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A study of the metabolism, pharmacological properties and disposition of substance P.

Renate Ingrid Uzubalis

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Awarded 1995

This thesis is dedicated to my
Grandmother,
Leonija Bušs.

Although you are no longer with us,
you taught me never to give up
and above all to believe in myself.

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Abstract

The primary aim was to determine whether levels of the endogenous peptide substance P (SP) would parallel and reflect the reported increased levels of the trophic agent nerve growth factor (NGF) which is associated with the development of sympathetic hyperinnervation (and ultimately hypertension) in the genetic animal model for hypertension, the spontaneously hypertensive rat (SHR). Initial studies developed radioimmunoassay (RIA) and ion exchange chromatographic (IEC) techniques to isolate and quantify SP from tissue samples. The RIA was developed to be sensitive for SP within the 10pg to 100pg range, although the antibody did cross-react with various C-terminal fragments. After initial characterization of the IEC procedure (utilizing HPLC techniques), anomalous data were observed and detailed studies suggested that there had been a change in the inherent characteristics of the IEC resin, resulting in an altered elution profile of SP. This resin was substituted for another in the later IEC experiments. Plasma degradation studies revealed that the endogenous form of the peptide was more stable than radiolabelled or synthetic SP. Moreover, dialysis studies suggested the existence of a plasma binding protein for substance P with a binding capacity of 61.9 ± 4.4 pg/ml which was saturable and reversible and was sufficient to protect endogenous plasma SP from enzymatic degradation. Human serum albumin was also found to bind SP (11.7 ± 1.1 pg/ml), but only accounted for approximately 20% of the binding capacity of whole plasma. The identity of the principal SP binding protein remains unknown. The C-terminal fragments SP 4-11, SP 5-11 and SP 6-11 were found to produce biological effects on the vasculature and gut which were similar to those observed for SP. Human plasma determinations revealed high circulating levels of SP 4-11 and SP 5-11, although the endogenous concentrations appeared insufficient to elicit a response in these systems. Finally, tissue determination of SP in tissues from SHRs and WKYs (normotensive, control Wistar Kyoto rats) revealed no significant difference between them. Thus, the original hypothesis was not supported, *i.e.*, tissue levels

of SP in the SHR do not reflect the reported enhanced levels of NGF in these animals.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Data from this thesis have been presented to Meetings of the Australian Society of Clinical and Experimental Pharmacologists in Melbourne 1990, Sydney 1991 and Perth 1992 and at special seminars at the Ruprecht-Karls-Universitat, Heidelberg, Germany (January 29, 1991) and the Institut fur Pharmakologie und Toxikologie, Bonn, Germany (January 30, 1991).

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Abbreviations used in this Thesis

<	Less than
>	Greater than
>>	Much greater than
%	Percent
AII	Angiotensin II
Ab	(Substance P) Antibody
ACE	Angiotensin I converting enzyme
ACE-I	ACE-inhibitor therapy
ACN	Acetonitrile
ad libitum	According to pleasure (literal translation from Latin)
NKA	Neurokinin A
NKA 4-10	Neurokinin A fragment 4-10
ANOVA	Analysis of variance
Arg	*L*-Arginine
Asn	*L*-Asparagine
Asp	*L*-Aspartic acid
Av	Average
BK	Bradykinin
BP	Blood pressure
BSA	Bovine serum albumin
Bzl	Benzyl
^{o}C	Degrees Celcius
CD	Cathepsin D
CHF	Severe congestive heart failure
Ci	Curie
cm	Centimetres

CNS	Central nervous system
cpm	Counts per minute
CTCE	C-terminal cleaving enzyme
D-	Dextrorotatory
DB	Diluent buffer
D-Lys(Nic)	*N*-Nicotinoyllysine
DNA	Deoxyribonucleic acid
DOPEG	Dihydroxyphenylethylene glycol
D-Phe(Cl_2)	3,4-Dichloro-*D*-phenylalanine
DPM	Disintegrations per minute
DPP IV	Dipeptidyl aminopeptidase IV
DRG	Dorsal root ganglia
DSAB	Double strength assay buffer
EC_{50}	Effective concentration to yield 50% response
ED_{50}	Effective dose to yield 50% response
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example [*exempli gratia*]
et al.	And others [*et alii*]
fmol/ml	Femtomoles per millilitre
FSH	Follicle stimulating hormone
g	Gram
G	Acceleration of free fall (due to gravity)
G-protein	Guanine nucleotide binding protein
GDP	Guanosine diphosphate
GDW	Glass distilled water
Gln	*L*-Glutamine
Gly	Glycine

Glu	*L*-Glutamic acid
GPI	Guinea pig ileum
GTP	Guanosine triphosphate
^{3}H	Tritium
HCl	Hydrochloric acid
HCOOH	Formic acid
5-HIAA	5-Hydroxyindole acetic acid
HHL	Hippuryl-histidyl-leucine
HMWC	High molecular weight complex
HPLC	High performance liquid chromatography
^{3}H-SP	Substance P,[2-*L*-prolyl-3,4-^{3}H(N)]-
HR	Heart rate
5HT	Serotonin
^{125}I	^{125}Iodine
i.c.v.	Intracerebroventricular
i.e.	That is (to say) [*id est*]
IEC	Ion exchange chromatography
in vitro	In glass (literal translation from Latin)
in vivo	In a living thing (literal translation from Latin)
i.p.	Intraperitoneal
^{125}I-SP	[^{125}I-Tyr^{8}]-substance P
i.v.	Intravenous
K	distribution coefficient (in IEC)
K^{+}	Potassium ion
kDa	kilodaltons
Krebs	Krebs-Henseleit Buffer
L	Litre

L-	Laevorotatory
Leu	*L*-Leucine
Leu-Enk	Leucine enkephalin
LH	Luteinizing hormone
lm/mp	Longitudinal muscle/myenteric plexus
Lys	*L*-Lysine
M	Moles per litre (molar)
mA	Milliamperes
MAO	Monoamine oxidase
MAP	Mean arterial pressure
MeOH	Methanol
Met	*L*-Methionine
Met-Enk	Methionine enkephalin
mg	Milligram
mg/kg	Milligrams per kilogram
mg/ml	Milligrams per millilitre
min	Minute
ml	Millilitre
ml/min	Millilitres per minute
mm	Millimetres
mM	Millimoles per litre
mmHg	Millimeters of mercury (pressure)
mmol/L	Millimoles per litre
mRNA	Messenger ribonucleic acid
MW	Molecular weight
N	Sample size
NA	Noradrenaline (norepinephrine)
NaCl	Sodium chloride

NaOH	Sodium hydroxide
N/C	Nitrocellulose paper
NEP	Neutral endopeptidase 24.11
NGF	Nerve growth factor
ng/ml	Nanograms per millilitre
NHF	National Heart Foundation (Australia)
NK_1	Neurokinin-1
NK_2	Neurokinin-2
NK_3	Neurokinin-3
NKA	Neurokinin A
NKB	Neurokinin B
Nle	*L*-Norleucine
nmol	Nanomoles per litre
NPK	Neuropeptide K
NPY	Neuropeptide Y
NRS	Normal rabbit serum
NSA	Normal serum albumin
NSAID	Nonsteroidal anti-inflammatory drug
p	Probability
Pal(3)	3-(3-pyridyl)alanine
PE	Prolyl endopeptidase
PEG	Polyethylene glycol 6,000
pg	Picogram
pg/ml	Picograms per millilitre
pGlu	Pyroglutamic acid
pH	Potential of hydrogen
Phe	*L*-Phenylalanine
pK	Ionization constant

pmol/g	Picomoles per gram
pmol/L	Picomoles per litre
PPT-1	Preprotachykinin-1
PRL	Prolactin
Pro	*L*-Proline
R	Range
RIA	Radioimmunoassay (for substance P)
RNA	Ribonucleic acid
Sar	Sarcosine
SARS	Sheep anti-rabbit immunoglobulin
SCG	Superior cervical ganglion
SCYI	Scyliorhinin I
SCYII	Scyliorhinin II
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
sec	Seconds
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rat
SHR-SP	Stroke-prone SHR
SP (SP 1-11)	Substance P
SPDE	Substance P degrading enzyme
SPLI	Substance P-like immunoreactivity
SSAB	Single strength assay buffer
TC	Total counts
TFA	Trifluoroacetic acid
Trp	*L*-Tryptophan
Tyr	*L*-Tyrosine
Tyr^{8}-SP	$Tyrosine^{8}$-Substance P
U/g	Units per gram

u	Microns
uCi	Microcuries
ug	Microgram
ug/g	Micrograms per gram
ul	Microlitre
μM	Micromoles per litre
U.S.A	United States of America
V	Volts
viz.	Namely [*videlicet*]
v/v	volume per volume
WKY	Wistar Kyoto rat
w/v	weight per volume

And where:

kilo (k) denotes 10^{3}
centi (c) denotes 10^{-2}
milli (m) denotes 10^{-3}
micro (μ) denotes 10^{-6}
nano (n) denotes 10^{-9}
pico (p) denotes 10^{-12}
femto (f) denotes 10^{-15}

CHAPTER 1

GENERAL INTRODUCTION.

1.1 BACKGROUND

The existence of substance P first came to light from experiments conducted by von Euler and Gaddum (1931). They noted that extracts taken from equine small intestine and brain possessed physiological effects unrelated to those of acetylcholine. These extracts produced a significant hypotensive effect in rabbits and caused contraction in isolated rabbit jejunum. Both effects were lost after alkali pretreatment of the extract, but were unaltered by atropine pretreatment, suggesting the presence of another active substance distinct from acetylcholine. Von Euler and Gaddum showed that the active principle was not histamine, adenosine or kallikrein and named it preparation P.

Further research on substance P was hampered by the use of crude extracts whose purity could not be assured. A major breakthrough occurred in 1971 when Chang and colleagues (Chang *et al.*, 1971) discovered and published the amino acid sequence of substance P (Fig. 1.1).

Arg^{1}-Pro^{2}-Lys^{3}-Pro^{4}-Gln^{5}-Gln^{6}-Phe^{7}-Phe^{8}-Gly^{9}-Leu^{10}-Met^{11}-NH_2

Fig. 1.1. The amino acid sequence of substance P.

Also reported at the same time was a solid-phase procedure for the synthesis of substance P (Tregear *et al.*, 1971). With a chemically pure form of the peptide becoming available, studies of this compound have increased over the years. This is evidenced by the growing array of reports regarding substance P (SP1-11) as summarized by Otsuka (1993).

The remainder of this chapter is devoted to presenting an overview of the developments and discoveries associated with substance P, in addition to outlining the theoretical considerations which have led to the present study.

1.2 THE PREPROTACHYKININ-1 GENE

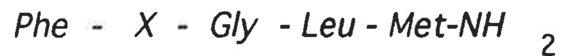
Substance P is a peptide consisting of 11 amino acids (Chang *et al.*, 1971) and may be referred to as SP 1-11 (or SP). It is a member of the tachykinin family which shares the common C-terminal sequence Phe-X-Gly-Leu-Met-NH_2 and is one of the mammalian tachykinins (Fig. 1.2a) along with neurokinin A (NKA; also known as substance K, neuromedin L, α-neurokinin or neurokinin α), neurokinin B (NKB; also known as neuromedin K or neurokinin β) and neuropeptide K (NPK) (Erspamer, 1981; Mussap *et al.*, 1993). The mammalian tachykinins share a similar spectrum of actions in various physiological systems, although SP is the most prominent of these (Mussap *et al.*, 1993; Otsuka and Yoshioka, 1993).

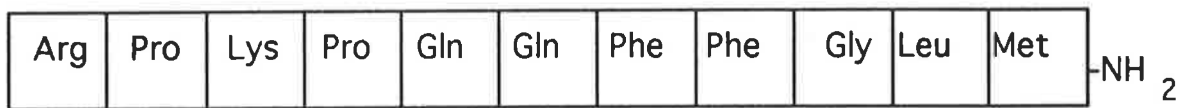
Nonmammalian tachykinins include physalaemin, kassinin, uperolein, eledoisin and scyliorhinin I (SCYI), as well as the novel cyclic tachykinin scyliorhinin II (SCYII) (Fig. 1.2.b) (Erspamer, 1981; Mussap *et al.*, 1993. The nonmammalian tachykinins also possess biological activities similar to substance P but tend to be utilized more as tools for tachykinin receptor characterization studies.

Substance P is processed initially within the framework of a larger precursor molecule known as a preprotachykinin-1 (PPT-1). Early work on bovine brains revealed the primary structure of two types of bovine substance P precursors, designated α- and β-PPT-1, as well as the structural organization of the bovine PPT-1 gene (Nawa *et al.*, 1983; Nawa *et al.*, 1984; Nakanishi, 1986). β-PPT-1 was found to contain the sequences for substance P and NKA (in exons 3 and 6 respectively), whilst α-PPT-1 only contained the sequence for substance P (exon 6 had been deleted) (Fig. 1.3). α-PPT-1 mRNA levels were 2-3 times higher than β-PPT-1 mRNA in the nervous system, yet in the peripheral tissues this trend was reversed (Nawa *et al.*, 1984). The expression of the two PPT-1 mRNAs was therefore regulated in a tissue-specific manner via different RNA splicing events.

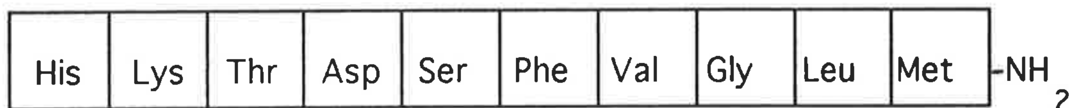
Nawa and colleagues also predicted the existence of a third PPT-1 mRNA, γ-PPT-1 which was confirmed by Kawaguchi and associates (1986). γ-PPT-1

Mammalian Tachykinins

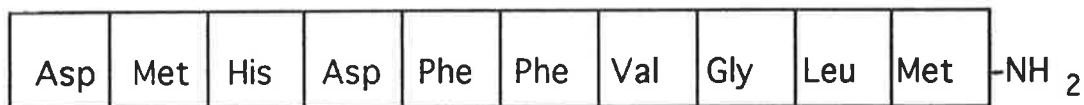




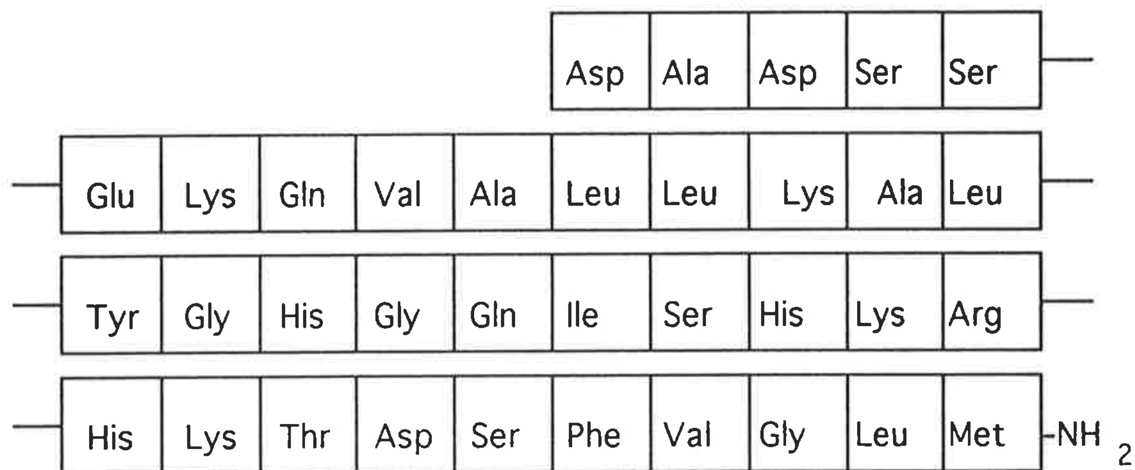
Substance P



Neurokinin A



Neurokinin B

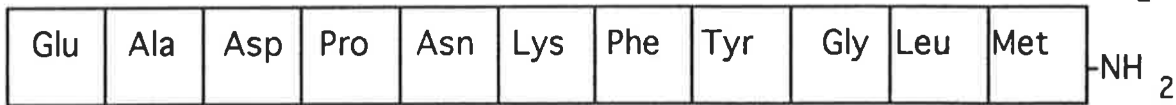


Neuropeptide K

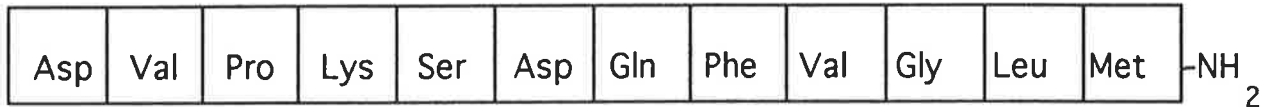
Fig. 1.2.a. Amino acid sequences for the mammalian tachykinins substance P, neurokinin A, neurokinin B and neuropeptide K.

Nonmammalian Tachykinins

Phe - X - Gly - Leu - Met-NH 2

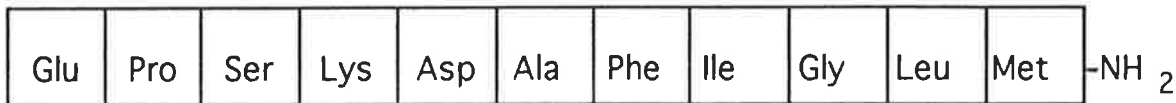


Physalaemin

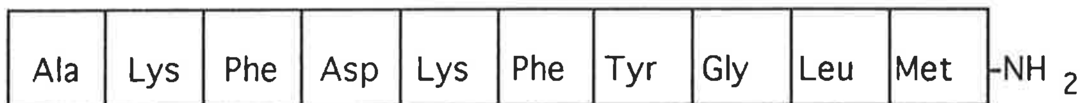


Kassinin

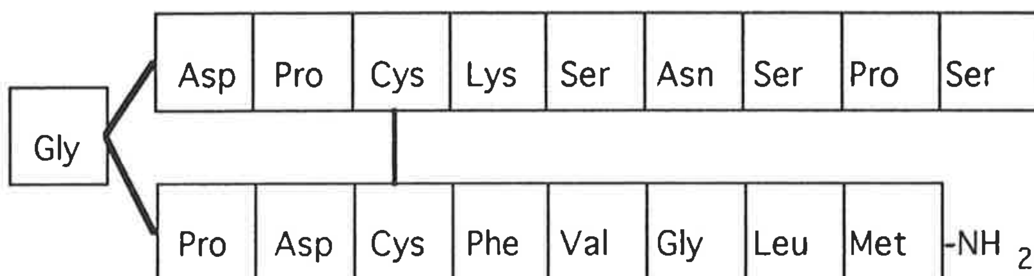
Uperolein



Eledoisin



SCYI



SCYII

Fig. 1.2.b. Amino acid sequences for the nonmammalian tachykinins physalaemin, kassinin, uperolein, eledoisin, scyliorhinin I (SCYI) and the novel cyclic scyliorhinin II (SCYII).

The Preprotachykinin-1 Gene

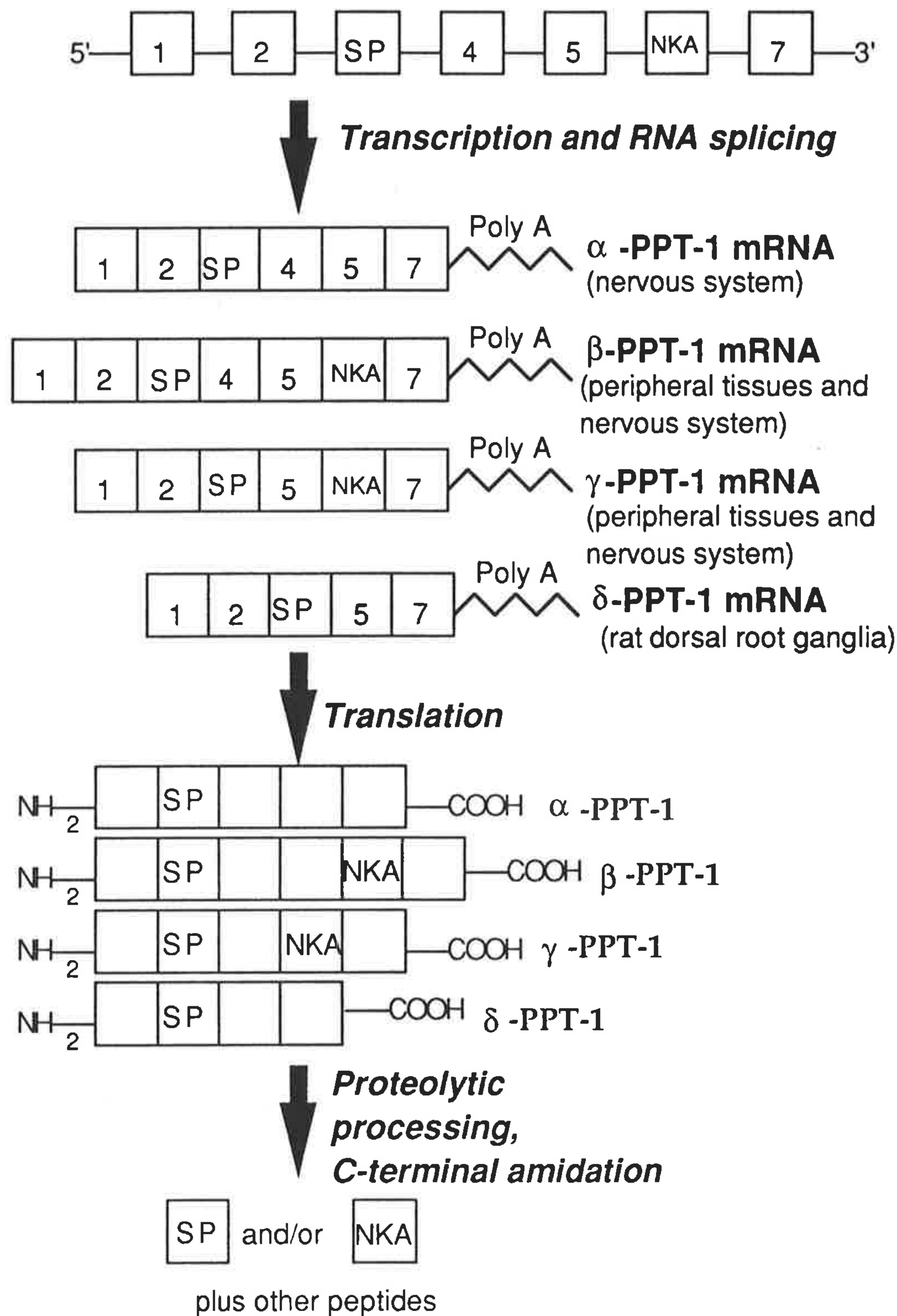


Figure 1.3. Schematic representation of the expression and structural organization of the PPT-1 mRNAs. Numbered boxes and boxes labelled SP and NKA stand for corresponding exons and exons encoding substance P (SP; exon 3) and neurokinin A (NKA; exon 6) respectively.

mRNA resulted fron the specific exclusion of exon 4 (the spacer sequence) by alternative RNA splicing (Fig. 1.3).

