enzyme would display a similar affinity for peptides closest to thyroliberin in structure i.e. acid thyroliberin and the thyroliberin analogue, <Glu-(Me)His-ProNH_2 which were both hydrolysed by the enzyme. However, they consistently had amongst the highest Ki values indicating a lower affinity for these peptides.

The high affinities and the narrow substrate specificities displayed by the guinea-pig synaptosomal PAP II (O'Connor and O'Cuinn, 1985) and the rabbit synaptosomal PAP II (Wilk and Wilk, 1989) appear to be characteristics common to the bovine synaptosomal PAP enzyme also.

4.2.8.4. Effects of Inhibitors on the Activity of Synaptosomal PAP

The effects of a range of inhibitors on the synaptosomal PAP activity from bovine brain are shown in Table 3.17. The most interesting features of this inhibitor profile is the almost

complete inhibition of the purified PAP activity by 2-iodoacetamide, iodoacetate and PCMB, and to a lesser extent, N-ethylmaleimide, which at 10mM concentration also inhibited the enzyme. These sulphydryl reagents, having such an effect on the enzyme's activity, indicate that the bovine synaptosomal PAP must have an essential thiol group for its activity.

The metal chelator, 1,10-phenanthroline, also inactivated the enzyme at high concentrations, suggesting that the enzyme has a requirement for a metal at its active site. Why these results are of particular interest is that all previously characterised synaptosomal PAP activities were found to be 'classical' metalloenzymes - inhibited by all the metal chelators (O'Connor and O'Cuinn, 1985; Wilk and Wilk, 1989). EDTA and 8-hydroxyquinoline, known to completely inactivate the guinea-pig particulate PAP at even 1mM concentration, did not inactivate the bovine synaptosomal PAP even at a concentration of 10mM. The particulate PAP from rat brain was identified as having a requirement for a metal for activity in its active site, most probably Zn^{2+} (Czekay and Bauer, 1993). Due to the bovine enzyme's inactivation by the metal chelator, 1,10-phenanthroline, it is possible that this enzyme has a similar requirement for a metal ion in its active site.

The noninvolvement of serine at the active site is indicated by the failure of PMSF and benzamidine to inactivate the enzyme activity. N-acetylimidazole, previously shown to react with tyrosine, histidine or cysteine residues, (Means and Feeney, 1971), had no effect on the enzyme's activity, suggesting that none of these residues play an important role in the bovine synaptosomal PAP's active site. Tyrosine, arginine and histidine were all identified as essential residues in the active site of guinea-pig synaptosomal PAP (O'Connor and O'Cuinn, 1987). These findings were in agreement with the findings that the active sites of metalloproteases usually contain a tyrosine residue and basic amino acids which are essential for their activity(Czekay and Bauer, 1993). Due to the apparent requirement for a thiol in its active site, the effect of DTT on the synaptosomal PAP activity was examined. As can be seen in Fig. 3.46, the enzyme's activity increases by 60% once even 1mM DTT is added. The combination of DTT and EDTA has a similar effect on the enzyme's activity, while EDTA on its own does not effect the enzyme's activity, negatively or positively. After 2mM DTT, increasing the concentration of DTT does not result in a corresponding increase in enzyme activity however. The effect of a range of 1mM metal ions was examined following the findings of the inhibitor studies on synaptosomal PAP activity. Zn^{2+}, Co^{2+}, Fe^{2+}, and Ag^{2+} were found to have the most dramatic effect on the enzyme's activity, causing upto 100% loss in activity (Fig. 3.43). Czekay and Bauer (1993) found that the rat particulate PAP activity was a typical metallopeptidase as it was inhibited by the transition metal ions Zn^{2+}, Hg^{2+}, Cd^{2+} and Cu^{2+}. The particulate PAP activity was inhibited by Zn^{2+} immediately whereas the others inhibited the activity in a time dependent manner. For the bovine synaptosomal PAP activity, while it was inhibited by 1mM Zn^{2+}, the other transition metals assayed, Cu^{2+} and Cd^{2+} did not cause any significant inhibition. The rat particulate PAP activity, as with many zinc metalloproteases, was increased by Co^{2+}, while the bovine synaptosomal PAP activity was completely inhibited by the addition of Co^{2+}. Since the bovine synaptosomal PAP activity is inhibited by thiol-modifying reagents, the

inhibition by some of the metal ions, notably Zn^{2+}, Co^{2+}, Fe^{2+} and Ag^{2+} could be due to interactions of the metals with essential thiol groups. Some of the metals caused an increase in enzyme activity, notably Fe^{3+}, Li^{+}, Ca^{2+} and K^{+}. It is possible that one of these metals may be present in the active site of the bovine synaptosomal PAP.