More recently, a fourth PPT-1 mRNA has been identified in rat dorsal root ganglion, δ-PPT-1 mRNA (Harmar *et al.*, 1990). δ-PPT-1 mRNA lacks both exon 4 and exon 6 (the exon for NKA) (Fig. 1.3). Thus, alternative RNA splicing of the mammalian PPT-1 gene can yield four forms of PPT-1 mRNA; α-PPT-1, β-PPT-1, γ-PPT-1 and δ-PPT-1 (Fig. 1.3) resulting in the synthesis of different peptide products in various tissue systems (Harmar *et al.*, 1986; Krause *et al.*, 1987; Nakanishi, 1987; MacDonald *et al.*, 1988; Krause *et al.*, 1989; Lindh *et al.*, 1989; MacDonald *et al.*, 1989; Sternini *et al.*, 1989; Carter and Krause, 1990; Harmar *et al.*, 1990; Somers and Beckstead, 1990; Walker *et al.*, 1990).

The various PPT-1 forms are highly conserved between species. Nucleotide sequences of bovine γ-PPT-1 mRNA and its rat equivalent have shown 91% homology (Kawaguchi *et al.*, 1986). Similarly, 96% sequence homology has been found with the rat and human PPT-1 mRNAS (MacDonald *et al.*, 1988) and 92% between bovine and human PPT-1 mRNAs (Harmar *et al.*, 1986).

More recently, it has been suggested that a homeostatic mechanism is involved in maintaining a consistent balance between β- and γ-PPT-1 (Marchand *et al.*, 1993). The precise mechanics of this homeostatic control of the processed tachykinins remains unknown and research continues.

Various factors are believed to regulate or modulate the transcription/translational processes involved in the tissue-specific production of the various PPT-1 products. Hormonal control mechanisms have been suggested, including serotonin (Walker *et al.*, 1990; Riley *et al.*, 1991; Riley *et al.*, 1993), certain excitatory amino acids (Somers and Beckstead, 1990), dopamine (Sivam and Krause, 1990; Bannon *et al.*, 1991), nerve growth factor (Gilchrist *et al.*, 1991a; Gilchrist *et al.*, 1991b; Vedder *et al.*, 1993), and interleukin-1 (Jonakait *et al.*, 1991), as well as osmotic stimuli (Larsen *et al.*, 1993), fetal substantia nigra grafts (Mendez *et al.*, 1993) and neuronal/support cell interactions (Lentz *et al.*, 1993).

Recently, putative transcription factor binding sites have been identified within the 5' flanking DNA sequence of the rat PPT-1 gene, although these results are extremely preliminary (Chapman *et al.*, 1993).

The proteolytic enzymes involved in the final stages of SP biosynthesis have not yet been identified. It is not known whether a single enzyme or a more complicated multiple enzyme system exists to liberate the various tachykinins which may be generated from the original PPT-1 gene.

Since the relatively recent discovery of the PPT-1 gene in the early 1980s, much research has been directed towards unravelling the factors which regulate the expression of SP in various tissues. However, the mechanisms involved in the regulation of the PPT-1 gene remain largely undiscovered.

1.3 THE DISTRIBUTION OF SUBSTANCE P

The widespread dispersion of mRNAs for PPT suggests that substance P (and neurokinin A) may be found throughout the central and peripheral nervous systems in many species (including man). Even the nervous systems in the insect world possess substance P-containing neurons (Nassel *et al.*, 1990).

1.3.1 The Central Nervous System (CNS).

One of the most extensively studied species for evaluating the distribution of substance P has been the rat. Warden and Young (1988) revealed an uneven distribution of substance P within the central nervous system (CNS) of the rat. This pattern of distribution suggests specific and different functional roles within the CNS.

De Felipe and associates (1989) examined the effect of a substance P antiserum on 2 day old rats (the controls received treatment with non-specific immunoglobulins). Substance P levels were significantly decreased in regions of the spinal cord and periaqueductal grey matter. There was no change in the number of substance P and opioid receptor binding sites of these animals, but

their antinociceptive response and hypertensive responses to substance P were inhibited. Thus, neonatal administration of anti-substance P treatment inhibits the production of and responses to substance P without affecting the receptors.

A review by Otsuka and Yoshioka (1993) acknowledges that substance P does share a similar pattern of distribution in the CNS (and the periphery) with neurokinin A, as may be predicted from molecular genetic studies. Nevertheless, there is a trend for a lower concentration of neurokinin A in the same region when compared with substance P. Neurokinin A is predominantly colocalized with substance P, but there are many substance P-containing neurons which do not necessarily contain neurokinin A. Otsuka and Yoshioka (1993) have also noted that neurokinin B is barely detected in the periphery and exhibits a different pattern of distribution from neurokinin A/substance P in the CNS.

1.3.2 Colocalization of Tachykinins with Other Neurotransmitters or Substances.

In many cases, two or more neurotransmitters may coexist in the one neuron. Other transmitters or substances which may be colocalized with substance P include serotonin, thyrotropin releasing hormone, neurokinin A, calcitonin gene-related peptide, gamma-aminobutyrate (GABA), choline acetyltransferase, glutamic acid decarboxylase, tyrosine hydroxylase, somatostatin, bombesin, enkephalin, corticotrophin-releasing factor and dynorphin (Helke *et al.*, 1991; Otsuka and Yoshioka, 1993). Many of these peptides have been shown to coexist with substance P in human trigeminal ganglion (Del Fiacco *et al.*, 1991) and human celiac/superior mesenteric ganglionic complex (Del Fiacco *et al.*, 1993). The significance of transmitter colocalization is not fully understood, but may allow for finer control of the final response.

1.3.3 Human Studies.

Edvinsson and colleagues (1983) examined the localization of substance P in cerebral artery, dura mater and choroid plexus specimens taken during routine autopsies. Cerebral arteries had higher levels of substance P than either the choroid plexus or dura mater. There was also regional variation in the concentration of substance P within the cerebral arteries. Post-mortem studies on human infant specimens also show regional variations in the substance P content of the medulla oblongata (Rikard-Bell *et al.*, 1990) and tractus solitarius (Yew *et al.*, 1990).

The main thrust towards researching the distribution of substance P in adult humans has occurred through attempts to determine the pathogenesis of disease states in which substance P has been implicated. Neurodegenerative disorders such as Parkinson's Disease, Alzheimer's Disease, Huntington's Disease and amyotrophic lateral sclerosis feature prominently (Pezzoli *et al.*, 1984; Agid and Javoy-Agid, 1985; Kostyk *et al.*, 1989; Halliday *et al.*, 1990; Anand *et al.*, 1991; Barker, 1991; Cramer *et al.*, 1991; Quigley *et al.*, 1991; Sivam, 1991; Kowall *et al.*, 1993; Otsuka and Yoshioka, 1993).

Other disease states in which substance P may play a role include schizophrenia (Heikkila *et al.*, 1990), systemic sclerosis (Matucci-Cerinic *et al.*, 1990), asthma (Ollerenshaw *et al.*, 1991), psoriasis (Eedy *et al.*, 1991), ophthalmological conditions (Pearson and Rose, 1991), Raynaud's phenomenon (Vaeroy *et al.*, 1988; Bunker *et al.*, 1991) and arthritis and other inflammatory conditions (Nakamura-Craig and Smith, 1989; Da Silva and Carmo-Fonseca, 1990; Mapp *et al.*, 1990; Basbaum and Levine, 1991) as well as familial dysautonomia, Hirschsprung's Disease, chronic bowel inflammation and constipation, headache, Herpes Zoster and carcinoid syndrome (Otsuka and Yoshioka, 1993). The role of substance P in various disease states and conditions continues to be an active field of research which has been aided by the development of improved agonists and antagonists.

1.3.4 *The Peripheral Nervous System.*

There is a widespread (but uneven) distribution of substance P in the CNS and peripheral tissues of humans and other animals including cats, rabbits, guinea-pigs and chickens (Bucsics *et al.*, 1983; Pernow, 1983; Kage *et al.*, 1988). A review on substance P by Pernow (1983) provides excellent information regarding its distribution in both the CNS and peripheral nervous system in experimental animals and man. However, substance P is mainly localized in two areas; *i.e.* the gastrointestinal neural network (Duckles, 1985; Scott *et al.*, 1989; Kwok and McIntosh, 1990) and primary sensory neurons (Hökfelt *et al.*, 1975). Substance P has also been found in some carcinoid tumours (Conlon *et al.*, 1985). The distribution of substance P in primary sensory neurons will be discussed further, as this topic is related to some of the theoretical considerations which led to the present study.

1.3.4.i *Primary Sensory Neurons.*

An early study on the localization of substance P in primary sensory neurons revealed staining to substance P in cell bodies, peripheral nerves and central nerves which corresponded closely with the distribution of primary sensory neurons (Hökfelt *et al.*, 1975). While these findings gave credence to the idea that substance P may act as a transmitter in some of the primary sensory neurons, the possibility that it may act as a regulator of motor or sensory function was not discounted. A more recent study has suggested that substance P-containing primary sensory neurons may mediate both sensory transduction and peripheral effects such as mediation of nociceptive information as well as being potential effectors of peripheral actions such as plasma extravasation and vasodilation (Cuello *et al.*, 1993).

The regulation of expression of substance P in sympathetic neurons appears to be a complex area. Co-culture of sympathetic neurons with Schwann cells, leukaemia inhibitory factor, or glucocorticoids (as well as other factors or conditions) modifies substance P expression (Kessler and Freidin, 1991). In

addition, SP itself may exert direct effects on sensory neurons (Spigelman and Puil, 1991).

1.3.4.ii Nerve Growth Factor (NGF) and Primary Sensory Neurons.

NGF is a prerequisite for the normal growth and development of primary sensory neurons (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). Manipulations of NGF with respect to the growth and development of sensory neurons have led to subsequent alterations in substance P content.

Administration of NGF to newborn rats dramatically increases ganglionic substance P levels and prevents the inhibition of substance P development which occurs after forelimb amputation (Kessler and Black, 1980). Similarly, Otten and associates (1980) found a substantial increase in substance P levels in NGF treated rodent neonates. Conversely, anti-NGF antibody administration significantly decreases substance P levels when compared with controls. This trophic effect of NGF on developing substance P-containing neurons also occurs in mature sensory ganglia (Goedert *et al.*, 1981).

The neuronal increases in substance P levels caused by NGF are due to preprotachykinin regulation by NGF. Adult rat dorsal root ganglion neurons were cultured in the presence and absence of NGF (Lindsay and Harmar 1989). NGF treatment increased both the PPT mRNAs and concentration of substance P-like immunoreactivity, whilst the absence of NGF resulted in a time-dependent decline of PPT mRNA levels and decline in the substance P-like immunoreactivity. Similar conclusions have been drawn in relation to substance P expression in sensory neurons (Lindsay *et al.*, 1989; MacLean *et al.*, 1991).

Recently, Gilchrist and colleagues (1991a and 1991b) have identified sequences within the bovine PPT gene which are responsive to NGF. It appears that there are two regions designated AR1 and AR2 which enhance the effects of NGF on PPT gene transcription. However, the authors acknowledge that these sequences may not be specific for NGF and that other trophic factors should be tested.

1.4 PHYSIOLOGICAL ACTIONS OF SUBSTANCE P

The actions attributed to substance P are numerous and reflect the varied distribution of this peptide throughout both the central and peripheral tissue systems. An arbitrary separation into the central and peripheral effects will be used in the following discussion.

1.4.1 Central Actions.

The discovery that substance P was distributed unevenly throughout the central nervous system suggested discrete physiological roles (Bucsics *et al.*, 1983; Pernow, 1983; Kage *et al.*, 1988; Warden and Young, 1988; Otsuka and Yoshioka, 1993).

1.4.1.i Neurotransmission and Nociception.

Substance P is believed to act as a transmitter within primary sensory neurons. Supporting evidence includes synthesis of the peptide in the dorsal root ganglia, the storage and release of substance P from primary afferent neurons, the presence of postsynaptic substance P receptors, the excitatory action of substance P on motorneurons and spinal dorsal horn neurons, replication of these effects by the application of exogenous substance P, inhibition of these effects by specific antagonists and the inactivation of substance P by various tissue systems (Pernow, 1983; Otsuka and Yanagisawa, 1987). The sensory modality believed to be mediated by substance P is pain perception. The nociceptive action of substance P is dose-dependent; low doses elicit analgesia (mediated via endogenous opioids), whilst higher doses elicit hyperalgesia (Pernow, 1983).

Other peptides such as neurokinin A, calcitonin gene-related peptide and somatostatin are also released during peripheral noxious stimulation. Neurokinin A has an excitatory action similar to substance P on spinal neurons, but somatostatin and galanin exert an inhibitory action on spinal neurons whilst calcitonin gene-related peptide enhances the release of substance P from primary afferents (Otsuka and Yoshioka, 1993).

Two common methods for determining nociceptive effects are the hot-plate test and tail-flick test for rodents. Administration of substance P increases reaction time in the tail-flick test after one minute, but not at later times (Post and Folkers, 1985). Behavioural effects of substance P in mice included biting and licking in an obsessive and hyperactive manner. Whether this was a direct effect or secondary to pain induction by substance P cannot be ascertained. However, this effect is known to be dose-dependent and pretreatment of the mice with naloxone shifts the dose-response curve to the left, indicating an interaction between endogenous opioids and substance P (Lei *et al.*, 1991).

Attempts have been made to determine the part of the pain response in which substance P is involved (Ohkubo *et al.*, 1990). The Formalin test is known to induce an early nociceptive response manifested via direct nerve stimulation followed by a late phase of approximately 20 minutes associated with an inflammatory response. Administration of substance P antagonists inhibited only the first phase of the response, indicating a role in the early phase of nociception. Other data obtained from hot-plate tests and tail pinch tests further suggest that substance P is involved in short-lasting pain, but not the subsequent inflammatory response. Thus, mediation of nociceptive neurotransmission is an important function of substance P.

1.4.1.ii Hormone Regulation.

Substance P is believed to play a role in the regulation of various hormones in the CNS. Microinjection of substance P into the medial preoptic area of male rats resulted in decreases in the plasma concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL) (Picanco-Diniz *et al.*, 1990). Treatment with an antagonist or substance P-directed antibody increased plasma LH and FSH, although levels of PRL were decreased. This and other evidence suggests that endogenous substance P in the medial preoptic area exerts tonic control over LH and FSH in an inhibitory manner and excitatory tonic control on PRL. Endogenous hypothalamic substance P has also been shown to

regulate LH and FSH (Arisawa, De Palatis *et al.*, 1990) and PRL (Arisawa, Snyder *et al.*, 1990).

Substance P has also been associated with the stress response and osmoregulation (Chowdrey *et al.*, 1990). Substance P produces an antidiuretic effect lasting for at least 30 minutes with an associated increase in plasma arginine vasopressin and a decrease in plasma adrenocorticotropin in rats. Other papers have also suggested a role for substance P in stress and catecholamine metabolism (Oehme *et al.*, 1982; Richter *et al.*, 1989).

1.4.1.iii Regulation of Muscle Tone.

Substance P may also mediate the regulation of muscle tone (Turski *et al.*, 1990). Microinjections of a substance P antagonist into genetically spastic rats reduced muscle tone in a dose-dependent and time-dependent manner. These effects were blocked by the co-administration of substance P. Thus, substance P may be involved in the control of muscle tone.

1.4.1.iv Actions on Blood Pressure.

A central control mechanism for the regulation of blood pressure (BP) by substance P has been suggested. *I.c.v.* injection of substance P causes a dose-dependent increase in BP and heart rate (HR) in rats (Trimarchi *et al.*, 1986; Takano *et al.*, 1991). Hall and associates (1987b) went on to characterize the hypertensive response to the central administration of substance P as a biphasic BP response, consisting of an initial drop in BP (maximal at approximately 19 seconds), followed by a slower but stronger hypertensive effect (lasting a number of minutes). It was concluded that substance P may influence BP control by acting as a neurotransmitter in the baroreceptor arc.

I.c.v. injection of neurokinin A and senktide (a neurokinin B analogue) are also known to increase blood pressure and heart rate (Otsuka and Yoshioka, 1993). Thus, other tachykinins may play a role in the CNS in maintaining a consistent blood pressure.

1.4.2 Peripheral actions.

1.4.2.i Hypotensive Effect.

Peripheral administration of substance P results in a dose-dependent vasodilatory response (via an endothelium-derived relaxing factor) accompanied by a decrease in BP and tachycardia. This has been demonstrated in dogs, rats and rabbits (Bury and Mashford, 1976 and 1977b; Maggi *et al.*, 1985). This hypotensive/vasodilatory response has also been observed in humans (Fuller *et al.*, 1987; Evans *et al.*, 1988; Benjamin and Webb, 1990).

1.4.2.ii Enhancement of Salivation/Gastrointestinal Contraction.

Substance P is known to be a potent sialogogue, and causes dose-dependent increases in salivation (Pernow, 1983; Maggi *et al.*, 1985). This property is often exploited as a bioassay for substance P. Neurokinin A also induces salivation in the rat and ferret, but is less potent than substance P (Otsuka and Yoshioka, 1993). Another well-used bioassay preparation for substance P is the guinea pig ileum (GPI). Substance P is known to be an extremely potent agent for eliciting strong contractile responses in the GPI preparation (Bury and Mashford, 1976; Escher *et al.*, 1982; Stavropoulos *et al.*, 1991). This also appears to be the case with humans (Grider, 1989; Otsuka and Yoshioka, 1993). Neurokinin A also produces contraction of intestinal muscle, while the relative potencies of neurokinin A and substance P vary depending on species and sites of the gut (Otsuka and Yoshioka, 1993).

1.4.2.iii Respiratory Function.

Tachykinins are among the most potent bronchoconstrictor agents known. Neurokinin A is more potent than substance P and neurokinin B in both the guinea pig and human, suggesting a more important role for neurokinin A than substance P in this action (Otsuka and Yoshioka, 1993).

Nevertheless, substance P has been implicated in the pathogenesis of asthma, although there was no evidence of bronchoconstriction in asthmatics after

inhalation of substance P (Fuller *et al.*, 1987). It remains possible that substance P may be involved in a subepithelial mechanism for example, or by an alteration in the innervation of the airways which results in bronchial hyperreactivity. However, substance P has been observed to significantly increase mucus secretion in human bronchi and this has been thought to contribute to the development of asthma (Rogers *et al.*, 1989).

In contrast, *i.v.* infusion of substance P causes bronchoconstriction in guinea pigs (Shore *et al.*, 1988) and bronchodilatation in humans (Evans *et al.*, 1988).

An adverse reaction of severe cough has been noted to occur in some patients taking antihypertensive drugs which inhibit the enzyme conversion of angiotensin I to angiotensin II (*i.e.*, the angiotensin converting enzyme inhibitors or ACE-I) (Sesoko and Kaneko, 1985; Webb *et al.*, 1986; Fuller and Choudry, 1987; Moric *et al.*, 1987). Substance P may be involved in the pathogenesis of this condition, although there is no difference in plasma substance P levels in hypertensive individuals before and after the commencement of ACE-I therapy, regardless of cough (Thysell *et al.*, 1988).

1.4.2.iv Other Peripheral Actions and Summary.

Substance P plays a major role in modulating inflammatory and immune responses. Leukocytes, lymphocytes, mast cells, macrophages, cytokines, neutrophils and proliferation of connective tissue cells may all be regulated by substance P (McGillis *et al.*, 1987; Payan and Goetzl, 1987; Louis *et al.*, 1989; Wiedermann *et al.*, 1989; Wozniak *et al.*, 1989; Kimball, 1990; Lam and Ferrell, 1990; McGillis *et al.*, 1990).

Substance P has also been shown to modulate the activation of K^+ channels (Mayer *et al.*, 1989), accumulation of inositol phospholipids (Suman-Chauhan *et al.*, 1990), to play a role in the mediation of miosis in the eye and a transmitter in the retina (along with neurokinin A; Otsuka and Yoshioka, 1993) and effector functions of platelets (Damonneville *et al.*, 1990). Further research on substance P

will no doubt continue to add to the already long list of actions attributed to this peptide (Table 1.1).

Central actions of substance P
Neurotransmitter
Nociceptive agent
Regulation of hormones (*LH, FSH, PRL*)
Osmoregulation (*arginine vasopressin, adrenocorticotropin*)
Stress/catecholamine metabolism
Regulation of muscle tone
Influence on behaviour/memory
Regulation of blood pressure (*hypertensive agent*)

Peripheral actions of substance P
Regulation of blood pressure (*hypotensive agent*)
Potent sialogogue
Regulation of gastrointestinal tone and motility
Regulation of respiratory function
Mediator of inflammatory responses
Mediator of immunoregulatory responses
Activation of K^+ channels
Accumulation of inositol phospholipids
Modulation of platelet effector function
Mediator of miosis in the eye
Transmitter in the retina

Table 1.1. A summary of the central and peripheral actions of substance P.

1.5 RECEPTORS, ANALOGUES, AGONISTS AND ANTAGONISTS.

Analogues of active peptides possessing either agonistic or antagonistic properties are important tools for characterization of the mechanisms of action and effects of peptides, transmitters and hormones. In the particular case of substance P, further classifications may separate the various analogues into two distinct classes; i) fragments of substance P itself and ii) analogues obtained via substitutions and/or additions/deletions of amino acids within the parent peptide.

1.5.1 Fragment Nomenclature.

Current popular nomenclature used to describe substance P fragment combinations refers to the amino acid sequence of the new fragment. Thus, cleavage of the peptide between amino acids 4 and 5 (*i.e.*, between proline and glutamine) yields the N-terminal fragment substance P 1-4 (SP 1-4, or the N-terminal tetrapeptide) and the C-terminal fragment substance P 5-11 (SP 5-11, or the C-terminal heptapeptide; see Fig. 1.4). Hence, this nomenclature may be used to describe all of the fragment combinations possible.