Taking all these parameters into account, the bovine synaptosomal PAP appears to be a thiol protease with an essential metal in its active site.

4.3. Prolyl Endopeptidase

4.3.1. Demonstration of two prolyl endopeptidase activities in different subcellular fractions of bovine brain

The unusual nature of the bovine brain thyroliberin-degrading activities was further demonstrated when both the cytosolic and particulate fractions were assayed for PE activity. While PE activity in the cytosol has been well documented (Tate, 1981; Yoshimoto et al., 1983), there have only been infrequent and scant references to the presence of a 'particulate prolyl endopeptidase' activity (Dalmaz et al., 1986; Dresdner et al, 1982). However, from the levels of PE activity seen in Table 3.2, it is clear that there are high levels of PE activity detected when the bovine brain homogenate is divided initially into its cytosolic and particulate fractions. After the inclusion of DTT and EDTA in the homogenisation buffer, the total cytosolic PE activity increased by 15%, while the level of PE activity in the particulate fraction remained the same. Cytosolic PE has been reported to have an essential thiol residue and its activity is increased by the inclusion of ß-mercaptoethanol or DTT (Kato et al., 1980; Kalwant et al., 1991) and so the increase in bovine cytosolic PE activity is in keeping with those findings.

4.3.2. Effect of osmotic shock on the levels of PE activity in the particulate fractions

In view of the fact that a particulate PE activity has not been previously purified or characterised from any source, it was important to ensure that the activity under investigation was indeed associated with the membranes, and was not a loosely-bound, non-specifically associated soluble enzyme activity. The criteria set out by many researchers for what actually constitutes a membrane enzyme were examined with reference to the particulate PE activity in bovine brain. Van Amsterdam et al., (1983) suggested that the lack of washing steps caused many reports of membrane-bound activities, when in fact the activity was merely non-specifically associated with, or entrapped within, the membranes. An initial salt-wash removed 36% of the PE activity from the original crude pellet, this activity was due mainly to residual soluble activity loosely attached to the membranes. By resuspending the pellet in distilled water with gentle hand homogenisation, the membranes are osmotically-shocked and release the contents of any

vesicles that may have been formed by the membranes during homogenisation. 0.6% of the PE activity was released from the vesicles by this step, leaving nearly 65% of the original crude particulate PE activity still in the pellet. Such a high percentage of activity remaining in the pellet suggests that the activity is more that just a non-specific soluble activity loosely attached to the membranes, and that it may in fact be a membrane-associated activity.

4.3.3. Effect of repeated salt-washing of the membranes

The membranes were then repeatedly washed by a 4M salt solution, in order to futher remove any soluble PE activity from the membranes. This step proved to be very effective as a means of removing excess loosely attached soluble PE activity, and ensuring that the resulting PE activity is indeed a specific membrane-associated activity.

4.3.4. Subcellular localisation of the particulate PE activity

Both previous references to a possible PE activity associated with the particulate fraction comment on its low activities, its probable presence in the membranes solely as an occluded activity, and of its location in the crude mitochondrial fraction (Dresdner et al., 1982; Dalmaz et al., 1986). Following a combination of differential centrifugation and isopynic ultracentrifugation, the PE specific activity was seen to increase nearly 2-fold in the synaptosomal membrane fraction, but there was no enrichment of PE activity in either the myelin or the mitochondrial fractions.

4.3.5. Phase-partitioning study on the particulate PE activity

As discussed in section 4.2.5., the mode of association of a membrane protein with the bilayer is an important feature of that protein. The two types of membrane proteins are integral and peripheral, and these differ by their mode of attachment to the phospholipid bilayer. While integral membrane proteins are integrated into the hydrophobic phase and require disruption of the phospholipid bilayer for their release, peripheral membrane proteins are associated with the membranes surface through interactions with other proteins or exposed regions of phospholipids. The assay developed by Bordier (1981) exploits the hydrophobic nature of membrane proteins, to determine whether an enzyme is an integral or peripheral membrane protein. The assay can only be used as an effective means of determining the association of a membrane protein with the membrane once all non-specifically loosely-bound activities have been removed. The intensive salt-washing and osmotic shock steps which preceeded this step ensure that any remaining PE activity is membrane-associated. The phase-partitioning step showed that 87% of the PE activity was associated with the detergent-depleted phase, indicating that the PE is a peripheral membrane enzyme.