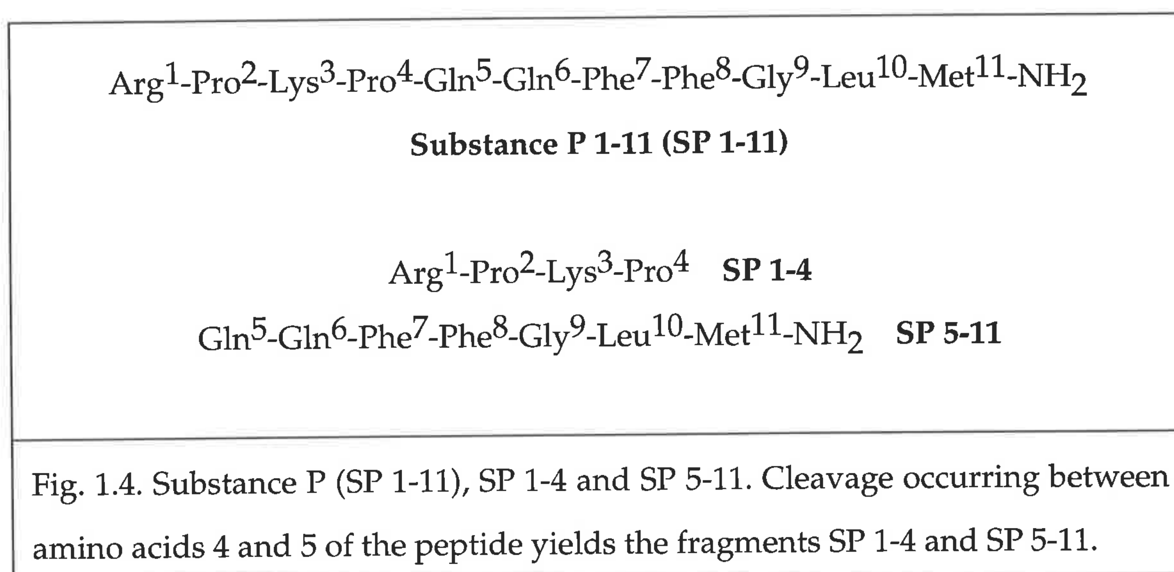


Fig. 1.4. Substance P (SP 1-11), SP 1-4 and SP 5-11. Cleavage occurring between amino acids 4 and 5 of the peptide yields the fragments SP 1-4 and SP 5-11.

1.5.2 Neurokinin Receptors.

Early research revealed subtypes of the substance P receptor although numerous receptor classification schemes complicated matters. Classifications included the SP.E, SP.P, NKA, NKB, SP_1, SP_2, SP.K, SP-N and NK-P subtypes (Brown and Hill, 1983; Buck and Burcher, 1986; Buck *et al.*, 1988; Regoli *et al.*, 1989; Guard and Watson, 1991). Since 1986 the receptor classifications of NK_1, NK_2 and NK_3 have been used to indicate the subtypes with the highest affinity for the ligands substance P, neurokinin A and neurokinin B respectively. An outline of current thoughts on tachykinin receptor characterization has been presented by Burcher and colleagues (1991) and Mussap and associates (1993).

NK_1 receptors are present in both the CNS and peripheral tissues, NK_2 receptors are found mainly in peripheral tissues and NK_3 receptors are present in high amounts in the CNS and in lesser amounts in the gastrointestinal tract (Otsuka and Yoshioka, 1993).

1.5.2.i The NK_1 Receptor.

The tachykinin receptors, including the NK_1 receptor, are members of the G-protein coupled superfamily of receptors (Helke *et al.*, 1990; Krause *et al.*, 1990; Mousli *et al.*, 1990). Other G-protein-coupled receptors include the α_1, α_2, β_1 and β_2 adrenergic receptors, muscarinic receptors, serotonin $5HT1_a$ and $5HT1_c$ and substance K receptors.

Members of this superfamily share a common structural feature; *i.e.*, seven α-helical membrane spanning domains. Homology comparisons and hydrophobicity plots (evidencing a high degree of conservation in these regions) support the existence of such domains, although functional roles of the domains have not been fully elucidated (Krause *et al.*, 1990).

Another feature of G-protein coupled receptors is the presence of potential glycosylation sites. Porcine brain NK_1 receptors have an abundance of β-galactose and N-acetylglucosamine galactose residues, but limited amounts of α-mannose, fucose and N-acetylgalactosamine (Liu and Quirion, 1991). This contrasts with the

distribution of these carbohydrate moieties found in other G-protein receptors where the β-galactose and N-acetylglucosamine galactose residues are rarely encountered. Thus, Liu and Quirion (1991) suggest that the presence of these carbohydrates may mediate specific NK_1 receptor functions.

Detailed characterization studies suggest the possibility of further neurokinin receptor subtypes, although this is not yet fully established (Buck, Fanger *et al.*, 1991; Buck, Van Giersbergen *et al.*, 1991; Burcher *et al.*, 1991; Floch *et al.*, 1993; Guard and Watson, 1991; Takeda and Krause, 1991; Watling *et al.*, 1993).

1.5.2.ii Distribution of Neurokinin Receptors.

The distribution of tachykinin receptor subtypes has been studied using specific analogue agonists and antagonists of substance P. Table 1.2 summarizes some of the receptor subtypes associated with different tissue systems.

Tissue Type	*Receptor Subtype*
Dog carotid artery	NK_1
Guinea pig ileum	NK_1, NK_2, NK_3
Rabbit pulmonary artery (without the endothelium)	NK_2
Rat duodenum	NK_1 and NK_2
Rat vas deferens	NK_2
Isolated human bronchus	NK_2
Hamster (urinary) bladder	NK_2 and NK_3
Rat portal vein	NK_3

Table 1.2. A summary of the neurokinin receptor subtypes found in different tissues as reported by Regoli *et al.* (1989).

This list is likely to expand as additional selective tachykinin receptor agonists and antagonists become available.

1.5.3 Substance P Analogues.

Novel analogues of substance P have been developed with the aim of improving receptor selectivity, to allow better characterization of these receptors or to enhance or inhibit a physiological action attributed to substance P itself. However, it is impossible to include all of the (published) analogues in the following overview. Thus, a small selection has been chosen to represent the group and its associated actions.

1.5.3.i Spantide.

Two analogues of substance P (*viz.* spantide and senktide) are commonly used in the classification of receptors. Spantide (which was introduced in 1984), is an analogue of substance P with the structure [*D*-Arg^1, *D*-$Trp^{7,9}$, Leu^{11}]SP, whilst spantide II has the structure [*D*-$Lys(Nic)^1$, $Pal(3)^3$, *D*-$Phe(Cl_2)^5$, Asn, *D*-$Trp^{7,9}$, Nle^{11}]SP (Folkers *et al.*, 1990). Both spantide and spantide II are substitution analogues of substance P, although it is believed that the presence of *D*-Arg^1 and Lys^3 in spantide contributed towards its neurotoxicity (Folkers *et al.*, 1990). The replacements with *D*-$Lys(Nic)^1$ and $Pal(3)^3$ to give spantide II resulted in higher potency and decreased side-effects when compared to its forebear.

1.5.3.ii Senktide.

Senktide is a substitution analogue of the fragment SP 6-11 which possesses agonistic properties. It has the structure succinyl[Asp^6, $MePhe^8$] substance P 6-11 and is highly specific for the NK_3 receptor subtype (Wormser *et al.*, 1986; Guard *et al.*, 1990).

1.5.3.iii Other Analogues.

Other SP 6-11 analogues have also been shown to possess agonistic properties. For example, [pGlu^6, *L*-Pro^9]SP 6-11 displayed selectivity for the NK_1 receptor whilst [pGlu^6, *D*-Pro^9]SP 6-11 was selective for the NK_2 receptor subtype (Suman-Chauhan *et al.*, 1990). Evidence was also provided to support the potent and selective NK_2 antagonistic properties of the nonpeptide antagonists L-659,874 and L-659,837 (Suman-Chauhan *et al.*, 1990).

Many other agonists and antagonists (either peptide substitutions or nonpeptide analogues) act on the various tachykinin receptor subtypes. Some of these agents have been listed in Table 1.3. (next page). An interesting sideline to the development of substance P agonists or antagonists is their possible use as therapeutic aids in substance P implicated diseases and conditions. These include antinociceptive effects in mice (Post and Folkers, 1985) and the inhibition of growth of small cell lung cancer cell lines (Woll and Rozengurt, 1990).

Research into tachykinin antagonists is progressing at a rapid rate, due largely to their possible clinical utility as a new generation of analgesics, anti-asthmatics and anti-rheumatics. Papers by Regoli and colleagues (1991) and Maggi and associates (1991) briefly outline various representative antagonists and their respective actions, whilst a paper by Seelig and Doelz (1991) profiles the basic physical characteristics of SP with some of its antagonists. More information on the conformational/structural aspects of both analogues and antagonists is presented in papers by Elguero *et al.*, (1989), Seelig (1990), Convert *et al.*, (1991) and Snider *et al.*, (1993).

Receptor	*Analogue*	*Action/Reference No.*
NK_1	spantide	antagonist 1
NK_1	[*D*-Pro^4,*D*-Trp^{7,9},Nle^{11}]SP 4-11	antagonist 1
NK_1	[*D*-Tyr^4,*D*-Trp^{7,9},Nle^{11}]SP 4-11	antagonist 1
NK_1	MM-150	antagonist 1
NK_1	CP 96345	antagonist 1
NK_1	[Pro^9]SP sulphone	agonist 2
NK_2	[β-Ala^8]NKA 4-10	agonist 2,4
NK_3	[MePhe^7]NKB	agonist 2,4
NK_2	MEN 10,207	antagonist 2
NK_1	[*D*-Pro^2]SP	agonist 3
NK_1	[*D*-Pro^4]SP	agonist 3
NK_1	[*D*-Gln^5]SP	agonist 3
NK_2	MDL 28,564	agonist 4
NK_1	[Sar^9]SP sulphone	agonist 4
NK_2	MEN 10,367	antagonist 4
NK_2	L 659,877	antagonist 4
NK_2	R 369	antagonist 4
NK_1	ranakinin	agonist 5
NK_1	[Sar^9, Met$(O_2)^{11}$]SP	agonist 5
NK_1	[Glu(OBzl)^{11}]SP 6-11	agonist 6
NK_1	[Glu(OBzl)^{11}]SP 5-11	agonist 6

Table 1.3. Some agonists and antagonists of the tachykinin receptors NK_1, NK_2 and NK_3. Reference numbers correspond to the following articles; 1 = Jukic *et al.*, 1991, 2 = Maggi *et al.*, 1990, 3 = Duplaa *et al.*, 1991, 4 = Giuliani *et al.*, 1991, 5 = O'Harte *et al.*, 1991 and 6 = Stavropoulos *et al.*, 1991.

1.5.4 Fragments of Substance P.

Fragments of substance P may possess some agonistic or antagonistic activities of their own. The biological activity of substance P and the C-terminal sequences (to SP 9-11) on hind limb blood flow of the dog, contractile activity in the isolated guinea pig ileum (GPI) and constrictor activity in the isolated rabbit ear vein has been determined (Bury and Mashford 1976). SP 4-11 was more potent than substance P in all of the systems. Other fragments more potent than substance P were SP 3-11 and SP 5-11 (dog femoral artery), SP 3-11, SP 5-11 and SP 6-11 (GPI preparation) and SP 3-11 (rabbit ear vein preparation). The fragments SP 7-11, SP 8-11 and SP 9-11 possessed little or no activity when compared with substance P. Thus, the fragments SP 3-11 to SP 6-11 appeared more active than the parent peptide (with SP 4-11 significantly more potent), whilst fragments smaller than SP 7-11 appeared to be inactive in these systems.

Alterations in blood pressure have been obtained by the administration of SP 1-7, SP 1-7, SP 1-4, SP 1-9 and the pyroglutamyl forms of SP 6-11 and SP 7-11 to rats, whilst SP 2-7, SP 1-6, SP 1-2, 1-8, and 3-7 did not affect the blood pressure of rats (Hall, Miley *et al.*, 1987).

Similarly, the relative affinities (potencies) of the C-terminal fragments SP 4-11, 5-11 and 6-11 were greater than substance P in the guinea pig ileum, whereas a decreased relative affinity was observed in the dog carotid artery (Regoli *et al.*, 1989). Substance P had low activity in the rabbit pulmonary artery, hamster urinary bladder and rat portal vein preparations and this was reflected in the relative affinities of the fragments.

Administration of substance P or the fragments SP 4-11, SP 5-11 ($pGlu^5$-substance P 5-11, the pyroglutamyl form of SP 5-11) and SP 6-11 ($pGlu^6$-substance P 6-11) to mice alters grooming, scratching, rearing and sniffing behaviour (Hall, Grantham *et al.*, 1987). The fragments SP 7-11 and SP 8-11 only altered rearing behaviour and sniffing behaviour was reduced with SP 7-11 but not SP 8-11. Rearing behaviour in mice is increased with SP 1-4, SP 1-6, SP 1-7 and SP 2-7 but unchanged in animals given SP 1-2, SP 1-8 or SP 3-7.

The activities of the fragments SP 1-9 and SP 3-11 on human polymorphonuclear leukocyte and monocyte locomotion have been examined in *in vitro* cell migration assays (Wiedermann *et al.*, 1989). SP 1-9 significantly increased human polymorphonuclear leukocyte migration whilst SP 3-11 increased the locomotion of human monocytes, although the sample sizes were very small (N=3 and N=2 respectively).

The cytotoxic activity of substance P and some of its fragments has been assessed in platelets towards larvae of the parasite *Schistosoma mansoni* (Dammoneville *et al.*, 1990). Substance P directly induced the cytotoxic activity of platelets to the larvae in a dose-dependent manner. SP 3-11 and 4-11 retained approximately 40% of this cytotoxic activity whilst SP 5-11 was inactive. Of the N-terminal fragments, SP 1-7 and 1-4 were approximately 15-20% active at the doses tested, but did not stimulate the platelets and were thus judged as having no effect on the cells.

In conclusion, the fragments of substance P appear to possess (sometimes enhanced) intrinsic biological activities of their own. The results of some studies suggest that the C- and N-terminal fragments may even possess opposing actions in some biological systems, yet, in most cases, only one type of fragment is usually active in any one biological system. Further studies into the actions may reveal regulatory roles for these fragments or significant biological actions in their own right.

1.6 METABOLISM OF SUBSTANCE P

A definitive knowledge of the mechanisms regulating the breakdown of this peptide is lacking. Reports in the literature suggest that a combination of non-specific enzymes may be responsible for the degradation process rather than a single substance P specific enzyme. A short overview of some of the major enzyme systems implicated in the *in vivo* metabolism of substance P follows.

1.6.1 Neutral Endopeptidase 24.11 (NEP).

Neutral endopeptidase 24.11 (otherwise known as enkephalinase, EC 3.4.24.11 or NEP) was discovered in the kidney, but is known to be distributed throughout the body including specific structures in the CNS, lung, male genital tract and the intestine as well as in neutrophils, fibroblasts and epithelial cells (Erdos and Skidgel, 1989; Iwamoto *et al.*, 1991; Nadel and Borson, 1991; Mussap *et al.*, 1993). NEP is bound to the plasma membrane in tissues and cells, but is also present in a soluble form in urine and blood, although its concentration in vascular endothethial cells is very low (Erdos and Skidgel, 1989). NEP may also be found in many (but not all) Schwann cell membranes in peripheral nerves (Erdos and Skidgel, 1989).

In addition to being the major enzyme responsible for the elimination of enkephalins, NEP has other substrates including bradykinin, substance P, neurotensin, chemotactic peptide, gastrin and atrial natriuretic peptide (Erdos and Skidgel, 1989).

The kinetics of substance P inactivation in rat brain fractions and human plasma has been determined with the use of [Phe-^{3}H,Nle^{11}]substance P (Berger *et al.*, 1979). Degradation velocity plots determined that a single enzyme system was responsible for the degradation of the substrate in these systems. Degradation was inhibited by reducing the temperature (to $0^{o}C$) and was optimal within the pH range of 7.0 and 8.0. Other effective inhibitors were *o*-phenanthroline and bacitracin, whilst aprotinin, dithiothreitol, EDTA and trypsin inhibitor were less efficient inhibitors. The authors concluded that the enzyme was a neutral

metallopeptidase found in both the rat brain and human plasma, but could not identify this enzyme further.

Incubation of substance P with a neutral/ metalloendopeptidase from human brain yields fragments obtained by cleavage between Gln^{6}-Phe^{7}, Phe^{7}-Phe^{8} and Phe^{8}-Gly^{9} (Lee, 1982). The enzyme had an optimal pH of 7.0 to 9.0 and was strongly inhibited by EDTA, 1,10-phenanthroline and dithiothreitol, but not by phosphoramidon, thiorphan, captopril or many others. Thus, although this enzyme may be a member of the neutral metalloendopeptidase family, it is not NEP, as thiorphan and phosphoramidon are known to be specific inhibitors of NEP (Erdos and Skidgel, 1989). Erdos and Skidgel (1989) also suggested that there may not be a specific enzyme for the metabolism of substance P *in vivo*.

NEP purified from C6 glioma cells from rats cleaves substance P at the following bonds; Gln^{6}-Phe^{7}, Phe^{7}-Phe^{8} and Gly^{9}-Leu^{10} (Endo *et al.*, 1989). Addition of phosphoramidon almost completely inhibited the generation of the fragments. The plasma membranes of the C6 cells also degraded substance P (this process was inhibited by phosphoramidon and EDTA). The enzyme inhibitors captopril, bestatin, diisopropyl fluorophosphate and *p*-chloromercuribenzenesulfonic acid had little or no effect on the degradation of substance P. Thus, rat glioma cells and their membranes may mediate the degradation of substance P by NEP.

Phosphoramidon has been found to potentiate the effect of substance P-induced mucus secretion in human bronchi *in vitro* (phosphoramidon itself did not alter basal mucus production) (Rogers *et al.*, 1989). Neurokinin A and neurokinin B were less effective at inducing an increase in mucus production, but phosphoramidon did not potentiate these effects. Thus, substance P may stimulate mucus secretion in human bronchi and NEP may play a major role in modulating its effects.

A similar result was seen with the *in vitro* ferret trachea preparation (Webber *et al.*, 1989). Substance P increased mucus volume, lysozyme and albumin outputs and these responses were all significantly increased by the

presence of phosphoramidon. It appears that a neutral endopeptidase exists in ferret trachea (most likely NEP) and modulates the actions of substance P.

A recent study to determine the binding of a new ligand to the NK_1 receptor of guinea pig lung membranes utilized thiorphan to establish the kinetics of binding (Aharony *et al.*, 1991). Results obtained with thiorphan, captopril, phenyl methyl sulfonyl fluoride, 1,10-*o*-phenanthroline and other inhibitors suggested the role of a metalloendopeptidase (particularly NEP) in the system.

1.6.2 Angiotensin I Converting Enzyme (ACE).

Angiotensin I converting enzyme (peptidyldipeptidase, EC 3.4.15.1 or ACE) occurs in all vascular beds, but only in endothelial cells (Rogerson *et al.*, 1989; Mussap *et al.*, 1993). Tissues with relatively high concentrations of ACE include the lung, testis, kidney and brain (Rogerson *et al.*, 1989). ACE has been shown to be an effective proteolytic enzyme in the dog carotid artery, rabbit pulmonary artery and rat portal vein (Rouissi *et al.*, 1990a) and in the rat, guinea pig and hamster bladder (Rouissi *et al.*, 1990b).

The inhibitors of ACE include captopril and enalapril (enalaprilat, enalaprilic acid) and are commonly used in the treatment of human essential hypertension and heart failure. Captopril has been shown to be completely effective in inhibiting the degradation of substance P and some C-terminal fragments (SP 4-11 and SP 6-11) in rat plasma, although it was believed that this effect was due to an unidentified endopeptidase which was inhibited by captopril (Couture and Regoli, 1981). Thus, captopril may not be as specific for ACE as is commonly assumed.

Substance P induces salivation in rats and pretreatment with captopril potentiates this response (Cascieri *et al.*, 1984). In the same study, substance P was incubated with ACE and the major metabolite determined by high performance liquid chromatography. The major site of initial hydrolysis was between the amino acids Phe^8-Gly^9 yielding the fragments SP 1-8 and SP 9-11. The authors thus suggested that ACE may facilitate the actions of substance P *in vivo*.

This site of hydrolysis by ACE has been confirmed in another study by Yokosawa and colleagues (1985). Results indicated the major cleavage product of substance P was the fragment SP 9-11 with SP 7-8 and SP 1-7 also observed. It is believed that Phe^{8}-Gly^{9} may be the primary site of action for ACE.

The importance of ACE in substance P regulation *in vivo* has been assessed by determining the alterations in human forearm blood flow mediated by substance P (Benjamin and Webb, 1990). Infusion of substance P resulted in a dose-dependent increase in blood flow. The ACE inhibitor enalaprilat caused no discernible change in blood flow and did not inhibit the increase in blood flow observed with substance P. Thus, ACE located in the blood vessel endothelium may not play a major role in the metabolism of substance P in the forearm vessels *in vivo*.

The effect of ACE inhibitors on substance P induced bronchoconstriction in the *in vivo* guinea pig model has also been studied (Subissi *et al.*, 1990). Bronchoconstriction induced by substance P was significantly potentiated by captopril or enalaprilic acid. Thus, ACE may modulate the effects of substance P in the respiratory tract *in vivo* and the inhibition of substance P metabolism may be partly responsible for the adverse reactions of cough and bronchial hyperreactivity observed in some patients on ACE-inhibitor therapies.

Thiorphan (as well as captopril) has also been examined in this model (Shore *et al.*, 1988). Essentially, both inhibitors were effective in potentiating the bronchoconstrictor response and increasing the recovery of immunoreactive substance P from the circulation *in vivo*, although captopril was more effective in increasing plasma substance P recovery. It was suggested that NEP may be the primary enzyme for substance P degradation in the respiratory tract *in vivo* and that ACE may be the primary enzyme for the metabolism of substance P in the vasculature *in vivo*, although the latter is in conflict with the results obtained by Benjamin and Webb (1990).

The wheal response induced by intradermal injection of substance P in humans is unaffected by the administration of enalapril or sulindac (a

nonsteroidal anti-inflammatory drug [NSAID]) either alone or in combination (Ferner *et al.*, 1991). Thus, the metabolism of substance P in the epithelium *in vivo* may not be primarily mediated by the actions of ACE.

Some of the N-terminal fragments of substance P may exert an inhibitory effect on the activity of ACE (Rogerson *et al.*, 1989). The inhibition of ACE in rat lung and brain was dose-dependent and equipotent for all tested fragments of substance P including the parent peptide (*i.e.*, SP 1-11, SP 1-3, SP 1-4, SP 3-4, SP 1-6 and SP 1-7). [Note: SP 1-3 did not alter lung-derived ACE activity but was more potent at inhibiting brain-derived ACE activity]. It appears that this inhibition of ACE activity by substance P is most likely contained in the N-terminus of the peptide and that the differing potency of the fragment SP 1-3 may suggest that brain-derived ACE and lung-derived ACE are isoenzymes.

1.6.3 Dipeptidyl Aminopeptidase IV (DPP IV).

Dipeptidyl aminopeptidase IV (postproline dipeptidyl aminopeptidase, EC 3.4.14.5 or DPP IV) may be found in hog kidney brush border and purified vascular plasma membrane (Palmieri and Ward, 1983). Incubation of substance P with hog DPP IVs yielded the N-terminal Arg^{1}-Pro^{2} (SP 1-2) and Lys^{3}-Pro^{4} (SP 3-4) fragments, theoretically resulting in SP 5-11 (which was not detected). However, it is possible that SP 5-11 was further degraded rapidly within the incubation medium.

DPPIV exhibits particularly high activity in kidney (Barelli *et al.*, 1993; Mussap *et al.*, 1993), followed by lung, liver and adrenal glands (Pernow, 1983). Palmieri and Ward (1983) have demonstrated that DPPIV occurs on the vascular surface membrane of hog mesenteric artery and aorta and they believe that DPPIV may be found on the vascular endothelium of numerous organs.

Incubation of substance P with human plasma suggests that DPP IV may be responsible for the inactivation of this peptide in plasma (Conlon and Sheehan, 1983; Conlon and Goke, 1984). *In vitro* incubations of plasma with exogenous

substance P sequentially yielded the fragments SP 3-11 then SP 5-11, followed by an unidentified C-terminal fragment (Conlon and Sheehan, 1983). Further characterization of the activity of the enzyme responsible suggested that the enzyme may be DPP IV.

A similar study (Conlon and Goke, 1984) obtained the same result; *i.e. in vitro* incubation of substance P in human plasma yielded the fragments SP 3-11 first followed by SP 5-11, consistent with the actions of DPP IV. Yet, in plasma samples obtained from anaesthetized rats following infusion of substance P, neither the peptide nor any C-terminal fragments were detected, despite the significant induction of vasodilatation. It seems that the exogenous substance P was rapidly cleared from the rodent circulation. Hence, *in vitro* human plasma degradation of substance P may be mediated by DPP IV whilst the enzyme (or enzymes) involved in the rapid *in vivo* degradation of the peptide in rodents has not been identified.

Substance P is not the only substrate for DPP IV, just as angiotensin I is not the only substrate for ACE. Other substrates for this enzyme include monomeric fibrin, chorionic gonadotropin, pancreatic polypeptide and gastrin releasing peptide (Nausch *et al.*, 1990).

1.6.4 Other Enzymes.

Other enzymes may inactivate substance P *in vivo*. Those which may be physiologically important include acetylcholinesterase (Goebel and Pourcho, 1992a), substance P degrading endopeptidase (Goebel and Pourcho, 1992b) and cathepsin G (Skidgel *et al.*, 1991), cathepsin D, prolyl endopeptidase, substance P degrading enzyme, C-terminal cleaving enzyme (Mussap *et al.*, 1993) and more may yet be discovered.

1.6.5. Comparative Studies.

A number of other studies have been conducted to compare the effectiveness of various enzyme inhibitors on the degradation of substance P (and other substrates) in different physiological systems. Some of these have been briefly summarized in Table 1.4 (next page).

1.6.6 Summary.

The various *in vitro* sites of inactivation of this peptide (and hence the fragments produced) are dependent upon the enzyme system involved. NEP and ACE act between the amino acid bonds 6 to 10 inclusive, whilst DPP IV attacks the bonds between amino acids 2 to 5 inclusive. Figure 1.5 summarizes the sites of action for the major substance P proteolytic enzymes NEP, ACE and DPP IV and other minor enzymes.

From the data presented it appears that distinct enzymes are responsible for the inactivation of substance P in diverse tissues and organs. NEP is important in the kidney, CNS, lung, male genital tract, intestine, neutrophils, fibroblasts and epithelial cells (section 1.6.1), ACE may be found in vascular endothelial cells, particularly in lung, testis, kidney and brain (section 1.6.2) and DPPIV is effective in kidney, lung, liver and adrenal glands (section 1.6.3). However, more research into the *in vivo* inactivation of substance P is required before any final conclusions may be made.

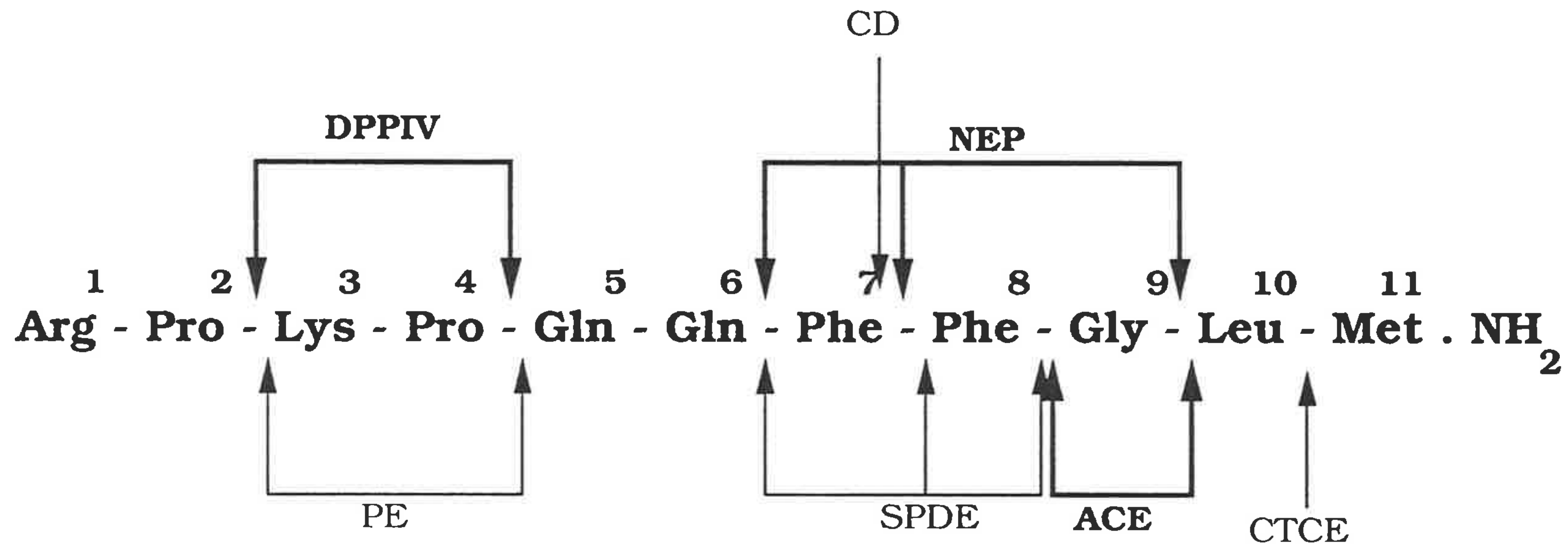


Fig.1.5. Inactivation sites for substance P by the enzymes neutral endopeptidase 24.11 (NEP), angiotensin I converting enzyme (ACE), dipeptidyl aminopeptidase IV (DPPIV), prolyl endopeptidase (PE), substance P degrading enzyme (SPDE), C-terminal cleaving enzyme (CTCE) and cathepsin D (CD). [From Mussap and colleagues, 1993].

Enzyme Inhibitors	*Physiological System*	*Results and References*
thiorphan, captopril, bestatin, leupeptin	*In vitro* rabbit tracheal epithelium ciliary activity.	NEP mediation. Kondo *et al.*, 1990.
acetorphan, captopril	*In vivo* guinea pig lung resistance.	NEP mediation. Lotvall *et al.*, 1990.
thiorphan, captopril, bestatin	*In vitro* guinea pig ileal circular muscle.	Inhibitors had no effect. Maggi *et al.*, 1990.
captopril, thiorphan, SCH 32615, leupeptin, bacitracin, bestatin, chymostatin	*In vitro* guinea pig airway opening pressure.	NEP mediation. Martins *et al.*, 1990.
captopril, thiorphan, bestatin, bacitracin, chymostatin, mergetpa, phosphoramidon	*In vitro* dog carotid artery, rabbit pulmonary artery and rat portal vein.	Primarily ACE mediation. Rouissi *et al.*, 1990a.
captopril, thiorphan, bestatin, bacitracin, chymostatin, phosphoramidon, mergetpa	*In vitro* guinea pig, hamster and rat urinary bladder.	Mainly ACE in rat. Multiple enzymes in the hamster and guinea pig. Rouissi *et al.*, 1990b.
bestatin, captopril, thiorphan	*In vitro* human colon circular muscle.	Inhibitors had no effect. Giuliani *et al.*, 1991.
captopril, thiorphan, kelatorphan, bacitracin, enaliprilat	*In vitro* rat spinal cord.	Primarily a bacitracin-sensitive enzyme. Mauborgne *et al.*, 1991.
thiorphan, captopril, bestatin, leupeptin, bacitracin	*In vitro* guinea pig sinus.	Mainly NEP mediation. Tramonata *et al.*, 1991.

Table 1.4. A summary of some studies which compared the effectiveness of several enzyme inhibitors on substance P inactivation in various biological preparations.

1.7 BACKGROUND AND AIMS OF THE PRESENT STUDY

1.7.1 Hypertension and Cardiovascular Disease.

Hypertension is a major contributing factor in the development of cardiovascular morbidity and mortality. Manifestations of cardiovascular disease which may be fatal or permanently debilitating include myocardial infarction and cerebral ischemia or cerebrovascular accident.

The National Heart Foundation of Australia (NHF) attempted to determine the level of hypertension and the importance of certain risk factors in various representative sections of the Australian community through the Risk Factor Prevalence Study surveys (National Heart Foundation of Australia, 1990). Risk factors assessed were high blood pressure, raised blood cholesterol and triglyceride levels, cigarette smoking, being overweight and dietary behaviour, diabetes mellitus, alcohol intake and psychological stress.

Hypertension, defined as a diastolic blood pressure of greater than 95 mmHg and/or the presence of antihypertensive therapy, was present in 17% of men and 13% of women. The incidence of hypertension increased steadily with age.

Assessment of known *major* risk factors (listed above) revealed 42% of men surveyed and 35% of women had at least one of the three major risk factors, whilst 8% of men and 5% of women had multiple risk factors (either two or all three of the risk factors) with the incidence peaking in the 50-54 years age range.

It is obvious from this report that hypertension is a serious condition which is prevalent in a large proportion of the community. This incidence is not specific for Australia, as cardiovascular disease (often precipitated by hypertension) is the leading cause of death in the United States (Westfall, 1990). It is therefore extremely important that a thorough understanding of the aetiology of the condition and predictive tests for hypertension be developed to combat the condition prior to its onset and also to develop novel treatment strategies for hypertension.

1.7.2 The Aetiology of Hypertension.

The development of hypertension in some instances, is a consequence of other pre-exisiting conditions in the individual. For instance, certain renal conditions may result in the development of high blood pressure as would pheochromocytoma (a tumour which secretes abnormal amounts of catecholamines). Treatment of the pre-exisiting condition also removes the driving influence for hypertension.

Essential hypertension (or primary hypertension) is the most predominant form of hypertension in the community. The aetiology and pathogenesis of this condition in humans remains largely unknown, although it is believed to be an inherited condition in many instances.

1.7.3 The Animal Model for Hypertension.

The development of a genetic animal model for human essential hypertension, the spontaneously hypertensive rat (or SHR; Okamoto and Aoki, 1963) has enabled research to be conducted into the underlying mechanisms which ultimately result in the increase in blood pressure seen in this condition. It is this particular rat and its normotensive cousin the Wistar Kyoto rat (WKY), which acts as the normotensive control, which have made it possible to study the various morphological and physiological alterations associated with the development and maintenance of hypertension in an animal model. These animal models have been employed in the current study.

1.7.3.i Noradrenaline Levels in the SHR.

Early studies concentrated on measuring levels of the sympathetic neurotransmitter noradrenaline (NA or norepinephrine), a potent and well known naturally-occurring vasoconstrictor agent. The NA concentration in mesenteric arteries from SHRs was significantly greater than those seen in WKYs (7.4 ± 1.1 μg/g and 4.4 ± 0.7 μg/g respectively; Head *et al.*, 1984). It was initially believed that there had been an alteration in MAO (monoamine oxidase) activity to account

for this difference, but measurement of the deaminated metabolite of NA (dihydroxyphenylethylene glycol or DOPEG) from the same mesenteric arteries revealed no difference in DOPEG formation between the two strains of rats. Thus, the increased levels of NA observed in SHR arteries was not a result of impaired neuronal metabolism of the transmitter NA.

Endogenous turnover of NA in SHRs and WKYs has also been evaluated by inhibiting the conversion of tyrosine to dihydroxyphenylalanine (a precursor of NA) by tyrosine hyroxylase with the use of α-methyl-*para*-tyrosine (Cassis *et al.*, 1988). In untreated animals, NA levels in caudal and mesenteric arteries were significantly higher in SHRs compared to WKYs, but treatment with α-methyl-*para*-tyrosine decreased NA to similar levels in the SHRs and WKYs. Thus, although there was a larger pool of NA in the mesenteric arteries, the turnover was similar to that seen in vessels from WKYs.

NA levels have been shown to be elevated in mesenteric arteries from both SHRs and SHR-SPs (the stroke-prone variant of the SHR) when compared with WKYs (Mano *et al.*, 1992). In contrast, NA levels were significantly higher in cardiac tissue in WKYs and in coeliac ganglia from SHRs, although similar NA levels were observed for all strains in the superior cervical ganglia. Thus, the increased NA observed in the blood vessels of the two hypertensive strains was not reflected in cardiac tissue or ganglia. However, NA levels in vascular tissue parallelled the increase in blood pressure observed in the hypertensive strains when compared with the normtensive WKY.

1.7.3.ii Histochemical Determinations of NA.

Histochemical techniques (in addition to other methods) have revealed significantly greater levels of NA in SHRs than WKYs in all regions of the caudal artery (Cassis *et al.*, 1985). Qualitative fluorescence histochemistry revealed a greater intensity of fluorescence in caudal arteries taken from SHRs when compared with WKYs. An increase in the number of axon bundles, as a result of

an enhancement of sympathetic innervation (hypernoradrenergic innervation) seems the most likely explanation for this result (Cassis *et al.*, 1985).

More recently, a review article (Head, 1989) has outlined evidence suggesting that sympathetic innervation of selected vascular beds in the SHR is greater than in the WKY, especially in the mesenteric vascular bed and caudal artery. However, enhanced sympathetic innervation is not a characteristic of all blood vessels of the SHR (Head, 1989).

1.7.3.iii Morphological Studies.

Closer inspection of the various morphological characteristics of blood vessels taken from SHRs and WKYs has revealed significant structural differences between the two strains (Warshaw *et al.*, 1980; Cassis *et al.*, 1985; Lee, 1985; Lee *et al.*, 1987; Owens *et al.*, 1988).

Electron microscopic analysis of the adventitial-medial border of sections of the caudal arteries taken from SHRs and WKYs revealed that both the total number of nerve bundles and the total area occupied by these nerves were greater in SHRs (Cassis *et al.*, 1985). Moreover, 7 to 9 muscle cell layers were observed in the caudal artery walls of WKYs , whilst 10 to 15 layers were observed in vessel walls from SHRs.

Vascular morphological alterations become evident prior to the development of hypertension in the SHR, as shown in an extensive study by Lee (1985). Light and electron microscopic techniques were used to obtain morphometric measurements of three categories of mesenteric vessels (arteriolar, elastic and muscular vessels) from *prehypertensive* SHRs and age-matched WKYs. Both species recorded similar tail-cuff blood pressure readings (48.0 $\pm$ 4.2 mmHg for WKYs and 43.0 $\pm$ 2.1 mmHg for SHRs), but structural alterations of blood vessels were already apparent in the SHRs. The structural differences (outlined in Table 1.5, next page) are thought to be one of the contributing factors leading to the development of hypertension in the SHR, rather than a secondary event.

	MESENTERIC VESSEL TYPE		
STRUCTURE	***SUPERIOR MESENTERIC ARTERY***	***LARGE BRANCHES***	***SMALL BRANCHES***
Intima and internal elastic lamina	no change	increase	no change
Media	no change	increase	no change
Adventitia	no change	increase	no change
Lumen	no change	no change	decrease
Smooth muscle cell layers	no change	increase	increase
Nerve density	no change	increase	no change

Table 1.5. Summary of the structural alterations observed in various representative mesenteric arteries taken from prehypertensive SHRs when compared with the vessels from age-matched WKYs (Lee, 1985).

The effect of neonatal sympathectomy on mesenteric artery morphology has been examined by Lee and colleagues (1987). Administration of anti-NGF and guanethidine for the first 4 weeks after birth to SHRs and WKYs abolished the development of hypertension in the SHRs at 28 to 30 weeks of age. Sympathectomy did not prevent hypertrophy of vascular smooth muscle cells, but did prevent hyperplasia in large and small mesenteric arteries from SHRs. Sympathectomy also reduced the number of smooth muscle cell layers in large and small mesenteric arteries from WKYs. It was suggested that the presence of hypernoradrenergic innervation prior to the development of hypertension in the SHR exerts some sort of trophic effect on the vasculature. Futhermore, this trophic effect then causes a hyperplastic change in smooth muscle cells of reactive and resistance vessels, ultimately contributing to the development of hypertension in older SHRs (Lee *et al.*, 1987).

Owens and associates (1988) have shown that hyperplasia rather than hypertrophy or hyperploidy, is the major mechanism responsible for increasing the smooth muscle content of SHR resistance vessels.

1.7.3.iv Time Course of Hyperinnervation.

NA levels were determined in SHRs and WKYs from very young animals (2-day-olds) to mature animals (119-day-olds), in a variety of both vascular and nonvascular tissues (Donohue *et al.*, 1988). From 46 to 119 days of age, the mean systolic blood pressure of SHRs was significantly greater than that of the WKYs. The NA content of the various tissues studied *generally* increased with age for both strains of rats. However, NA content in the mesenteric arteries taken from SHRs was significantly greater than in WKYs for all ages. A similar trend was observed in the kidneys (Donohue *et al.*, 1988).

For caudal arteries, no differences were observed in NA content between the ages of 20 and 40 days for both strains of rats, but at 80 and 120 days, a higher NA content was observed in the SHRs. No significant difference was observed in the NA content of the heart over the age range of 2 to 119 days between the SHRs and WKYs. Generally low levels of NA were seen in aortae taken from both strains, but significantly higher values were seen at 81 and 119 days for SHRs (Donohue *et al.*, 1988).

In contrast, NA content in spleen taken from SHRs was significantly greater up to and including 20 days of age. Thereafter, no difference in NA levels between the 2 strains was observed, although total NA content increased with time. However, NA determinations for the adrenal glands revealed a significantly higher level in tissue from SHRs at 46 and 80 days of age, but not at 120 days of age (Donohue *et al.*, 1988).

Apparently, hyperinnervation of the mesenteric artery has already occurred soon after the birth of SHRs (Donohue *et al.*, 1988). Moreover, it was suggested that hypernoradrenergic innervation *precedes* the elevation of blood pressure observed in the SHRs. Yet the enhanced sympathetic innervation in the caudal artery of SHRs followed the development of hypertension, as did the NA content in aortae from SHRs. The enhanced innervation of the kidney may contribute to the development of hypertension in SHRs. If the NA level is used as an index of sympathetic innervation, it seems that certain vascular and

nonvascular tissues exhibit hypernoradrenergic innervation *prior* to the development of hypertension (Donohue *et al.*, 1988).

1.7.3.v Influence of Nerve Growth Factor.

The presence of the trophic agent, nerve growth factor (NGF) is known to be a prerequisite for the normal growth and development of primary sensory neurons (1.3.4.ii *Nerve growth factor (NGF) and primary sensory neurons*). Studies suggest that NGF may mediate the hypernoradrenergic innervation observed in the SHR, in addition to the growth of peripheral sympathetic nerves in general.

The NGF levels of mesenteric arteries and aortae from young (20-day-old) and adult (6-month-old) SHRs and WKYs have been determined (Donohue *et al.*, 1989). At 20 days of age, the NGF content of the mesenteric artery and aorta was approximately 50% greater in SHR tissues. Yet, at 6 months of age, no significant differences were observed in NGF content of either tissue between the SHRs and WKYs. It was suggested that the enhanced NGF levels in vascular tissues from young SHRs may be involved in the hypernoradrenergic innervation of blood vessels observed in this model of hypertension.

The *i.c.v.* infusion of NGF into adult rats induced axonal sprouting of mature, uninjured axons in association with the internal carotid artery (Isaacson *et al.*, 1990). The total number of axons associated with the vessel wall increased three-fold following NGF administration when compared with control animals. The results suggest that NGF may mediate hyperinnervation in the CNS.

An association of NGF with vascular smooth muscle has also been examined as the presumed source of NGF for sympathetic nerves in the target tissue (Creedon and Tuttle, 1991). Dissociated superior cervical ganglion (SCG) neurons were co-cultured with either exogenous NGF or vascular smooth muscle cells (from rat thoracic aorta) or alone for 72 hours. Neuronal counts revealed a low survival rate for SCG neurons cultured alone. Incubation of SCG neurons with vascular smooth muscle cells promoted neuronal survival equivalent to 50 ng/ml NGF. Upon addition of anti-NGF to the co-cultures, the vascular smooth

muscle cell survival rate of SCG neurons was reduced by 52-99%. Apart from promoting neuronal survival, the vascular smooth muscle cells served as a receptive substrate for neuronal outgrowth. Molecular biology techniques established the presence of an NGF mRNA in the vascular smooth muscle cells. The authors suggested that vascular smooth muscle cells may produce and secrete the NGF which supports the SCG neurons *in vitro* and that this effect may occur *in vivo*.

The effect of chronic NGF treatment of the normotensive WKYs on vascular innervation and blood pressure has also been studied (Zettler *et al.*, 1991). Newborn WKYs received NGF (or saline) for 8 weeks and their blood pressure was monitored between 5 to 8 weeks of age (at 8 weeks the animals were sacrificed). All animals remained normotensive, but in NGF treated animals the sympathetic ganglia were greatly enlarged and superior cervical ganglion neuronal numbers were increased by 200%. Catecholamine levels in various sympathetically innervated tissues were similar to levels observed in the SHRs. Histochemical analysis revealed a significant hyperplastic response in the smooth muscle wall of the caudal artery. This suggests that NGF may mediate the hyperinnervation and hyperplasia observed in the SHR, but not necessarily the associated elevation in blood pressure (Zettler *et al.*, 1991).