4.3.6. Release of the synaptosomal PE activity from the membranes

Van Amsterdam et al. (1983) queried the claims of many researchers when identifiying enzymes as being membrane-bound, while not examining the possibility of the membranes forming vesicles which entrap soluble enzyme activity. They urged the use of salt-washing, osmotic shock and higher concentrations of detergent, condemning low levels such as 0.05-0.1% (v/v) as being insufficient. They claimed that such low concentrations of detergent are not effective in detaching particle-bound enzymes, but are just capable of disorganising membraneous structures and causing the release of the soluble enzyme activities entrapped within the membranes. However, by assaying the synaptosomal membranes in the presence of a series of Triton X-100 concentrations (0-0.5% (v/v)), it was found that only with 0.3-0.4% Triton X-100 was the majority (82.5%) of the PE activity released. A requirement for such high detergent concentrations for effecting the release of the enzyme from the synaptosomal membranes, added to the repeated salt-washing and the osmotic shock step, further confirms its status as a membrane-associated activity.

4.3.7. Purification of the synaptosomal PE activity by column chromatography

A series of column chromatography steps was used to complete the purification of the synaptosomal PE activity. In this case, gel filtration, hydroxylapatite and hydrophobic interaction chromatography were found to be the most efficient methods for its purification 1417-fold, which resulted in a 23% recovery of the PE activity. The use of the Pharmacia Biopilot system for the gel filtration step meant that larger than normal quantities of enzyme could be purified at a faster rate. The gels shown in Figs. 3.20 show how the enzyme was purified to homogeneity and from the SDS-gel, the molecular weight was estimated to be between 80 and 90kDa. This MW was confirmed to be 87kDa by running MW markers over the S-200 gel filtration column. This weight is slightly larger than the MW estimated for cytosolic bovine PE by Tate (1981) of 65kDa and by Yoshimoto et al., (1983) of 76kDa. The larger size may be due to this enzyme being membrane-associated and could be due to some form of anchor or linkage to the membrane.

4.3.8. Characterisation of the synaptosomal PE activity from bovine brain

4.3.8.1. Stability Studies on the Synaptosomal PE Activity

The purified synaptosomal PE activity proved to be quite stable under a variety of storage conditions. The enzyme lost only 5% of its activity when stored for 100 hours at -20°C in its purified state. It was slightly less stable at 4°C, loosing 15% activity over the same time period, and its activity dropped by 50% if the purified enzyme was allowed remain at room temperature. The addition of glycerol to the purified enzyme did not effect its stability at -20°C (Fig. 3.38) and it appeared to be relatively equally stable in either potassium phosphate, HEPES or imidazole buffers. The only buffer in which it was unstable wasTris-HCl, after 100 hours at -20°C its activity

decreased by 21%. Overall it was an extremely stable enzyme regardless of its storage conditions.

4.3.8.2. Substrate Specificity Studies on the Synaptosomal PE Activity

The cytosolic PE has been reported to have a broad substrate specificity, cleaving proline-containing neuropeptides of length between 3 and 30 amino acids, including thyroliberin and LHRH, but it cannot hydrolyse longer peptides (O'Cuinn et al., 1990). However it will also cleave at alanine residues in oligoalanine peptides at low efficiency (Welches et al., 1993). PE was also shown to cleave at an Ala-Ser bond in which the serine was phosphorylated in an undecapeptide mimicking a phosphorylation site in proteins (Rosen et al., 1991). The failure of PEs to cleave at this same site of the unphosphorylated peptide was of interest because it suggests that PE's specificity and protein peptides phosphorylation could function as a method of control for peptide processing (Welches et al., 1993). The specificity of the synaptosomal PE was examined for both the membrane-associated and the purified enzyme to ensure that no physiological change occurred during the enzyme's solubilisation from the membrane. Both the membrane-associated and the purified synaptosomal PE displayed a very broad substrate specificity and cleaved all of the proline-containing peptides. Bombesin, the only non-proline-containing peptide, was included as a control and was not cleaved.

However, the enzyme did display some specificity with regard to its position of cleavage in the case of peptides with more than one proline moiety. For both bradykinin and substance P, the enzyme cleaved at certain sites and ignored others (see Table 3.15). In bradykinin there are three possible cleavage sites - Pro^2-Pro^3, Pro^3-Gly^4 and Pro^7-Phe^8. The PE cleaves the latter two bonds, but ignores the Pro^2-Pro^3 bond. Likewise in Substance P, it will cleave the Pro^4-Gln^5, but not the Pro^2-Lys^3 bond. This demonstrates a certain level of specificity, which had previously been reported for the cytosolic PE, by Camargo et al. (1979).