1.7.3.vi Current Hypothesis.

The previous data suggest that there is sympathetic hyperinnervation of the vasculature of the SHR and that this is reflected in an increase in the NA content of the blood vessels. Stimulation of these nerves would result in an increase in the amount of neurotransmitter released into the synaptic cleft (compared to the normotensive WKY), ultimately resulting in a larger contraction of the vascular smooth muscle cells and a greater increase in blood pressure. Furthermore, the development of hypertension in this model is associated with various vascular structural alterations such as smooth muscle cell hyperplasia and hypertrophy as well as increases in NGF. Consequently, it is thought that these

factors may interact and promote one another, ultimately producing the condition of hypertension.

Such a self-reinforcing cycle leading to the development of hypertension has been proposed by Head (1989) (Fig. 1.6). The theory suggests a relationship between vascular smooth muscle hyperplasia and sympathetic hyperinnervation (mediated by trophic factors), resulting in a potentiating cycle. NGF is generally believed to be produced by the target cells (to be innervated), thus the smooth muscle cells of the blood vessel wall produce this trophic factor. NGF in turn enhances the growth of the sympathetic nerves leading to hypernoradrenergic innervation. A trophic influence of sympathetic innervation on hyperplastic changes in vascular smooth muscle cells has been demonstrated (Lee *et al.*, 1987) and it is proposed that in the SHR the enhanced innervation induces abnormal vascular smooth muscle cell proliferation in the prehypertensive animal. Possible candidates for this trophic factor include angiotensin II and NA. It is suggested by Head that the initiation site of the cycle may be an increase in sympathetic activity (Head, 1989).

Thus, in the prehypertensive phase in the SHR, vascular smooth muscle cell hyperplasia and hypernoradrenergic innervation each exert trophic influences on the other, resulting in a potentiating cycle. These phenomena collectively induce enhanced contractile responses to sympathetic nerve stimulation, consequently leading to increased vascular resistance and increased blood pressure in the SHR. In response to the development of hypertension, hypertrophic changes now occur in the vascular smooth muscle cell population to compensate for the enhanced pressure and stress on the vascular walls. A final consequence to this scenario is believed to be an increase in the thickness of the medial layer of the blood vessel wall, thus supporting and maintaining the elevated blood pressure in this animal model. Whether human essential hypertension follows this sequence of events is unknown.

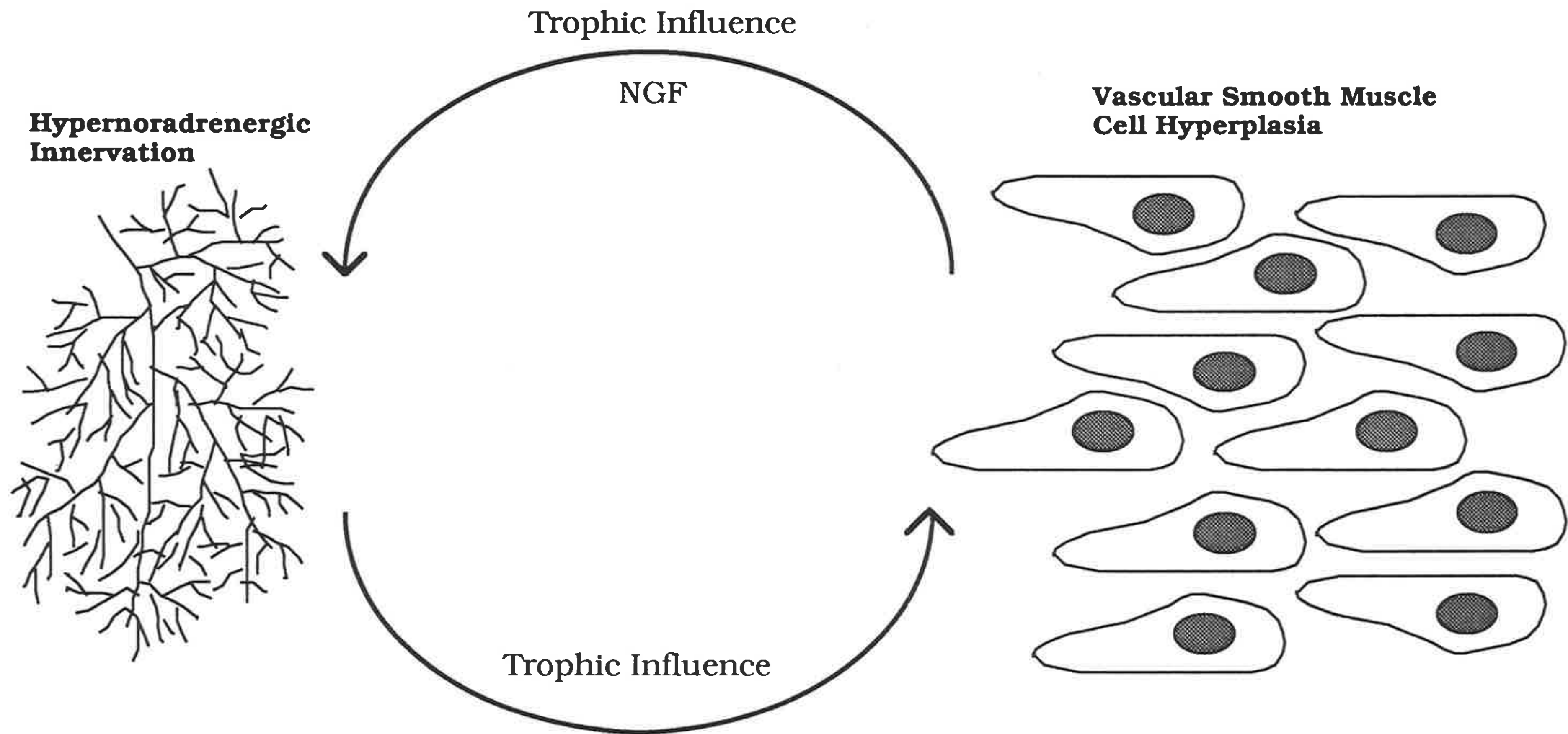


Figure 1.6. A schematic representation of the reinforcing effects of vascular smooth muscle cell hyperplasia and hypernoradrenergic innervation in the vasculature of the SHR rat as proposed by Head (1989). [Reproduced with permission].

1.7.4 Substance P and Hypertension.

Antihypertensive treatments currently available are effective in reducing the blood pressure of hypertensive individuals towards levels observed in normotensive individuals, although many require this treatment on a continuous basis. Release from treatment often results in the return of elevated blood pressure. A subpopulation of patients also experience adverse reactions associated with the antihypertensive treatment prescribed.

Current research has focussed on ways to prevent the development of hypertension. Several life-style factors have been identified which may aggravate the onset of hypertension and increase the risk of cardiovascular and cerebrovascular disease. Control of blood pressure may thus be achieved by changing life-style habits accordingly, but these changes are usually not completely effective on their own and additional drug therapy often needs to be prescribed, particulary in the more severe grades of hypertension.

Treatment of hypertensive individuals often commences upon the discovery of elevated blood pressure. If the condition is diagnosed in the early stages, less of the prescribed drug or appropriate life-style changes may arrest further development of hypertension. At present, no predictive test for the development of hypertension is available.

1.7.4.i Potential Biochemical Markers for the Development of Hypertension.

NGF is responsible for the abnormal growth of sympathetic nerves which contain NA as the neurotransmitter in the SHR. Theoretically, there should be at least two possible biochemical markers which may be used to predict the development of human essential hypertension *i.e.*, NA and NGF. Plasma NA determinations may not accurately reflect sympathetic nerve activity, as there is rapid re-uptake and metabolism of the catecholamine *in vivo*, although several studies suggest that NA is elevated in essential hypertension (De Champlain *et al.*, 1973; Franco-Morselli *et al.*, 1977; Buhler *et al.*, 1982; Howe *et al.*, 1986; Floras,

1992). NGF is either absent in plasma or occurs in concentrations below current limits of detection (Head, personal communication).

A direct link between NGF and substance P has been demonstrated (Kessler and Black, 1980; Otten *et al.*, 1980; Goedert *et al.*, 1981) whereby the application of NGF or antibodies to NGF to substance P-containing neurons was observed to significantly increase or decrease the levels of substance P in the neurons respectively. Furthermore, culturing adult sensory neurons in the presence or absence of NGF was found to significantly enhance or reduce the levels of substance P (Lindsay *et al.*, 1989) and PPT mRNA (Lindsay and Harmar, 1989) in the cultures respectively.

The most direct evidence for a link between NGF and substance P via primary sensory neurons has been provided by Gilchrist and associates (1991a). Through various biochemical manipulative techniques applied to the PPT gene, two functional elements within the gene were identified which conferred NGF responsiveness. It is believed that NGF acts via a transcription factor binding site on the PPT gene to increase gene transcription, resulting in an increase of PPT mRNA and ultimately an increase in substance P in the sensory neuron.

However, despite this link between increases in NGF and increases in substance P levels in primary sensory neurons, there is little reported in the literature with respect to this relationship in the hypertensive animal model. As mentioned previously, substance P is known to be mainly localized to the gastrointestinal neural network and primary sensory neurons, but may also be found throughout the entire peripheral nervous system (1.3.4 *The Peripheral Nervous System*). Furthermore, most tissues and organs are known to be innervated by substance P fibres (Leeman and Gamse, 1981 and Pernow, 1983). Thus, it may be possible that certain substance P tissue/organ levels may accurately reflect the levels of substance P in primary sensory neurons and therefore may offer the potential for reflecting abnormal levels of NGF in the hypertensive animal model, the SHR (Fig. 1.7).

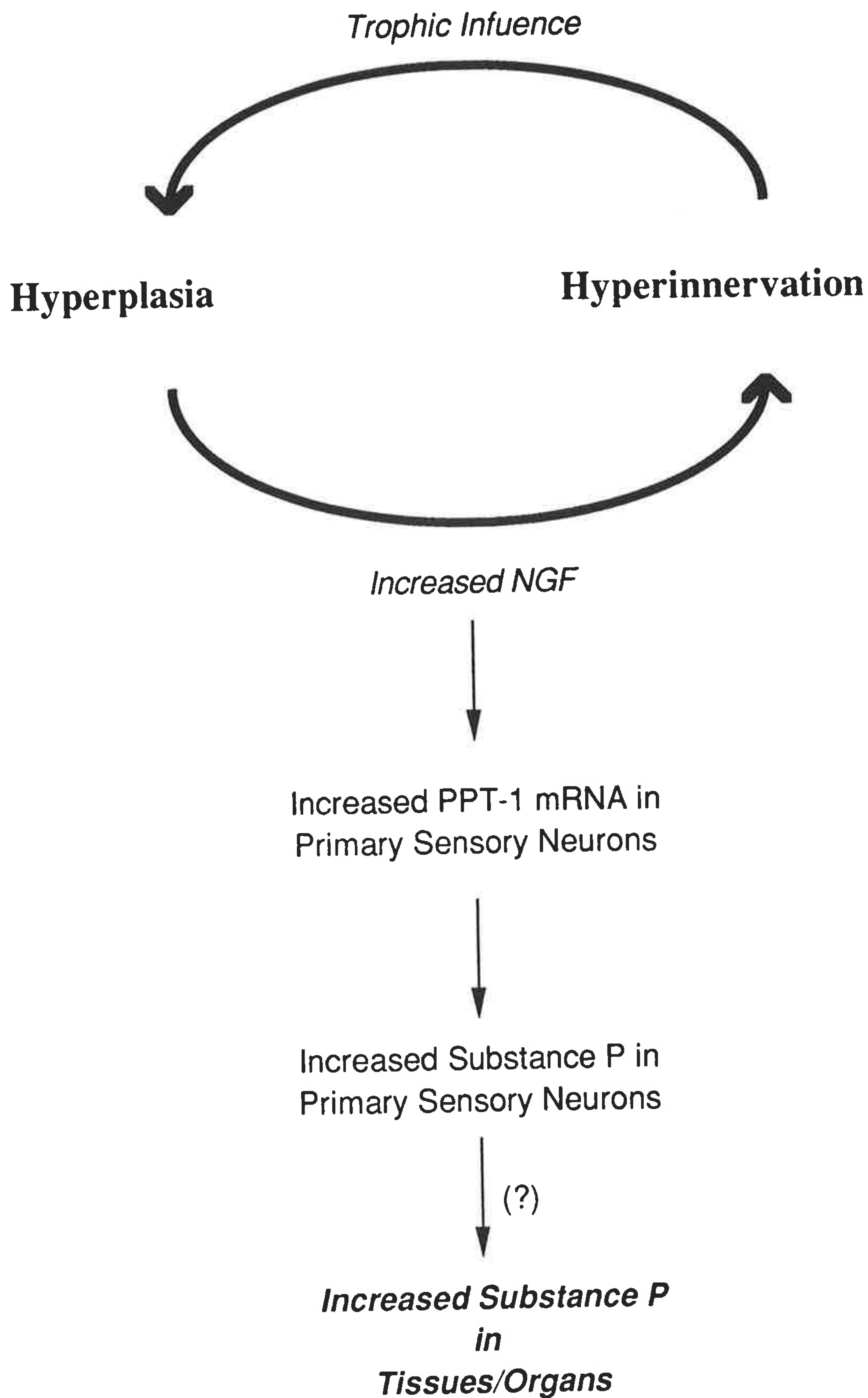


Figure 1.7. Schematic representation of the proposed hypothesis linking hypertension to increased plasma levels of substance P.

1.7.5 Aims of the Present Study.

The aims flow from the above discussion and address the possibility that substance P levels in certain tissues may be enhanced (due to a greater influence of NGF) in hypertension in the SHR. Preceding this primary aim however, is the need to review various assay methods to determine the most appropriate technique to measure substance P in tissues.

Much evidence exists in the literature for the quantification of substance P levels in plasma (Table 1.6, next page). However, reported plasma substance P levels vary greatly; ranging from a low of 1.3 pg/ml (Thysell *et al.*, 1988) to a high of 1200 pg/ml (Skrabanek *et al.*, 1976). Thus, it appears that substance P is present in the circulation, although there is no agreement on the concentration of the peptide in human plasma.

It is therefore necessary to explore the reasons why investigators using traditional assays have reported such variable values for substance P in human plasma (Table 1.6). The latter can only be investigated after a thorough analysis of the reliability of the substance P assay techniques and the influence of metabolism and degradation on plasma substance P. When these properties have been examined and optimum conditions have been developed for plasma substance P quantification, the method may then be tailored to suit the determination of substance P in tissues. The aims of the present study have been summarized on the page following Table 1. (*i.e.*, page 44).

Reported SP Concentrations	*Methodology*	*References*
Av. 180 pmol/L (243 pg/ml). R. 70-399 pmol/L (94-538 pg/ml)	RIA on plasma. No mention of any extraction techniques.	Nilsson *et al.*, 1975.
Control Av. 41±7 pg/ml. Hospital patient R. < 20 to 1200 pg/ml.	RIA on plasma. No mention of any extraction techniques.	Skrabanek *et al.*, 1976.
Male Av. 298 pg/ml. R. 120-840 pg/ml. Female Av. 251 pg/ml. R. 70-570 pg/ml.	RIA on plasma. No mention of any extraction techniques.	Yanaihara *et al.*, 1976.
Control R.15-280 pg/ml. Sleep disorder R. 2-100 pg/ml.(Taken from graphs).	RIA on plasma. No mention of any extraction techniques.	Oehme *et al.*, 1982.
Control Av. 45.4 ± 7.18 pg/ml. Patients with hypertension Av.13.6 ± 2.30 pg/ml.	RIA on plasma. No mention of any extraction techniques.	Faulhaber *et al.*, 1983.
Control Av. 13±2 fmol /ml. (17.5±2.7 pg/ml). Patients with hepatic coma 119±13 fmol/ml. (160±17.5 pg/ml).	Plasma extracted with acid acetone followed up with an RIA.	Hortnagl *et al.*, 1984.
Below detection. < 10 fmol/ml. (< 13.5pg/ml).	Sep-Pak extraction followed up with an RIA.	Conlon *et al.*, 1985.
Approximate Av. 1.0 pmol/L. (1.3 pg/ml). R. 0.1-4.0 pmol/L (0.1-5.4 pg/ml). (Taken from graphs).	RIA on serum. No mention of any extraction techniques.	Thysell *et al.*, 1988.
Controls 38.3 ± 7.7 pg/ml. Pre-term infants 27.2 ± 6.2 pg/ml.	RIA on plasma. No mention of any extraction techniques.	Scholle *et al.*, 1990.
Av. 2.2±1.1 pmol/L (3.0±1.5 pg/ml).	Sep-Pak extraction followed up with an enzyme immunoassay.	Takeyama *et al.*, 1990.
CHF 2.28 ± 0.30 pmol/L (3.1 ± 0.4 pg/ml). CHF and on ACE-I 4.05 ± 0.79 pmol/L (5.5 ± 1.1 pg/ml).	RIA on plasma. No mention of any extraction techniques.	Valdemarsson *et al.*, 1991.

Table 1.6. An overview of the range of SP plasma concentrations and sampling techniques which have been reported. Note; Av.=average; R.=range; RIA=radioimmunoassay; CHF=severe congestive heart failure; ACE-I=ACE-inhibitor treatment.

Aims of the present study;

1. To determine a reliable and accurate method for quantifying substance P in human plasma with specific reference to:

i) its metabolism/ degradation

ii) its interaction with other plasma components, especially metabolites of the parent peptide.

2. To examine whether the presence of substance P fragments affects the determination of the levels of the parent peptide.

3. To determine whether metabolic fragments of substance P are present in human plasma and whether these fragments have significant biological activity.

4. To apply the method for quantifying substance P in human plasma to animal tissue samples (namely SHR and WKY samples).

5. To determine and compare substance P levels (and metabolite levels if appropriate) in various selected tissues of the hypertensive SHR and normotensive WKY.

CHAPTER 2

PROPERTIES OF A RADIOIMMUNOASSAY (RIA) METHOD FOR THE DETERMINATION OF SUBSTANCE P.

2.1 BACKGROUND AND AIMS

Detection and quantification of endogenous peptides may be achieved by many diverse methods. In fact the physiological actions of substance P were discovered in a bioassay system designed to test for acetylcholine (von Euler and Gaddum, 1931). Other bioassay systems have since been employed to determine the concentration of substance P, with most exploiting the contractile effect of this peptide on guinea pig ileum (Bury and Mashford, 1977; Lembeck *et al.*, 1978; Couture and Regoli, 1981). Although this is an acknowledged and effective method for measuring the level of substance P present in the system, the downfall of this technique is the lack of sensitivity. High-performance liquid chromatography (HPLC) has also been used to quantify substance P, but is not sensitive enough to determine the physiological concentrations expected in plasma and tissue samples (Lembeck *et al.*, 1985; Ohno, *et al.*, 1989).

Other techniques developed to detect extremely low concentrations of substance P include a competitive enzyme-linked immunoassay (Takeyama *et al.*, 1993; Watanabe *et al.*, 1993) and cell immunoblot assay (Arita *et al.*, 1993). However, these techniques have only become available during the closing phases of this study.

The most frequently cited technique to detect substance P in various physiological fluids and tissue samples is the radioimmunoassay (RIA). Examples include the detection of substance P in human cerebrospinal fluid (Cramer *et al.*, 1988; Cramer *et al.*, 1989; Higa *et al.*, 1989), human plasma (Lundin *et al.*, 1988; Reynolds *et al.*, 1988; Tonnesen *et al.*, 1988), human saliva (Parris *et al.*, 1990), human ocular aqueous humor (Kieselbach *et al.*, 1993), human rectal mucosa (Lysy *et al.*, 1993), human synovial fluid (Menkes *et al.*, 1993) as well as in the human and

animal nervous system (Cooper *et al.*, 1987; Rao *et al.*, 1993; Singh *et al.*, 1993; Wilson *et al.*, 1993).

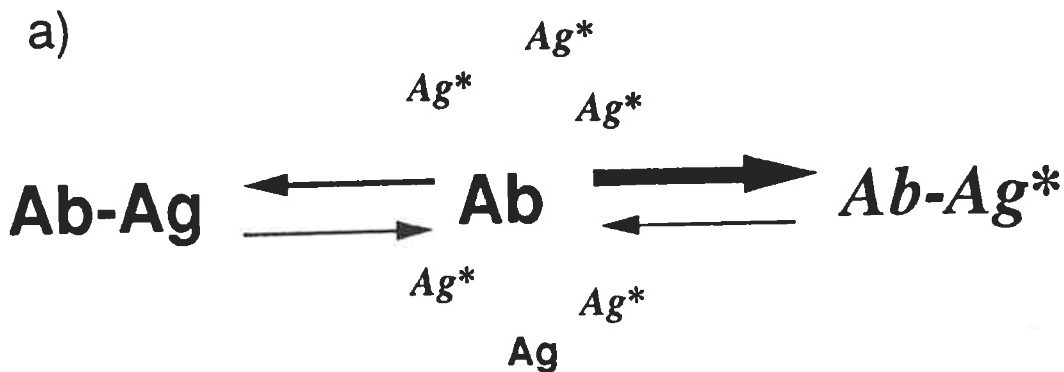
RIAs are often chosen as the technique of choice due to their a) high level of sensitivity, b) specificity for the particular peptide or hormone (dependent upon the choice of antibody) and c) relative ease and economy.

RIAs are competitive protein binding assays. They exploit the competition for a specific antibody occupancy site between a known concentration of radiolabelled peptide (the tracer) and the unknown concentration of non-radioactive peptide (the sample). Low concentrations of non-radioactive peptide (sample) result in high concentrations of the radiolabelled peptide (tracer) binding to the antibody. This in turn yields a large number of radioactive antibody-antigen complexes which can be separated from the solution and the resultant high level of radioactivity determined (Fig 2.1a). Conversely, high concentrations of non-radioactive peptide (sample) compete for the antibody site and displace the radiolabelled form of the peptide (tracer). This results in fewer radioactive antibody-antigen complexes forming, which after separating from the solution, yields a lower radioactive count (Fig 2.1b).

Once a standard curve has been produced (using known concentrations of unlabelled substance P), the subsequent level of radioactivity in the antigen-antibody complex formed from samples of unknown concentration may be converted to the corresponding substance P concentration via the standard curve.