Tate (1981) reported a bovine brain cytosolic PE which had a requirement for a basic amino acid residue prior to the proline, which would set that enzyme apart from all other PE activities previously characterised. He reported the cleavage of thyroliberin, LHRH, angiotensin II and neurotensin, but not of bradykinin, oxytocin or vasopressin, due to their lack of a basic amino acid prior to the proline moiety. However, this report has since been refuted by several researchers, including Yoshimoto et al. (1983). While also studying the cytosolic PE from bovine brain, Yoshimoto et al. claimed that the enzyme did not show any specificity for basic amino acids prior to the proline and showed the cleavage of bradykinin, oxytocin and vasopressin. In fact, Yoshimoto et al. (1983) were amongst the first researchers to use the synthetic specific PE substrate, Z-Gly-Pro2NNap, which incidently does not have a basic amino acid prior to the proline. In this study, a modified version of that same substrate was used, Z-Gly-ProMCA and detected both cytosolic and particulate bovine PE activity. This study also showed the cleavage of bradykinin by the membrane-associated PE, thereby suggesting that the bovine synaptosomal PE and the previously characterised cytosolic PE activities display a similarly broad substrate specificity.

4.3.8.3. Preliminary Kinetic Studies of the Synaptosomal PE Activity

As no particulate PE activity has been previously characterised, the only comparisons between kinetic constants can be made with the cytosolic enzyme. The synaptosomal PE was found to follow Michaelis-Menten kinetics using both the specific PE substrate, Z-Gly-ProMCA and <Glu-His-ProMCA as substrate. For Z-Gly-ProMCA, the enzyme had a Km of 60μM and a Vmax of 747.3nmol/min/mL. For the second substrate, <Glu-His-ProMCA, its Km was estimated at 72.6μM and its Vmax was found to be 418.4nmol/min/mL. The results from these kinetic studies are shown in Table 3.14. The most notable feature is the number of non-competitive inhibitiors, which were shown to be non-competitive regardless of the method used to plot the data. Considering that non-competitive inhibition is reported to occur only in rare cases (Cornish-Bowden, 1979), it is indeed remarkable that the majority of the naturally-occurring peptides (all hydrolysed by PE) were found to be non-competitive. Another interesting feature is the level of inhibition - all of the Ki values for the inhibitors are in the μM range and most of them are less than 1μM. For non-competitive inhibitors to display such low Ki values, the synaptosomal PE enzyme must have a very high affinity for all of these neuropeptides. In each case, thyroliberin and acid thyroliberin have the highest Ki values - in the case of the Lineweaver-Burk and Eadie-Hofstee plots, the Ki is nearly equivalent to the Km for Z-Gly-ProMCA suggesting that it has a similar affinity for both the synthetic substrate and for thyroliberin and acid thyroliberin. It also shows a high Ki for the substrate <Glu-His-ProMCA (72.6μM), in fact it has a higher affinity for this substrate than the synaptosomal PAP does.
The kinetic constants obtained with the bovine cytosolic PE by Tate (1981) are all in the μM range also. The enzyme displays a high affinity for angiotensin II (1.2μM), LHRH (4μM), neurotensin (1.8μM) and bradykinin (7μM). It has a very low affinity for acid thyroliberin (580μM) and thyroliberin (480μM). This low affinity for thyroliberin appears to be a feature of the cytosolic PE in all species. The Km for thyroliberin in rat brain was reported to be as high as 4100μM (Andrews et al., 1980), but most values range from 540-690μM (Yoshimoto et al., 1983). The kinetic constants shown by the bovine synaptosomal PE for both the <Glu-His-ProMCA substrate (72.6μM) and thyroliberin (Ki= 44.93μM) and its very low Ki values for the other neuropeptides examined sets this enzyme apart from its cytosolic counterpart.