A basic RIA technique to measure substance P in plasma had been developed by Mark Mano (CSIRO, Division of Human Nutrition, Adelaide) but still required minor refinements. Thus, the aim of this section of the project was to optimize conditions to produce a RIA technique which was most sensitive for concentrations of substance P in the 10-100 pg range (the expected sensitivity required for the physiological samples). This included determining the optimum concentration of antibody, selecting the most appropriate radiolabel (tracer) and examining the cross-reactivity of the antibody for metabolites of substance P and related peptides.

a)



b)

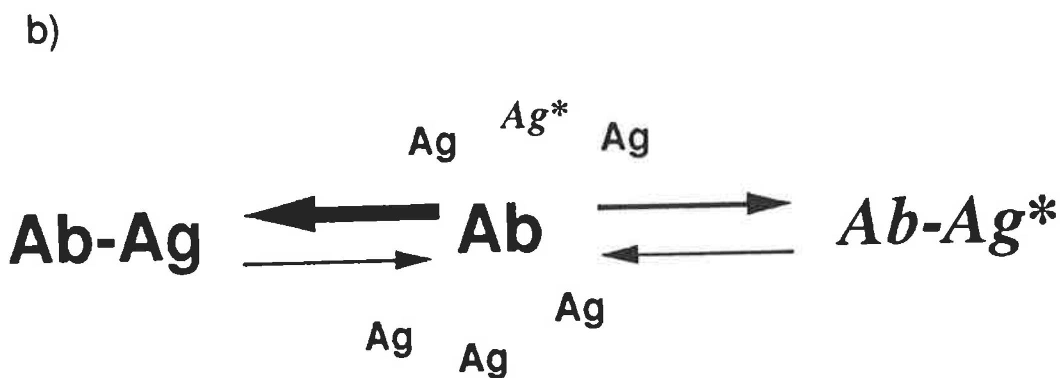


Fig. 2.1. Schematic representation of antigen (peptide)-antibody complex formation for a) low concentration of sample and b) high concentration of sample. It is the amount of Ab-Ag* complex formed from which the level of radioactivity is determined.

Note: Ab= antibody; Ag= peptide (sample);

Ag*= radiolabelled peptide (tracer).

2.2 MATERIALS AND METHODS

2.2.1 Materials and Chemicals.

2.2.1.i Materials and Chemicals for the Basic RIA.

Sodium chloride, acetic acid and di-sodium hydrogen orthophospate dodecahydrate ($Na_2HPO_4.12H_2O$) (Ajax Chemicals, Sydney, Australia). Substance P (triacetate), bacitracin (53500 U/g) and ethylenediaminetetraacetic acid (EDTA, di-sodium) (Sigma, Mo., U.S.A). Thiomersal (BDH Chemicals, Poole, England). Bovine serum albumin (BSA; Boehringer Fraction V, No. 735108). Substance P antibody (Auspep, Melbourne, Australia). Normal rabbit serum and anti-rabbit immunoglobulin (raised in sheep) (Silenus, Sydney, Australia). [^{125}I-Tyr^8]-Substance P (NEN, DuPont, C.A., U.S.A). Polyethylene glycol 6,000 (PEG) and 5ml polystyrene tubes (Lab Supply, Adelaide, Australia).

2.2.1.ii Additional Materials and Chemicals used in the Work Described in this Chapter.

Substance-P,[2-L-Prolyl-3,4-^{3}H(N)]- (*i.e.*, ^{3}H-Substance P; NEN, DuPont, C.A., U.S.A).

Tyr^8-substance P, nona-substance P (SP 3-11), octa-substance P (SP 4-11), hepta-substance P (SP 5-11), hexa-substance P (SP 6-11), penta-substance P (SP 7-11), substance P (1-4), substance P (1-6), substance P (1-7) and substance P (1-9) (Peninsula Laboratories, C.A., U.S.A).

Methionine enkephalin, leucine enkephalin, neurokinin A, neurokinin A fragment 4-10, neuropeptide K (porcine), neuropeptide Y (human) and angiotensin II ([Val^5]-angiotensin II) (Sigma, Mo., U.S.A) and bradykinin (Auspep, Melbourne, Australia). Scintillant (see *Appendix III*) and 5ml scintillation vials (Packard, Netherlands).

2.2.2 The Basic RIA Method.

Substance P RIA standards (aliquots of 100µl of 100µg/ml SP in 1% acetic acid (w/v)) were prepared in bulk and stored at -80 ^{O}C until required. Substance P concentrations for the standard curve were 0, 2, 5, 10, 20, 100, 200, 500 and 1,000 pg. Dilutions of the frozen standard to construct the standard curve have been summarized in *Appendix I*. *Appendix I* also gives details of the preparation of double strength assay buffer (DSAB), single strength assay buffer (SSAB), diluent buffer (DB), substance P antibody (Ab), normal rabbit serum (NRS), sheep anti-rabbit immunoglobulin (SARS), polyethylene glycol (PEG) and tracer (^{125}I-SP).

Each RIA run comprised the following basic design; total counts (TC) in triplicate; blanks in triplicate, standard curve (0-1,000 pg SP) in triplicate and unknowns (or samples) in duplicate.

Prior to the commencement of the RIA, any lyophilized samples were redissolved by adding 250µl of 1% acetic acid (v/v), vortexing and waiting 30 mins. Then 250µl DSAB was added, the solution vortexed again and briefly centrifuged (approximately 1,000-1,500G for around 5 minutes) before incorporating the unknowns into the RIA.

All RIAs were performed at between 2-5 ^{O}C (to promote formation of the antibody-antigen complex) in 5ml polystyrene tubes. Firstly, the blanks (100µl SSAB and 200µl DB) and standard curve were produced as outlined in *Appendix I*. Then 200µl of sample was added to each unknown tube. This was followed by the addition of 100µl of the diluted antibody (Ab; 1:50,000 dilution in SSAB) to the standard curve and unknown tubes only. Antibody was NOT added to the total count or blank tubes. Finally, 100µl of diluted tracer (^{125}I-SP; see *Appendix I*) was added to all RIA tubes before they were vortexed and incubated overnight at 4°C.

On the subsequent day, 100µl NRS (1:100 in SSAB) and 100µl SARS (1:25 in SSAB, see *Appendix I*) were added to all RIA tubes, which were then vortexed and incubated for at least 30 mins. 1.5ml of 5.5% PEG (see *Appendix I*) was later added to each tube with further vortexing followed by an incubation for at least 10 mins.

These compounds (NRS, SARS and PEG) bind to the antibody-antigen complex to increase its mass and allow it to form the pellet during centrifugation. The RIA tubes were then centrifuged (at 3,500 G for 20 mins. at $4^{O}C$) followed by immediate aspiration of the supernatant whilst taking care not to disturb the pellet. The radioactive content of the pellet was then determined using an LKB Wallac 1261 Multigamma γ-counter (Turku, Finland; counting efficiency 75%).

2.2.3 Determination of Optimum Antibody Concentration.

Standard curves (N=5) were obtained for three different concentrations of antibody (Ab). These were 1:50,000, 1:10,000 and 1:5,000 dilutions of the Ab. The tracer used for the determination of optimum Ab concentration was ^{125}I-SP. The standard curve consisted of triplicates of the following substance P concentrations; 0, 2, 5, 10, 20, 100, 200, 500 and 1,000 pg of SP. No unknowns were assayed during these runs. γ-radioactivity contained within the pellets was determined directly using an LKB Wallac 1261 γ-counter (as per the basic RIA method).

Results have been represented as the resultant standard curves obtained at each Ab dilution; *i.e.*, plots of substance P (pg) versus the bound radiolabelled form of the peptide (expressed as mean $\pm$ SEM cpm).

The selection of optimum Ab concentration was then based upon the concentration which yielded the most linear portion and steepest gradient of the standard curve between 10pg and 100pg SP (the expected physiological concentrations of SP).

2.2.4 Determination of Choice of Radiolabel; ^{125}I-Substance P versus ^{3}H-Substance P.

As two radioisotopes of substance P were commercially available for use as the tracer, both were examined to determine the best option for use in the current RIA. Hence, [^{125}I-Tyr^{8}]-substance P (^{125}I-SP) and substance-P,[2-L-Prolyl-3,4-^{3}H(N)]- (^{3}H-SP) (NEN, DuPont, U.S.A) were employed as the tracer for the standard curves which were run in parallel.

A total of three standard curves were obtained with each tracer (^{125}I-SP and ^{3}H-SP) using a different concentration of Ab (1:50,000, 1:10,000 or 1:5,000) each time. The standard curve consisted of triplicates of the following substance P concentrations; 0, 10, 100, 1,000 and 10,000 pg of SP. No unknowns were assayed during these runs.

γ-radioactivity contained within the pellets was determined directly for ^{125}I-SP tubes using an LKB Wallac 1261 γ-counter (as per the basic RIA method). Tritiated pellets (*i.e.*, those with ^{3}H-SP) were washed three times each (with vortexing) with 150μl glass distilled water (GDW), with the cumulative washes collected in 5ml scintillation vials. Scintillant (4.5ml) was added to each vial and mixed well before determination of β-radioactivity in an LKB Wallac 1218 Rackbeta Liquid Scintillation Counter (β-radiation counting efficiency was 30%).

Results have been represented as the resultant standard curves obtained from each tracer (^{125}I-SP and ^{3}H-SP) at each Ab dilution; *i.e.*, plots of substance P (pg) versus the bound radiolabelled form of the peptide (expressed as cpm).

Selection of the better tracer would be dependent upon performance in the standard curve as well as additional factors such as shelf-life and ease of measurement of radioactive content.

2.2.5 Antibody Affinity for Substance P, Its Fragments and Related Peptides.

The assessment of antibody affinity (or cross-reactivity) was determined by substituting SP with the compound of interest (*i.e.*, SP 4-11 or methionine-enkephalin) in the construction of the standard curve. Thus, the standard curve would consist of 0, 10, 100, 1,000 and 10,000pg of SP 4-11 or methionine-enkephalin. The 1:50,000 dilution of Ab was used and ^{125}I-SP was employed as the tracer.

Peptides tested for their Ab affinity (due to their amino acid sequence homology with SP) were: Tyr^{8}-substance P (Tyr^{8}-SP), nona-substance P (SP 3-11), octa-substance P (SP 4-11), hepta-substance P (SP 5-11), hexa-substance P (SP 6-11),

penta-substance P (SP 7-11), substance P (1-4), substance P (1-6), substance P (1-7), substance P (1-9), methionine enkephalin (Met-Enk), leucine enkephalin (Leu-Enk), neurokinin A (NKA), neurokinin A fragment 4-10 (NKA 4-10), neuropeptide K (NPK), neuropeptide Y (NPY) and angiotensin II (AII) and bradykinin (BK).

Results have been shown as standard curves for the representative peptide compared with a standard curve for SP which was obtained at the same time. (Note; standard curves have been plotted as "Substance P" (pg) [*i.e.*, the representative peptide in the particular assay (pg)] against bound ^{125}I-SP (percent cpm) [*i.e.*, the maximum cpm obtained at 0pg of peptide is equivalent to 100% cpm since absolute cpm at 0pg varied slightly between RIAs due to minor differences in added concentration of tracer, age of tracer, *etc.*].

2.3 RESULTS

2.3.1 Determination of Optimum Antibody Concentration.

Figure 2.2 displays the standard curves obtained when ^{125}I-SP was used as the tracer for the determination of the optimum Ab concentration. The exact values obtained from the RIA have been listed in Table 2.1 (next page). The 1:50,000 Ab dilution yielded a good "reverse S-shaped" standard curve, while the 1:10,000 and 1:5,000 Ab dilutions yielded flat lines between the 0 to 100pg SP range followed by a linear decrease in cpm from the 100pg SP concentration onwards.

As the 1:50,000 Ab dilution generated the most linear portion and steepest gradient between the 10pg and 100pg SP concentrations, it was the Ab concentration of choice. The gradient is an important component of the standard curve, as it enables better distinction between two very similar concentrations of SP samples; *i.e.*, a steep gradient may enable a clear distinction between 20pg and 22pg SP whilst a standard curve with a flatter gradient may be unable to clearly discriminate between these two concentrations.

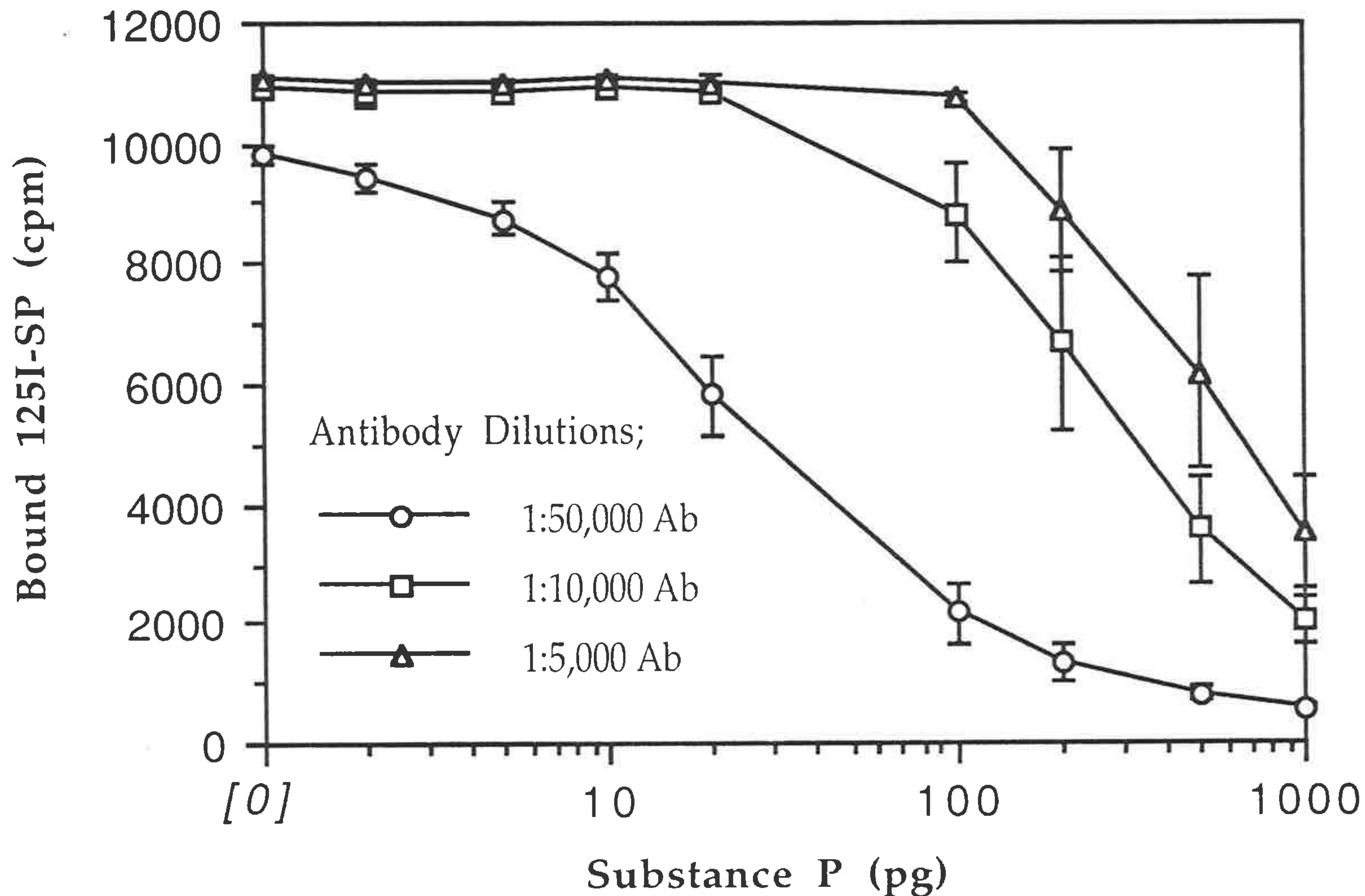


Fig. 2.2. Radioimmunoassay (RIA) standard curves using different dilutions of antibody (Ab).

Standards were prepared for concentrations of substance P (SP) ranging between 0 pg and 1,000 pg. The RIA was performed in the usual manner with one set of SP standards using the 1:50,000 dilution of Ab (N=5), one set using 1:10,000 Ab (N=5) and the last set using the 1:5,000 dilution of Ab (N=5). The tracer was ^{125}I-SP for all of the Ab dilutions. The figure shows the relationship between the radioactive content (bound ^{125}I-SP [mean ± SEM cpm]) of the final pellet to the concentration of SP (pg) for each of the Ab dilutions tested.

SP (pg)	1:50,000 Ab	1:10,000 Ab	1:5,000 Ab
0	9.80 ± 0.18	10.93 ± 0.16	11.10 ± 0.08
2	9.44 ± 0.23	10.82 ± 0.20	10.99 ± 0.08
5	8.76 ± 0.24	10.81 ± 0.11	10.96 ± 0.10
10	7.79 ± 0.41	10.88 ± 0.12	11.03 ± 1.18
20	5.82 ± 0.68	10.84 ± 0.10	11.02 ± 0.13
100	2.16 ± 0.53	8.83 ± 0.82	10.75 ± 0.10
200	1.32 ± 0.28	6.68 ± 1.46	8.87 ± 1.03
500	0.80 ± 0.10	3.56 ± 0.92	6.19 ± 1.6
1,000	0.58 ± 0.06	2.03 ± 0.42	3.47 ± 0.93

Table 2.1. RIA standard curve determinations for the different concentrations of Ab. Results have been expressed as the mean ± SEM cpm (N=5).

2.3.2 Determination of Choice of Radiolabel; ^{125}I-Substance P versus ^{3}H-Substance P.

Figure 2.3 displays the standard curves obtained when ^{125}I-SP was used as the tracer at the varying Ab concentrations. Both the 1:10,000 and 1:5,000 Ab dilutions yielded good "reverse S-shaped" standard curves, whilst the 1:50,000 dilution yielded a comparatively flat standard curve. However, the 1:10,000 and 1:5,000 Ab dilutions did display a linear and sharp gradient over the 10 to 100 pg SP range (Fig.2.3). (The difference observed with Figures 2.2 and 2.3 is discussed in section 2.4).

The standard curves generated with ^{3}H-SP at the various Ab concentrations have been shown in Figure 2.4. The curves using ^{3}H-SP as the tracer (Fig. 2.4) were much flatter than those obtained with ^{125}I-SP (Fig. 2.3) and

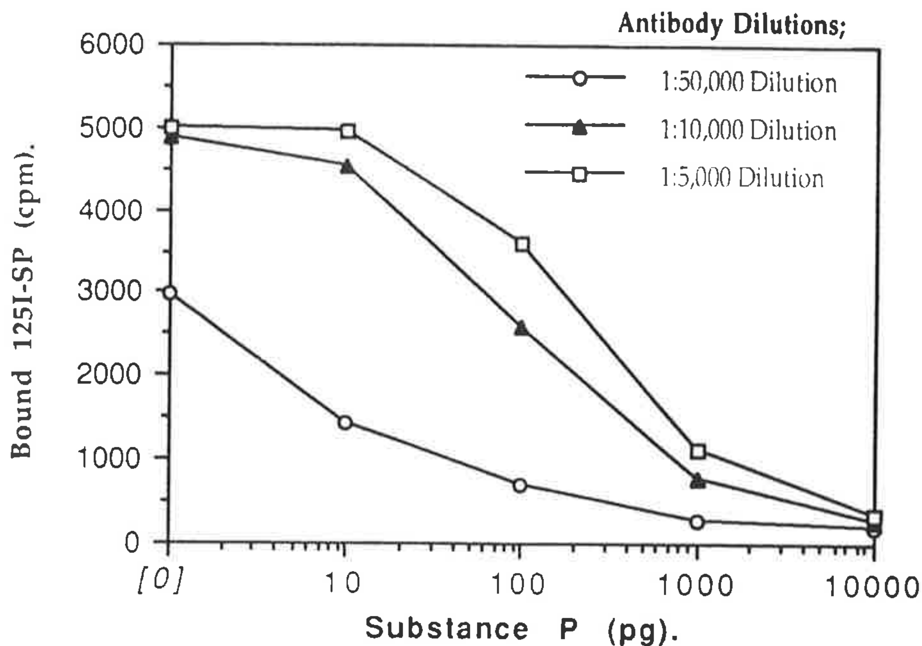


Fig. 2.3. Radioimmunoassay (RIA) standard curves using ^{125}I-SP as the tracer and different dilutions of antibody (Ab).

Standards were prepared for concentrations of substance P (SP) ranging between 0 pg and 1,000 pg. The RIA was performed in the usual manner using ^{125}I-SP as the tracer and each of the Ab dilutions (1:50,000 Ab, 1:10,000 Ab and 1:5,000 Ab) were used on one set of the SP standards. The figure shows the relationship between the radioactive content of the final pellet (bound ^{125}I-SP [cpm]) to the concentration of SP (pg) for each of the Ab dilutions tested (N=1).

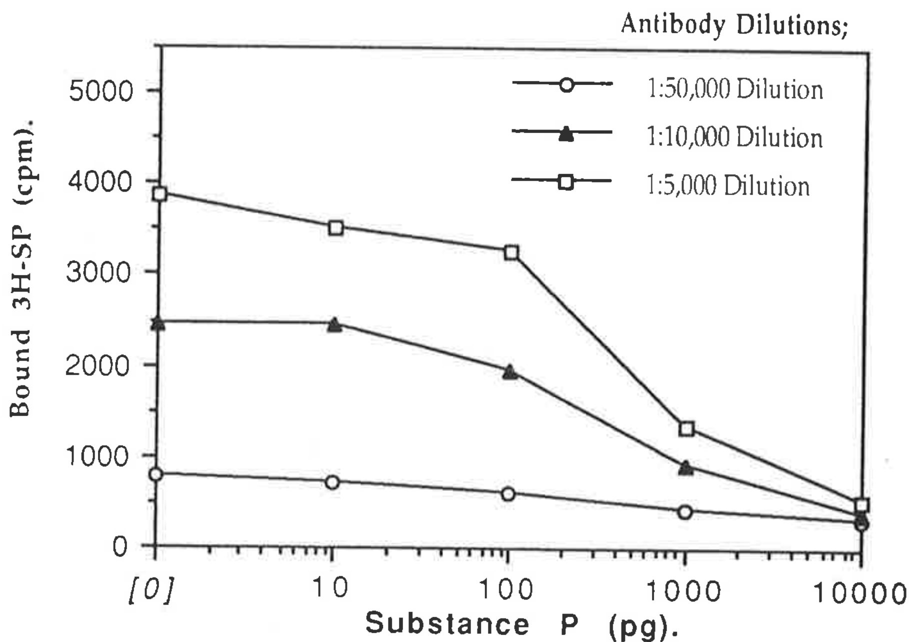


Fig. 2.4. Radioimmunoassay (RIA) standard curves using ^{3}H-SP as the tracer and different dilutions of antibody (Ab).