4.3.8.4. Effect of Inhibitors on PE Activity

The cytosolic PE has been well characterised from many sources including bovine, rat and rabbit brains. Due to this extensive charaterisation, it was of special interest to see if the inhibitor profile resulting from the investigation of the bovine synaptosomal PE activity was similar or very different to its cytosolic counterpart. There appears to be some conflict in the literature with regard to the exact nature of the cytosolic PE. It has been identified as a serine protease, with no metal requirement (Yoshimoto et al., 1977), it is reported to have an essential thiol group and its activity increased by DTT and 2-mercaptoethanol (Kato et al., 1980; Kalwant et al., 1991). Its inhibition by sulphydryl reagent also indicated the presence of a sulphydryl group necessary for expression of enzyme activity (Browne and O'Cuinn, 1983) and by benzyloxycarbonyl (CBZ)-

Pro-prolinal *in vitro* and *in vivo* (Wilk and Orlowski, 1983; Friedman et al., 1984). The bovine synaptosomal PE was found to be completely inhibited by PCMB at all concentrations, and by N-ethylmaleimide at 10mM concentration. At the lower concentrations, N-ethylmaleimide only inactivated 34% of the purified enzyme. The two other sulphydryl reagents did not cause as much inactivation of the enzyme, whether membrane-bound or purified. The inactivation by PCMB and N-ethylmaleimide of the synaptosomal PE activity, whether membrane-bound, Triton X-100-solubilised or purified, indicates that there is a thiol group present in the active site of the enzyme. The other major inhibitor was 1,10-phenanthroline, and to a lesser extent 8-hydroxyquinoline. EDTA, the third metal chelator, did not have any great effect on the enzyme's activity. This selective inhibition by both sulphydryl reagents and metal chelators is an unusual feature of the bovine synaptosomal PE' s activity. However, Tisljar and Barrett (1990) reported the presence of endopeptidase 24.15 in both rat and rabbit which was a thiol-dependent metallopeptidase. The enzyme was inhibited by the metal chelators, EDTA and 1,10-phenanthroline in a time-dependent manner, but this activity was restored with the addition of metal ions. The enzyme was activated by sulphydryl reagents such as 2-mercaptoethanol and DTT, and was inhibited by PCMB and other thiol blocking agents. Due to the apparent dual dependence of the enzyme on a thiol group and on an essential metal at its active site, the name 'thimet protease' was proposed for such an enzyme (Barrett and Brown, 1990). It is possible that the bovine synaptosomal PE is such an enzyme, as it is inhibited by the sulphydryl reagents PCMB and N-ethylmaleimide (at high concentrations), yet it is also inhibited by the metal chelator 1,10-phenanthroline. The cytosolic PE was reported to be inhibited by bacitracin (Browne and O'Cuinn, 1983), and this bovine synaptosomal PE activity was also inhibited by 62% by 500 units of bacitracin .

The report by Barrett and Brown (1990) claimed that even though the endopeptidase 24.15 was inhibited in a time-dependent manner in the presence by EDTA and 1,10-phenanthroline, that some metal ions were found to restore activity after such treatment. Zn^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} and Cd^{2+} were all seen to restore activity in a decreasing order of effectiveness. Other metal ions however were found to be inhibitory. Even though this type of assay was not performed on the bovine syanptosomal PE enzyme, the effects of a number of metal ions on enzyme activity were examined. Zn^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+} were all found to inactivate the enzyme by between 90 and 100%. The inhibition of metallopeptidases by transition metal ions such as Hg^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} is a common occurrance. The inhibition of the enzyme's activity by Hg^{2+}, Cd^{2+} and Cu^{2+} usually proceeds in a time-dependent manner. An excess of Zn^{2+} can often result in immediate inhibition. This difference may be explained by the different inhibition mechanisms of Zn^{2+}, which occupy an inhibitiory metal binding site in zinc-dependent proteases, whereas the inhibition by other transition metal ions is also due to the exchange of the catalytic Zn^{2+} ion. The enzyme' s activity was not increased by Co^{2+}, often an indicator of a zinc-metalloprotease, in fact it inhibited the enzyme's activity by 90%.
Taking into account both the effects of the inhibitors and the metal ions, the bovine

synaptosomal PE appears to have many of the features of a thimet protease as described by Barrett and Brown (1990).

The effect of EDTA and DTT on the purified synaptosomal PE activity was examined. EDTA showed no effect on the level of PE activity, and 1mM DTT only increased the level of activity by 5%. However, even this slight increase in enzyme activity is yet another indication of the presence of an essential thiol group in the prolyl endopeptidase's active site.