Standards were prepared for concentrations of substance P (SP) ranging between 0 pg and 1,000 pg. The RIA was performed in the usual manner using ^{3}H-SP as the tracer and each of the Ab dilutions (1:50,000 Ab, 1:10,000 Ab and 1:5,000 Ab) were used on one set of the SP standards. The figure shows the relationship between the radioactive content of the final pellet (bound ^{3}H-SP [cpm]) to the concentration of SP (pg) for each of the Ab dilutions tested (N=1).

none of the tested concentrations of Ab resulted in a standard curve which was sensitive to SP within the requisite 10pg to 100pg concentration range.

2.3.3 Antibody Affinity for Substance P, Its Fragments and Related Peptides.

Only four of the eighteen peptides assessed for affinity (or cross-reactivity) with the Ab exhibited a significant binding potential (see Fig. 2.5). One of these was a substitution analogue (Tyr^{8}-SP) where the phenylalanine in position eight has been replaced with tyrosine, whilst the remaining trio consisted of the C-terminal fragments of SP (SP 3-11, 4-11 and 5-11).

Tyr^{8}-SP generated a curve which very closely resembled that of the parent peptide, almost overlapping the SP curve (Fig. 2.5). The C-terminal fragments also followed the curve generated from SP, although the shorter the fragment the lesser binding affinity for the Ab (Fig. 2.5).

The remaining fourteen peptides displayed little or no significant affinity for the SP Ab (Fig.2.6a and 2.6b). These peptides may be separated into two categories; a) fragments of SP and b) related peptides. The fragments which had no relative affinity for the SP Ab were the C-terminal fragments SP 6-11 and 7-11 and the N-terminal fragments SP 1-4, 1-6, 1-7 and 1-9 (Fig. 2.6a). All the related peptides which were examined were found to be unresponsive towards the SP Ab. They were Met-Enk, Leu-Enk, NKA, NKA 4-10, NPK, NPY, AII and BK (Fig. 2.6b).

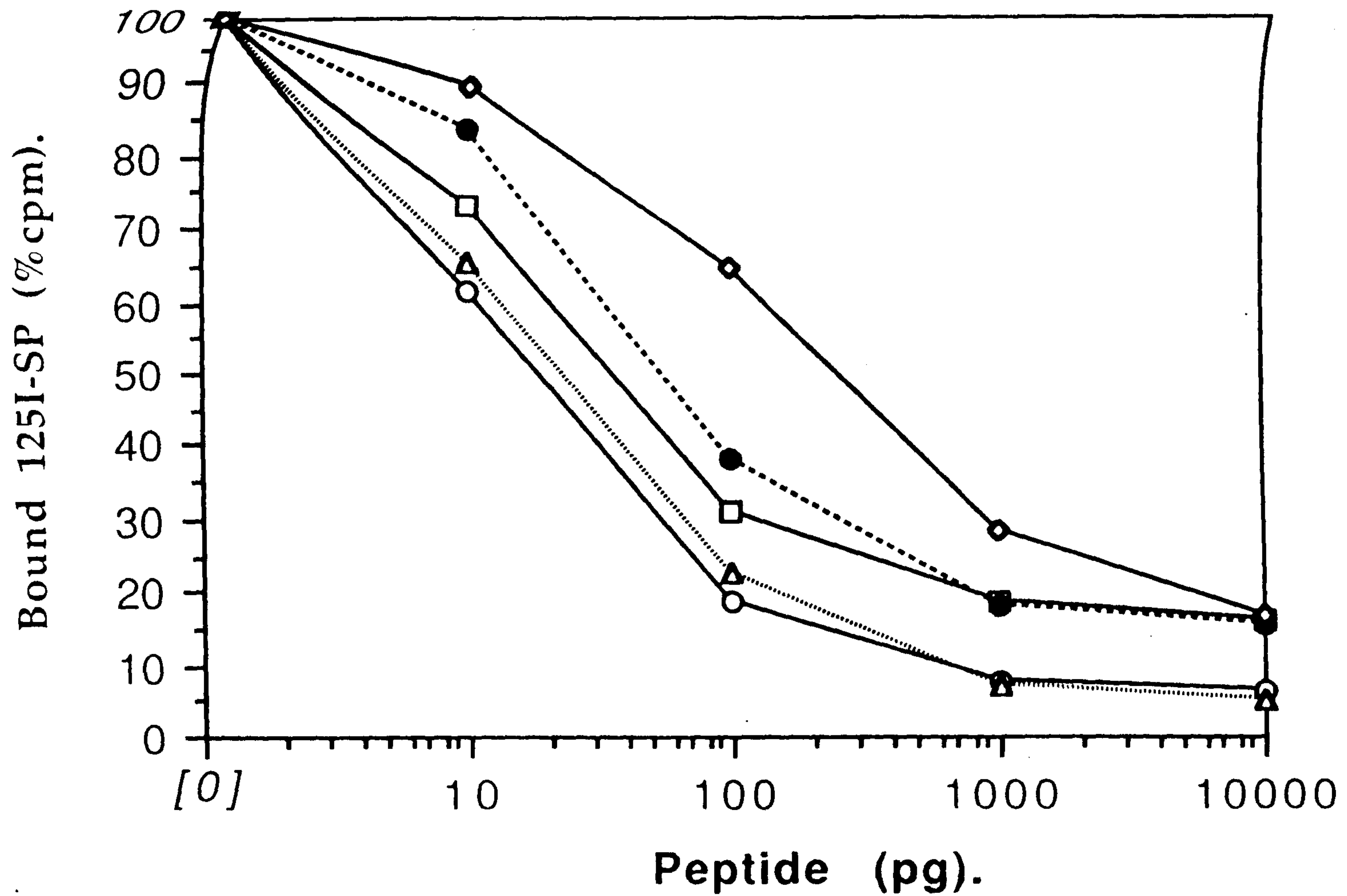


Fig. 2.5. Radioimmunoassay (RIA) standard curves of peptides with affinity for the antibody (Ab).

Standards were prepared for substance P, tyrosine^{8}-SP (Tyr^{8}-SP), nona-substance P (SP 3-11), octa-substance P (SP 4-11) and hepta-substance P (SP 5-11) within the concentration range of 0 pg to 10,000 pg. The RIA was then performed in the usual manner using ^{125}I-SP as the tracer and the 1:50,000 dilution of Ab (N=1 for each peptide). The figure shows the relationship between the radioactive content of the final pellet (bound ^{125}I-SP [%cpm]) to the concentration of the peptide (pg).

Legend; Substance P ——○——, Tyr^{8}-SP ·········▲·········, SP 3-11 ——□——, SP 4-11 - - - - -●- - - - - and SP 5-11 ——◇——.

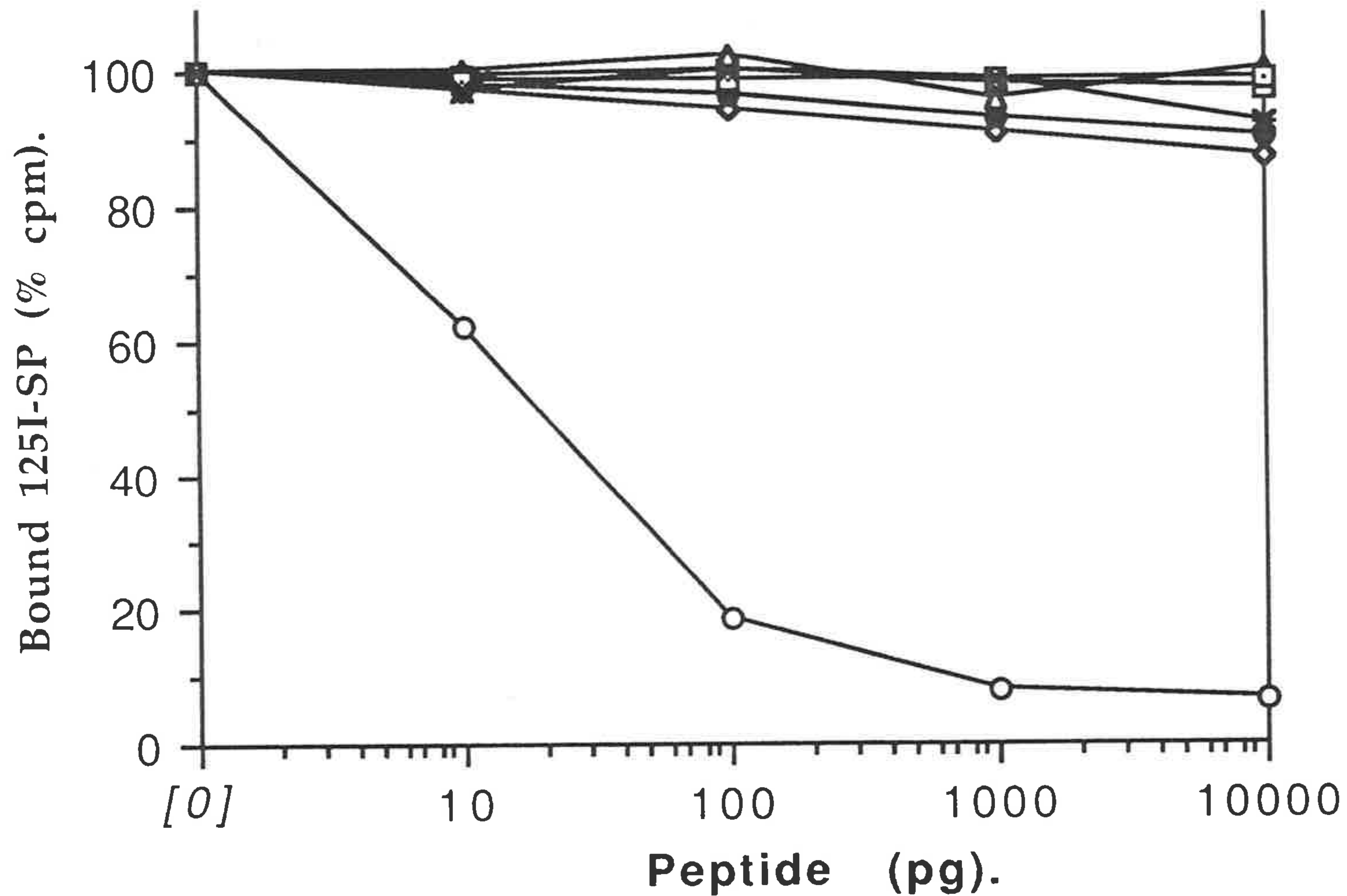


Fig. 2.6.a. Radioimmunoassay (RIA) standard curves of fragments of substance P with little or no affinity for the antibody (Ab).

Standards were prepared for substance P, hexa-substance P (SP 6-11), penta-substance P (SP 7-11), SP 1-4, SP 1-6, SP 1-7 and SP 1-9, within the concentration range of 0 pg to 10,000 pg. The RIA was then performed in the usual manner using ^{125}I-SP as the tracer and the 1:50,000 dilution of Ab (N=1 for each peptide). The figure shows the relationship between the radioactive content of the final pellet (bound ^{125}I-SP [%cpm]) to the concentration of the peptide (pg).

Legend; Substance P —o—, SP 6-11 —◇—, SP 7-11 —●—, SP 1-4 —□—, SP 1-6 —▼—, SP 1-7 —▣— and SP 1-9 —✱— .

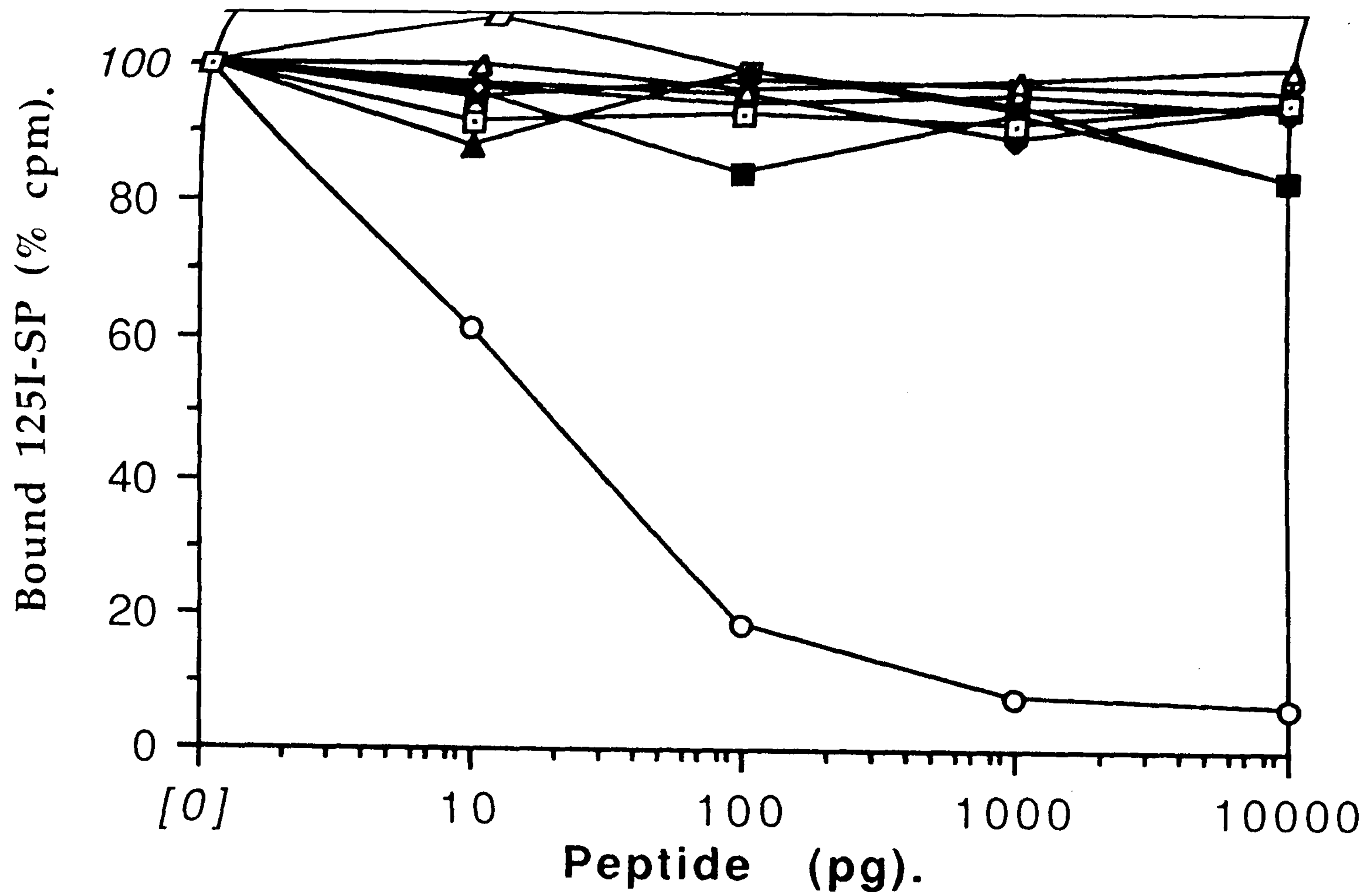


Fig. 2.6.b. Radioimmunoassay (RIA) standard curves of peptides with little or no affinity for the antibody (Ab).

Standards were prepared for substance P, neuropeptide K (NPK), neuropeptide Y (NPY), neurokinin A (NKA), neurokinin A 4-10 (NKA 4-10), bradykinin (BK), methionine enkephalin (Met-Enk), leucine enkephalin (Leu-Enk) and angiotensin II (AII), within the concentration range of 0 pg to 10,000 pg. The RIA was then performed in the usual manner using ^{125}I-SP as the tracer and the 1:50,000 dilution of Ab (N=1 for each peptide). The figure shows the relationship between the radioactive content of the final pellet (bound ^{125}I-SP [%cpm]) to the concentration of the peptide (pg).

Legend; Substance P —○— , NPK —◆— , NPY —✱— ,NKA —□— , NKA 4-10 —■— , BK —◇— , Met-Enk —▲— , Leu-Enk —△— and AII —▣— .

2.4 DISCUSSION

RIAs have often been the technique of choice for quantifying physiological concentrations of endogenous peptides and hormones, including SP (Cooper *et al.*, 1987; Cramer *et al.*, 1988; Reynolds *et al.*, 1988; Higa *et al.*, 1989; Parris *et al.*, 1990; Kieselbach *et al.*, 1993; Rao *et al.*, 1993; Wilson *et al.*, 1993). Mark Mano (CSIRO Division of Human Nutrition, Adelaide) had developed a RIA technique to measure SP in plasma and tissues. However, minor refinements were required to improve the sensitivity in the 10pg to 100pg SP range. This necessitated the determination of optimum Ab concentration, selection of the best tracer as well as discerning the selectivity of the Ab for SP.

Studies to determine the optimum concentration of Ab to use for the RIA revealed that the 1:50,000 Ab dilution was the best choice. This was the only Ab dilution tested which displayed the stereotypical reversed-S shaped curve, as well as containing the steepest linear portion bewteen the 10 to 100 pg SP range (Fig.2.2). This SP concentration was selected to represent the most likely range of endogenous SP concentrations. Thus, all of the subsequent RIAs which were performed in this body of work have used the 1:50,000 Ab dilution.

An interesting exercise was to condense the data points of fifty RIA standard curves obtained with the 1:50,000 dilution of Ab immediately following the experiments detailed in this chapter. It was shown that this concentration of Ab was sufficiently sensitive within the 10pg to 100pg SP range (Fig. 2.7). This dilution consistently yielded a good reversed S-shaped curve, with the most linear portion and steepest gradient occurring within the essential 10pg to 100pg SP range (the 5pg to 100pg SP range also fulfilled these criteria).

Figure 2.2 and Figure 2.3 have used the same tracer and the same Ab dilutions but with slightly different results; *i.e.*, the 1:50,000 dilution was superior in the 10 to 100 pg range in Fig. 2.2 but not Fig. 2.3. However, a relatively flat 1:50,000 Ab curve was observed for both ^{125}I-SP and ^{3}H-SP (Figs. 2.3 and 2.4) and it is suspected that this may be due to an error in this Ab dilution for these parallel

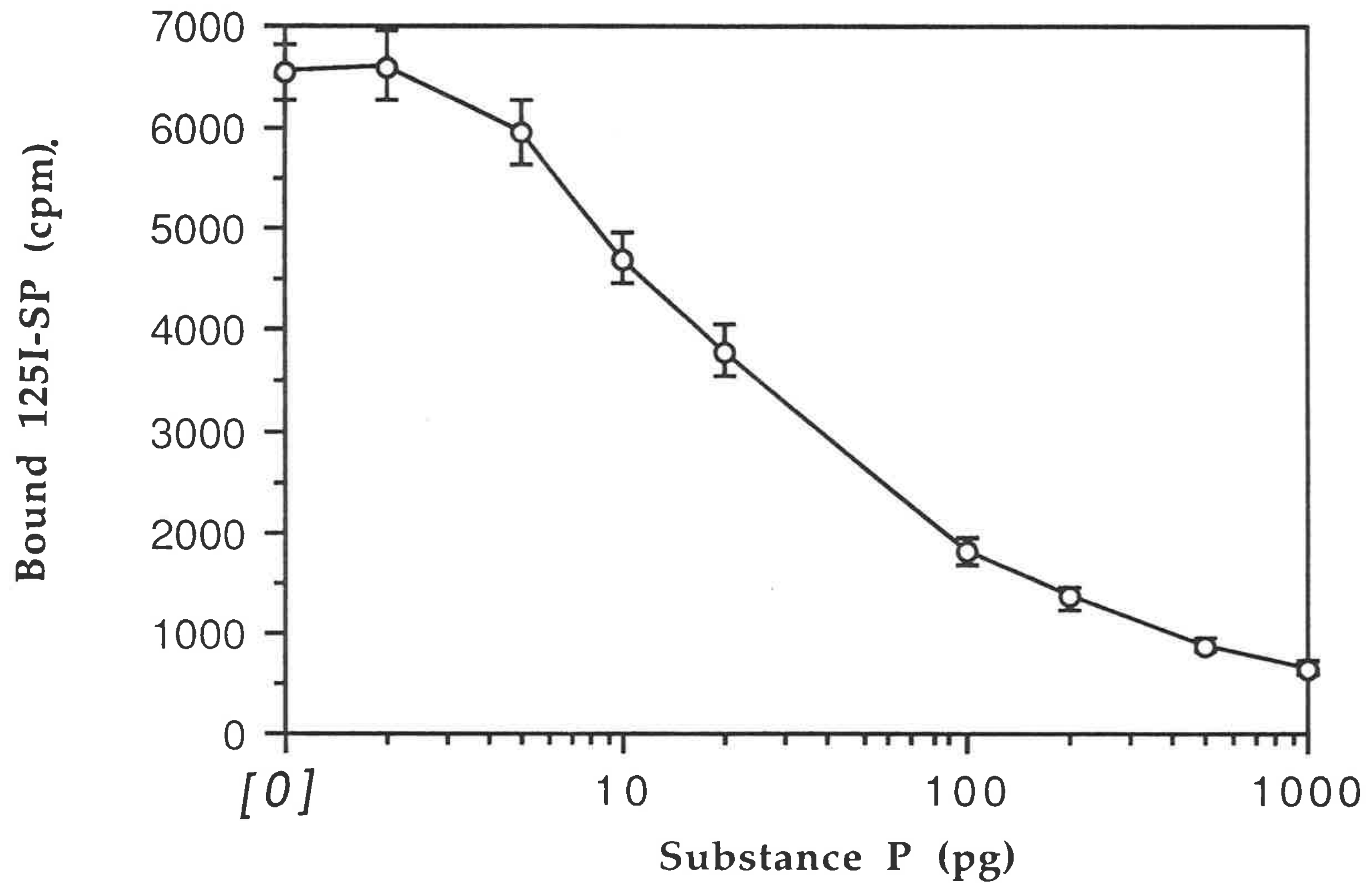


Fig. 2.7. The average standard curve obtained from fifty radioimmunoassays (RIAs).

The data points (i.e., the mean radioactive content [cpm] for each substance P standard concentration) of fifty RIAs performed during the course of this work were pooled and the mean and SEM of each data point was determined. This figure shows the standard curve (mean ± SEM) obtained from such an exercise (N=50).

experiments. These 1:50,000 Ab results (Figs. 2.3 and 2.4) did not affect the selection of optimum tracer or optimum Ab concentration.

With regard to the selection of the appropriate tracer, it was obvious when comparing standard curves obtained with ^{125}I-SP (Fig 2.3) with those using ^{3}H-SP (Fig. 2.4) that the former resulted in curves which were both more linear and possessed a steeper gradient within the 10pg to 100pg SP range. In addition to this, determination of γ-radiation (emitted by ^{125}I) may be obtained directly from the pellet acquired at the end of the RIA (with 75% efficiency), whilst detection of β-radiation (emitted by ^{3}H) requires the pellets to be redissolved, transferred to scintillation vials and be thoroughly mixed with scintillant before counting can occur. The counting efficiency of β-radiation was then only 30%. The only domain in which ^{3}H-SP may be considered to be superior to ^{125}I-SP was that of half-life. Tritium has a half-life of approximately 12 years, whereas ^{125}I has a half-life of only 60 days.