4.4. Conclusion

The initial part of this work concentrated on the development of two new fluorimetric assays for the detection of particulate pyroglutamate aminopeptidase (PAP Type II). Prior to this, there existed two assays for the enzyme's quantitation, the first, a complex assay based on radiolabelled thyroliberin being degraded by the particulate PAP (Bauer and Kleinkauf, 1980), and the second, a coupled-enzyme spectrophotometric assay using the synthetic substrate <Glu-His-ProNA (Friedman and Wilk, 1986). While both of these assays adequately detected particulate PAP activity, there were several drawbacks to their use. The radiolabelled thyroliberin assay was complex and tedious, and also very expensive, while the second assay involved the use of dipeptidyl aminopeptidase IV as the coupling enzyme. DAP IV is not available commercially, and so it had to be purified in the laboratory for use in the assay. The spectrophotometric substrate <Glu-His-ProNA had the added disadvantage of being sensitive only to the nanomolar range and also of using the known carcinogen, naphtylamide.

The development of a new fluorimetric substrate, <Glu-His-ProMCA, heralded a new opening for the development of an assay system for the detection of particulate PAP activity. The coupled enzyme fluorimetric assay developed using this substrate proved to be efficient, sensitive (up to the picomolar range), economical and easy to use. Its only disadvantage was its dependence on the coupling enzyme DAP IV for the cleavage of the Pro-MCA bond.

The spontaneous and non-enzymatic cyclisation of His-ProNH_2 is a well recorded phenomenon (Peterkofsky et al., 1982; Moss and Bungaard, 1990). The idea of exploiting this cyclisation mechanism led to the development of the second assay for the detection of particulate PAP activity. It was shown that by increasing the temperature of the sample after the reaction has been stopped by acid, the cyclisation of the His-ProMCA to cyclo(His-Pro) and free MCA is encouraged. This assay has many advantages over the coupled enzyme assay, the principal one being the assay does not require the coupling enzyme, DAP IV. It is as efficient, as economical and as easy to use as the coupled enzyme fluroimetric assay, but does not require DAP IV. Its only disadvantge is that the substrate tends to degrade at such high temperatures, but once this degradation is taken into account, the spontaneous cyclisation assay is fast, efficient and sensitive.

Particulate PAP activity has been studied from several different sources, guinea-pig (O'Connor and O'Cuinn, 1984), rat (Garat et al., 1985) and rabbit (Wilk et al.,1988) being the most researched. This study used bovine brain as its source of PAP activity as it was readily available,

large in size and proved to be rich in enzyme activity. The PAP activity, which was found to be localised on the synaptosomal membranes of the bovine brain, displayed many of the characteristics common to previously studied particulate PAP (PAP II) activities. Its location on the synaptosomal membranes, its large size, its narrow substrate specificity and its high affinity for its substrate thyroliberin, are all features common to the guinea-pig, rabbit and rat PAP II activities. However, the bovine brain particulate PAP also displayed some unusual features, such as an inhibition by thiol protease inhibitors, a requirement for DTT, no effect seen with EDTA and non-competitive inhibition by LHRH. These features are more in keeping with the cytosolic PAP activity, which is classed as a thiol protease, than with PAP II which is classed as a 'classical' metalloprotease . As mentioned already, LHRH non-competitively inhibits the particulate PAP activity. Non-competitive inhibition is unusual in itself, but PAP II had been shown to have a higher affinity for LHRH than for thyroliberin, although it does not cleave LHRH (O'Connor and O'Cuinn, 1985). While the bovine particulate PAP still has a high affinity for LHRH, it consistently has a higher affinity for thyroliberin. The exact nature of the enzyme activity is still unknown. It appears to be a 'hybrid' form of pyroglutamate aminopeptidase, displaying many of the characteristics of both the cytosolic and the particulate type II PAP activity. A clearer understanding into the exact nature of the enzyme would be possible following investigation into the regulation of the enzyme, as the modes of cytosolic and particulate PAP regulation are very different (Bauer, 1987; Scharfmann et al., 1990). The reason as to why another form of pyroglutamate aminopeptidase would exist is also unclear, but with the ubiquitous nature of thyroliberin, it may act as an extra control mechanism for thyroliberin levels *in vivo* and for the production of cyclo(His-Pro), known to be a bioactive peptide byproduct of the actions of PAP on thyroliberin and on the thyroliberin precursor (Miyashita, et al., 1993).

Prolyl endopeptidase (PE) activity has been widely characterised from many sources (Yoshimoto et al., 1983; Camargo et al., 1984). However despite some references to the possibility of there existing a particulate form of the enzyme, such an activity was never investigated. Dresdner et al. (1982) localised the enzyme in the cytoplasm, they remarked on how a sizeable proportion of the PE activity appeared to be associated with the particulate fraction, but took this activity to be due to entrapped cytosolic activity, as they had not adequately washed the membranes. Camargo et al. (1984) and Dalmaz et al. (1986) likewise noticed PE activity in the particulate fraction, but took it to be non-specifically associated or to be associated with the crude mitochondrial fraction. Therefore, it was with extreme interest that the particulate PE activity in bovine brain was examined. As with all claims of a membrane-bound activity, many precautions had to be observed prior to giving the enzyme the title 'membrane-bound'. Van Amsterdam et al. (1983) refuted many claims of membrane-bound activities by showing that following an osmotic shock step, and adequate washing of the membranes, much occluded enzyme activity is removed. This is soluble activity which has merely become entrapped within vesicles formed by the membranes during the homogenisation step. Van Amsterdam went on the say that most membrane-associated activities, even if they survived the washing and osmotic shock steps, would be removed from the membranes by a low

concentration of detergent (upto 0.1% v/v Triton X-100). The bovine particulate PE was washed with repeated 4M salt solutions, was subjected to an osmotic shock step as suggested by Van Amsterdam, and was then localised to the synaptosomal membranes. Following such a vigorous series of steps the bovine PE activity was solubilised from the synaptosomal membranes with 0.4% v/v Triton X-100. This indicates that the enzyme is at least specifically membrane-associated, if not membrane-bound. Using the method of Bordier (1981), particulate PE was found to exist as a peripheral membrane activity. The enzyme was purified and characterised for the first time from a particulate fraction. It has a molecular weight of 87kDa, a broad substrate specificity and a high affinity for thyroliberin (higher in fact than the affinity displayed by the particulate PAP) and many other neuropeptides. Its chemical characterisation proved to be unusual as it was inhibited by some of the thiol protease inhibitors and also by some of the metal chelators. Such a dual dependency on a thiol group and an essential metal ion has been recorded before (Barrett and Brown, 1990; Tisljar and Barrett, 1990) and has been classed as a 'thimet' protease. It is possible that the bovine synaptosomal PE activity is also a thimet protease.

The possible role of a particulate PE is of great interest. Its action on thyroliberin gives rise to acid thyroliberin which has been accredited with several effects of its own (Boschi et al., 1980; Webster et al., 1983). This biotransformation produces a relatively stable compound, His-ProOH, which is only slowly degraded into its constituent amino acids (Griffiths et al., 1983) and may have the ability to reamidate itself into thyroliberin (Webster et al., 1983). Based on the ubiquitous distribution of thyroliberin, acid thyroliberin and PE it is possible that a precursor/product relationship may exist between thyroliberin and acid thyroliberin similar to that for PAP (Prasad and Edwards, 1984). It is also possible that the particulate PE may act on the thyroliberin precursor and produce acid thyroliberin directly, in a manner similar to that shown for cyclo(His-Pro) production (Miyashita et al., 1993). Recent studies of angiotensin have shown that angiotensin (1-7) which can be produced by the action of prolyl endopeptidase on angiotensin, is also a bioactive fragment, activating vasopressin secretion (Schiavone et al., 1988), stimulating the neuronal exicitability within the vagal-solitary complex (Barnes et al., 1990), modulating the baroreflex (Campagnole-Santos et al., 1989) and releasing prostaglandins (Trachte et al., 1990). These biotransformations yielding such active by products, acid thyroliberin and angiotensin (1-7), strengthen the idea that these enzymes are multifunctional in degrading and producing neuropeptides and may be involved in many more as yet undiscovered biotransformations.

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5. References

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Appendix

1. Derivation of Eadie-Hofstee Equations for Enzyme Inhibition (Segel, 1975)

Michaelis-Menten Equation : $V = \frac{Vmax\,[S]}{[S] + Km}$

$$\frac{V}{[S]} = \frac{Vmax}{Km} - \frac{V}{Km}$$

By plotting V vs V/[S]:

Slope = -1/Km

Intercept on y-axis = Vmax /Km

1. Competitive Inhibition

$$\frac{V}{Vmax} = \frac{[S]}{Ks(1+ [I] / Ki) + [S]}$$

$$VKs(1 + [I] / Ki) + V[S] = [S]\,Vmax$$

$$V/[S]\ Ks(1 + [I] / Ki) + V = Vmax$$

$$V/[S] = \frac{Vmax}{Ks(1 + [I] / Ki)} - \frac{V}{Ks(1 + [I] / Ki)}$$

By plotting V vs V/ [S] :

$$\text{Slope} = \frac{-1}{Ks\,(1 + [I]/Ki)}$$

$$\text{Intercept on y-axis} = \frac{Vmax}{Ks(1 + [I] / Ki)}$$

2. Non-Competitive Inhibition:

$$\frac{V}{Vmax} = \frac{[S]}{Ks(1 + [I]/Ki) + [S]\,(1 + [I]/Ki)}$$

$$VKs(1 = [I] / Ki) + V[S]\,(1 + [I] / Ki) = Vmax\,[S]$$

$$V / [S] = \frac{Vmax}{Ks(1 + [I] / Ki)} - \frac{Vmax}{Ks}$$

By plotting V vs V/[S]:

Slope = -1/Ks

$$\text{Intercept on y-axis} = \frac{Vmax}{Ks(1 + [I] / Ki)}$$

2. Derivation of Hanes-Woolf Equations for Enzyme Inhibition:

Michaelis-Menten Equation: $V = \frac{Vmax\,[S]}{[S] + Km}$

$$\frac{[S]}{V} = \frac{[S]}{Vmax} + \frac{Km}{Vmax}$$

By plotting [S] vs [S]/V:

Slope = 1 / Vmax

Intercept = Km / Vmax

1. Competitive Inhibition:

$$\frac{V}{Vmax} = \frac{[S]}{Ks(1+[I]/Ki) + [S]}$$

$$VKs(1 + [I]/Ki) + V[S] = Vmax\,[S]$$

$$Ks(1 + [I]/Ki) + [S] = Vmax\,[S]/V$$

$$\frac{[S]}{V} = \frac{[S]}{Vmax} + \frac{Ks}{Vmax}(1+[I]/Ki)$$

By plotting [S] vs [S] / V:

Slope = 1/Vmax

Intercept on y-axis = $\frac{Ks}{Vmax}(1+[I]/Ki)$

2. Non-Competitive Inhibition:

$$\frac{V}{Vmax} = \frac{[S]}{Ks(1 + [I]/Ki) + [S](1 + [I]/Ki)}$$

$$VKs(1 + [I]/Ki) + V[S](1 + [I]/Ki) = [S]\,Vmax$$

$$VKs + V[S] = \frac{[S]\,Vmax}{(1 + [I]/Ki)}$$

$$V(Ks + [S]) = \frac{[S]\,Vmax}{(1+[I]/Ki)}$$

$$Ks + [S] = \frac{[S]}{V}(Vmax/(1 + [I]/Ki))$$

By plotting [S] vs [S]/V:

Slope = $\frac{(1 + [I]/Ki)}{Vmax}$

Intercept on y-axis = $\frac{Ks}{Vmax}(1 + [I]/Ki)$

3. Derivation of the Lineweaver-Burk Equation for Enzyme Inhibition:

Michaelis-Menten Equation: $V = \frac{Vmax\,[S]}{[S] + Ks}$

By taking the reciprocal of both sides:

$$\frac{1}{V} = \frac{Ks + [S]}{Vmax\,[S]}$$

$$\frac{1}{V} = \frac{Ks}{Vmax\,[S]} + \frac{[S]}{Vmax\,[S]}$$

$$\frac{1}{V} = \frac{1}{Vmax}\,\frac{Ks}{[S]} + \frac{1}{Vmax}$$

By plotting 1/[S] vs 1/V:

Slope = Ks / [S]

Intercept on y-axis = 1/ Vmax

1. Competitive Inhibition:

$$\frac{V}{Vmax} = \frac{[S]}{Ks(1 + [I]/Ki) + [S]}$$

$$\frac{1}{V} = \frac{Ks\,(1 + [I]/Ki) + [S]}{[S]\,Vmax}$$

$$\frac{1}{V} = \frac{Ks\,(1 + [I]/Ki)}{[S]\,Vmax} + \frac{1}{Vmax}$$

By plotting 1/ [S] vs 1/ V:

Slope = Ks/ Vmax (1 + [I] / Ki)

Intercept on y-axis = 1 / Vmax

2. Non-Competitive Inhibition:

$$\frac{V}{Vmax} = \frac{[S]}{Ks\,(1 + [I]/Ki) + [S]\,(1 + [I]/Ki)}$$

$$\frac{V}{Vmax/\,(1 + [I]/Ki)} = \frac{[S]}{Ks + [S]}$$

By taking the reciprocal of both sides:

$$\frac{1}{V} = \frac{Ks\,(1 + [I]/Ki)}{Vmax}\,\frac{1}{[S]} + \frac{1}{Vmax}\,(1 + [1]/Ki)$$

By plotting 1/ [S] vs 1/ V:

Slope = (Ks/ Vmax) (1+ [I] / Ki)

Intercept on y-axis = 1/ Vmax (1 + [I] / Ki)