In overview, ^{125}I-SP yielded more suitable standard curves for this purpose than its rival, ^{3}H-SP and quantification of γ-radiation could be made directly. Consequently, ^{125}I-SP was the tracer of choice and was subsequently used throughout the course of this study for the SP RIA.

The final consideration was the selectivity (cross-reactivity) of the SP Ab for fragments of the parent peptide or related peptides. Proprietary literature cites the cross-reactivity of the SP Ab as substance P (100%), eledoisin (0.1%), kassinin (<0.1%), neurokinin A (<0.01%), neurokinin B (<0.01%) and physalaemin (<0.01%) (Auspep Product Catalogue, 1992/1993). The cross-reactivity of the Ab for fragments of SP was not mentioned in the Auspep catalogue. The Auspep catalogue does refer to a paper by Morris and associates (Morris *et al.*, 1986) regarding the specifications of this particular SP Ab (RMSP-1), although no fragment cross-reactivity studies were performed. Yet, since it was proposed to quantify SP levels in tissues and plasma, it seemed quite likely that metabolites of the parent peptide (*i.e.*, fragments of SP) would be present in the samples. Thus, it

was imperative to deduce the cross-reactivity of the Ab for fragments of SP and other endogenous peptides which may interact with the Ab.

Only the C-terminal fragments SP 3-11, 4-11 and 5-11 (as well as Tyr^8-SP) possessed any substantial affinity for the Auspep SP Ab (Fig. 2.5). Interestingly, the C-terminal fragments SP 6-11 and 7-11 displayed little or no affinity for the Ab (Fig 2.6a). Additionally, not one of the N-terminal fragments (SP 1-4, 1-6, 1-7 and 1-9) demonstrated any affinity for the Ab (Fig. 2.6a).

The amino acid sequences of the fragments with affinity for the Ab and for those without affinity for the Ab are shown in Figure 2.8 and Figure 2.9 respectively. Curiously, the SP 5-11 fragment possesses a degree of affinity for the Ab whereas the fragment SP 6-11 has no obvious affinity whatsoever. This suggests that the fifth to eleventh amino acid sequence of SP is the minimum sequence homology requirement for binding to occur with this particular SP Ab. Yet, not one of the N-terminal fragments of SP, (not even SP 1-9), showed any affinity for the Ab (Fig 2.6a). Thus, an N-terminal fragment with nine amino acids in sequence with the total eleven amino acids of substance P (but lacking the concluding two amino acids, Fig. 2.9) still does not attain the required conformation to bind with the Ab. Consequently, it becomes apparent that the minimum sequence chain of amino acids from the structure of SP which are necessary for binding to this particular SP specific Ab constitutes the C-terminal fragment SP 5-11 (Fig. 2.8).

Extension of this fragment by adding extra SP amino acids (*i.e.*, SP 4-11 or 3-11) does appear to enhance the affinity of these C-terminal fragments for the peptide (Fig. 2.5). However, N-terminal fragments which lack the complete SP 5-11 amino acid sequence, such as SP 1-9 (Fig. 2.9) also appear to lack recognition with the Ab binding site (Fig. 2.6a). Yet, the substitution analogue Tyr^8-SP (Fig. 2.8) which carries a substitution within the seemingly important fifth to eleventh amino acid sequence (at position eight) almost mimics SP itself for affinity with the Ab (Fig. 2.5). Either this amino acid position is not very important for binding

Fig. 2.8. Amino acid sequence for peptides with affinity for the antibody.

Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	**SP 1-11**
Arg	Pro	Lys	Pro	Gln	Gln	Phe	Tyr	Gly	Leu	Met	Tyr^8 -SP
		Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	SP 3-11
			Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	SP 4-11
				Gln	Gln	Phe	Phe	Gly	Leu	Met	SP 5-11

Fig. 2.9. Amino acid sequence for fragments of SP 1-11 with little or no affinity for the antibody.

Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	**SP 1-11**
					Gln	Phe	Phe	Gly	Leu	Met	SP 6-11
						Phe	Phe	Gly	Leu	Met	SP 7-11
Arg	Pro	Lys	Pro	SP 1-4							
Arg	Pro	Lys	Pro	Gln	Gln	SP 1-6					
Arg	Pro	Lys	Pro	Gln	Gln	Phe	SP 1-7				
Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	SP 1-9		

Fig. 2.10. Amino acid sequence for peptides with little affinity for the antibody.

SP 1-11		**Arg**	**Pro**	**Lys**	**Pro**	**Gln**	**Gln**	**Phe**	**Phe**	**Gly**	**Leu**	**Met**
NPK ←		Arg		Lys				Phe		Gly	Leu	Met
NKA				Lys				Phe		Gly	Leu	Met
NKA 4-10								Phe		Gly	Leu	Met
BK		Arg	Pro						Phe			
NPY			Pro							Gly		
Met-Enk										Gly		Met
Leu-Enk										Gly		
AII	Asp	Arg						Phe				

Where SP 1-11 is substance P, NPK is the last 11 amino acids in neuropeptide K (the full amino acid sequence is given in Fig. 1.2a), NKA is neurokinin A, NKA 4-10 is the fragment neurokinin A 4-10, BK is bradykinin, NPY is neuropeptide Y , Met-Enk is methionine-enkephalin, Leu-Enk is leucine-enkephalin and AII is angiotensin II.

with the Ab, or the substitution of tyrosine for phenylalanine does not significantly alter the Ab recognition and binding properties.

It would be expected that none of the related peptides assessed for affinity with the Ab would bind to the SP Ab according to this apparent minimum sequence homology requirement for Ab recognition and binding to occur. This was in fact the case (Fig. 2.6b). Not one of these peptides contained the SP 5-11 amino acid sequence. The peptides with sequences which corresponded best with the SP 5-11 sequence were the other tachykinins which contained the common terminal sequence -Phe-X-Gly-Leu-Met, *i.e.*, NPK, NKA and NKA 4-10 (Fig. 2.10). NPK contained the greatest number of amino acids in sequence with SP (six in total), but these were scattered and not concentrated towards the influential terminal portion of the peptide (Fig. 2.10).

The remaining peptides, BK, NPY, Met-Enk, Leu-Enk and AII only incorporated one or two amino acids in sequence homology with SP (Fig. 2.10). This was not nearly enough to confer binding affinity with the SP Ab (Fig. 2.6b). Thus, none of the related peptides tested demonstrated any affinity for the SP Ab (Fig. 2.6b).

In conclusion, the most appropriate tracer and Ab dilution for the RIA were found to be ^{125}I-SP and the 1:50,000 dilution of Ab. These parameters have been adopted in the subsequent studies (unless otherwise specified). The Ab was not 100% specific for SP. Tyr^{8}-SP, SP 3-11, SP 4-11 and SP 5-11 all cross-reacted with the Ab to varying degrees, although Tyr^{8}-SP mimicked the binding potential of SP most closely. A generalization as to the binding affinity of the aforementioned C-terminal fragments tends to be that shorter fragments have less affinity for the Ab. Remaining C-terminal fragments (SP 6-11 and SP 7-11) and all tested N-terminal fragments (SP 1-4, SP 1-6, SP 1-7 and SP 1-9) displayed no cross-reactivity with the SP Ab. These data suggest the minimum amino acid sequence requirement for Ab recognition and binding to be those which are found within the SP 5-11 fragment. Finally, NPK, NPY, NKA, NKA 4-10, BK, Met-Enk, Leu-Enk and AII possessed no affinity for the Ab.

CHAPTER 3

EVALUATION OF AN ION EXCHANGE CHROMATOGRAPHIC ISOLATION PROCEDURE FOR SUBSTANCE P AND ITS FRAGMENTS.

3.1 BACKGROUND AND AIMS

It was noted in the *General Introduction* that there is little agreement in the literature regarding the endogenous plasma levels of substance P (*1.7.5 Aims of the Present Study*; Table 1.5). It seemed appropriate to consider a purification procedure for the samples (prior to their RIA determination) which would also reduce the cross-reactivity of other components normally found in the sample with the RIA antibody.

It was reported in the previous chapter (*Properties of a Radioimmunoassay Method for the Determination of Substance P*) that potential endogenously occurring fragments of substance P possess affinity for the antibody to SP in the RIA. As a consequence, it was particularly important to isolate SP from these fragments prior to RIA determination of the peptide. The most commonly used separation procedures for many peptides and proteins are the chromatographic techniques such as high-performance liquid chromatography (HPLC) and ion exchange chromatography (IEC) (Bergstrom *et al.*, 1983; Igwe *et al.*, 1988; Toresson *et al.*, 1988; Wallasch *et al.*, 1988; Martensson *et al.*, 1989; Ohno *et al.*, 1989; Cheung *et al.*, 1993; Rissler *et al.*, 1993).

Although the HPLC technique is a sensitive method which may be selectively manipulated to isolate very similar compounds, this procedure is sensitive only in the μg range. It was anticipated that the endogenous levels of substance P to be determined in the present study would be within the pg range (*i.e.*, one millionth of a μg) and therefore, the HPLC technique would not be suitable.

Ion exchange chromatography (IEC) is a process which can separate similar biological molecules from highly complex mixtures with high resolution and from low concentrations. An IEC procedure for the isolation of substance P from homogenized rodent brain tissue has been described and this technique was used (with slight modification) in the experiments presented in this thesis (Bergstrom *et al.*, 1983).

In initial experiments SP Trisacryl M was substituted for SP Sephadex C-25 (as used by Bergstrom *et al.*, 1983) as the IEC resin. This was due to the manufacturer's claims that SP Trisacryl M resin had superior resolution, flow rate (which is often a limiting step), pressure resistance and chemical, thermal and mechanical resistance (IBF Product Catalogue, 1987). Many of these factors can be a problem with IEC methods and therefore the SP Trisacryl M resin was considered a better choice than SP Sephadex C-25.

The basic aim of the present study was to determine the appropriate pre-RIA method for isolating substance P from biological samples. HPLC techniques were used to determine the elution profile of substance P and its fragments from the IEC column (since the RIA cannot distinguish SP from the fragments which possess an affinity for the antibody). An unusual phenomenon was then encountered with the use of the columns during the study and this has been examined in some detail. It must be noted, however, that the same basic IEC method was used throughout this thesis.

3.2 MATERIALS AND METHODS

3.2.1 Materials, Chemicals and Solutions.

3.2.1.i Materials and Chemicals for the Basic IEC.

5ml polypropylene tubes (Disposable Products, Adelaide, Australia), 2ml syringes and 21G x 1.5 needles (Terumo, Sydney, Australia). One-way stop-cocks (Vygon, France), glass microfibre filters (cut to size; 5.5cm, Whatman, England), SP Trisacryl M (IBF Biotechnics, France), SP Sephadex C-25 (Pharmacia, Uppsala,

Sweden). Methanol, hydrochloric acid, pyridine and formic acid (Ajax Chemicals, Sydney, Australia).

3.2.1.ii Additional Materials and Chemicals used in the Work Described in this Chapter.

Substance P (triacetate), trifluoroacetic acid (TFA) (Sigma, Mo., U.S.A), Tyr^8-substance P, nona-substance P (SP 3-11), octa-substance P (SP 4-11), hepta-substance P (SP 5-11), hexa-substance P (SP 6-11) (Peninsula Laboratories, CA, U.S.A). [^{125}I-Tyr^8]-substance P (^{125}I-SP; NEN, DuPont, CA, U.S.A). Acetic acid, acetonitrile, sodium dihydrogen phosphate (NaH_2PO_4), phosphoric acid, sodium chloride, sodium hydroxide (Ajax, Sydney, Australia). EDTA (di-sodium salt), dithiothreitol (molecular biology grade) (Sigma, Sydney, Australia), ascorbic acid (BDH, Sydney, Australia). 5ml polystyrene tubes (Lab Supply, Adelaide, Australia). 12 x 75mm Disposable Borosilicate Glass Culture Tubes (Kimble, Adelaide, Australia).

Spherisorb S10ODS2 reversed phase HPLC column (10 C18 30cm x 4.6mm) Spherisorb ODS5 reversed phase HPLC column (5 C18 30cm x 4.6mm) and Activon C18 guard column (10cm x 4.6mm; all packed by G. Crabb, Department of Clinical and Experimental Pharmacology, University of Adelaide). Helium (CIG Gas, Adelaide, Australia).

HPLC equipment for the analysis of SP Trisacryl M IEC fractions: Kortec K45 HPLC Gradient Controller, 2 x LC 1500 HPLC Pumps, Variable Wavelength HPLC UV Detector ETP Kortec K95 (all from ICI Instruments, Australia).

HPLC equipment for the analysis of SP Sephadex C-25 IEC fractions: LC 1600 Autosampler, DP 800 Data Interface, 2 x LC 1100 HPLC Pumps, LC 1200 UV/VIS Detector and ICI DP 800 Chromatography Data Station (all from ICI Instruments, Australia).

Materials and chemicals for the substance P RIA (see Chapter 2).

3.2.2 *Column Preparation.*

3.2.2.i *Resin Preparation*

Glass distilled water was added to SP Sephadex C-25 resin and the mixture was left overnight to allow for sufficient swelling (note: SP Trisacryl M was delivered in wet form [pre-swollen]). The resin was washed (in batches) to remove preservatives and impurities and reach IEC starting conditions (according to the method of Bergstrom *et al.*, 1983). This method has been outlined below in Table 3.1. The volume of solution used to wash the resin was ten times the volume of the resin. Thus, 100ml of resin was washed with 1L of each of the wash solutions.

WASH	***VOLUME***	***SOLUTION***
1	10 volumes	1:1 methanol/0.1M HCl
2	10 volumes	3M pyridine/1M formic acid
3	10 volumes	Glass distilled water
4	10 volumes	0.018M pyridine/0.1M formic acid

Table 3.1. Solutions used to wash the resin prior to use. (Note: see *Appendix II* for preparation of these solutions).

The required solution was added to the resin and the mixture stirred thoroughly. The resin was allowed to settle before the supernatant was decanted or aspirated. The next solution was then added in the same manner. At the final wash (which was also the first IEC method buffer, *i.e.*, 0.018M pyridine/0.1M formic acid) a layer of the solution, (about 2cm), was left above the resin and the resin refrigerated until required.

3.2.2.ii IEC Column Preparation.

A needle was attached to a one-way stop-cock (used to control the flow of the buffers through the column) and a 2ml syringe. A filter paper (cut to size) was placed in the base of the syringe (without the plunger) and a small volume of glass distilled water passed through (to prevent air bubble formation in the resin bed and ensure that the filter fitted tightly to the base of the syringe).

The prepared resin was loaded onto the column using a glass pipette (to a bed volume of 1ml). The resin was allowed to settle to ensure that the bed volume remained at 1ml (more resin was added if necessary).

Columns were stored in the refrigerator in air-tight containers for up to a week before use. On the day of the IEC, the excess buffer was drained from the column until a thin layer remained above the resin bed. The column was then ready for use. All columns used to obtain data for this thesis contained only freshly prepared and previously unused resin.

3.2.3 The Basic IEC Method.

The basic IEC method was based on the technique of Bergstrom and associates (1983) and involved the sequential application of pyridine/formic acid buffers in increasing concentration to isolate substance P.

The first step involved loading the required sample onto the column. For this study, the samples were either radiolabelled or synthetic forms of SP, fragments of SP or various biological samples (which had been through a preliminary extraction procedure) which contained SP. Once the sample was loaded (*i.e.*, once it had run through the column), the separation process was initiated. (Note: at no time was the column ever allowed to run dry as there was always a small proportion of buffer covering the resin. Flow was halted when required with the one-way stop-cocks).

The IEC elution method has been outlined in Table 3.2 (next page). All buffers were applied in two 2ml volumes and the resultant fractions were collected in 5ml polypropylene tubes (if required). The first buffer was 0.018M

pyridine/0.1M formic acid, which was followed by the 0.1M pyridine/0.1M formic acid buffer and so on, until the most concentrated buffer had passed through the column.

Number	*IEC Buffer*	*Volume*
1	0.018M pyridine/0.1M formic acid	2 x 2ml
2	0.1M pyridine/0.1M formic acid	2 x 2ml
3	0.35M pyridine/0.35M formic acid	2 x 2ml
4	0.8M pyridine/0.8M formic acid	2 x 2ml
5	1.5M pyridine/1.5M formic acid	2 x 2ml

Table 3.2. An outline of the sequence of application of IEC buffers to the column to isolate SP. It must be noted that the final buffer concentration used by Bergstrom *et al.*, (1983) was 1.6M pyridine/1.6M formic acid. The method of preparation of these buffers is reported in *Appendix II.*

The convention used to name the resultant IEC fractions for this thesis utilizes the pyridine concentration of the buffer; *i.e.*, the 0.8M fraction refers to the fraction obtained from the IEC column when the 0.8M pyridine/0.8M formic acid buffer had run through the column.

3.2.4 The IEC Elution Profile of SP Trisacryl M versus SP Sephadex C-25.

The IEC elution profile of SP alone and in the presence of some of its fragments (the "mixed" sample) was determined for both the SP Trisacryl M and SP Sephadex C-25 resins. For the SP only samples, 20μg of SP was dissolved in 500μl of 0.1% acetic acid and loaded onto a column containing either of the resins. The mixed sample consisted of 20μg of each of the following peptides; Tyr^{8}-SP, SP 3-11, SP 4-11, SP 5-11, SP 6-11 and SP in 500μl of 0.1% acetic acid. This mixture

was also loaded onto other columns containing either the SP Trisacryl M or SP Sephadex C-25 resin.

The IEC procedure was then performed in the usual manner (as outlined in 3.2.3). However, a 1.7M pyridine/1.7M formic acid IEC buffer was included to follow the 1.5M pyridine/1.5M formic acid buffer. All resultant fractions for each IEC buffer were collected in 5ml polypropylene tubes. The fractions were vacuum dried in a Savant Speedivac prior to HPLC analysis.

3.2.5 High Performance Liquid Chromatography (HPLC) Analysis of the IEC Fractions.

The IEC elution profiles for the two resins (SP Trisacryl M and SP Sephadex C-25) were not determined in the same time period. The Trisacryl study was performed first, on the then current standard laboratory HPLC equipment. In the interim (before the commencement of the Sephadex determinations) new HPLC equipment arrived in the laboratory and was subsequently utilized for the following Sephadex determinations.

3.2.5.i The HPLC method for the Analysis of the SP Trisacryl M IEC Fractions.

The HPLC technique for the analysis of SP Trisacryl M IEC fractions was based upon a modification of the method of Wallasch and co-workers (1988). A trifluoroacetic acid-acetonitrile (TFA/ACN) gradient buffer system was employed over a time period of 40 minutes. Buffer A was 0.1% TFA while buffer B was 60% ACN (in buffer A). The Kortec Gradient Controller regulated the flow rates and gradient for the method and the main HPLC column was the Spherisorb S100DS2. Table 3.3 summarizes the HPLC gradient program used for the Trisacryl IEC samples (next page).

Time (min)	*Flow (ml/min)*	*Buffer A %*	*Buffer B %*
0	1	100	0
40	1	0	100
42	1	0	100
43	1	100	0
49	1	100	0
50	0	100	0

Table 3.3. The gradient program used for the Trisacryl HPLC method. Buffer A = 0.1% TFA and buffer B = 60% ACN in A. Note: helium was bubbled into the buffers to displace dissolved oxygen and minimize the possibility of air bubbles disrupting an HPLC run.

After 100% buffer B was attained, this concentration continued for another two minutes to elute any remaining peptides from the column. The pumps then returned to 100% buffer A in preparation for the next HPLC sample.

Prior to the HPLC analysis of the Trisacryl IEC samples, it was necessary to determine the HPLC retention times of SP, Tyr^8-SP and the fragments SP 3-11, 4-11, 5-11 and 6-11. HPLC standards were made up of each of these peptides within the concentration range of 20µg/100µl and 60µg/100µl in 0.1% acetic acid (maintaining identical concentrations was not important for the determination of retention times).

100µl of the HPLC standard was injected onto the HPLC column and the gradient program initiated. The outflow was monitored by a Variable Wavelength HPLC UV Detector (wavelength 214nM). The absorbance peaks and retention times were recorded by the Kortec K45 HPLC Gradient Controller.

For the HPLC analysis of the Trisacryl IEC samples, the dried IEC fractions were reconstituted in 1.2ml 0.1% acetic acid and 500μl subsamples were injected onto the HPLC column for analysis. Peak times and areas were recorded by the Gradient Controller.

The results have been presented as the percentage of the *total* peak area recorded for each peptide. Many of the peptides eluted into more than one IEC fraction, although a major proportion of each peptide tended to elute into one particular IEC fraction, Thus, expressing the results as % (total) peak area readily reflected the disposition of the peptides in the different IEC fractions.

3.2.5.ii The HPLC method for the Analysis of the SP Sephadex C-25 IEC Fractions.

The HPLC method used on the SP Sephadex IEC fractions was based on a modification of the technique of Igwe and associates (1988). In essence, it consisted of a gradient system using buffer A (50mM sodium dihydrogen phosphate, NaH_2PO_4; adjusted to pH 3.0 with phosphoric acid [85% w/v]) and buffer B (60% ACN in buffer A). The gradient program adopted for this study has been summarized in Table 3.4 (see the next page) and was effectively controlled by the DP 800 Data Interface and ICI DP 800 Chromatography Data Station. The HPLC column used was a Spherisorb ODS5.

The remainder of the gradient program (*i.e.*, the time remaining after 40 minutes had elapsed) was a rapid column washing program followed by a return to starting conditions for the next sample.

Time (min)	*Flow (ml/min)*	*Buffer A %*	*Buffer B %*
0.0	1.0	100	0
40.0	1.0	0	100
40.5	1.0	0	100
41.0	1.0	100	0
46.5	1.0	100	0
47.0	0.0	100	0

Table 3.4. The gradient program used for the Sephadex HPLC method. Buffer A = 50mM NaH_2PO_4 (pH 3.0) and buffer B = 60% ACN in A. Note: helium was bubbled into the buffers to displace dissolved oxygen and minimize the possibility of air bubbles disrupting an HPLC run.

Preparation of the HPLC standards was the same as that outlined in the previous section. Standards were made up for SP, Tyr^8-SP and the fragments SP 3-11, 4-11, 5-11 and 6-11. 100µl of the HPLC standard was injected onto the HPLC column and the gradient program initiated. Outflow from the column was monitored by an LC 1200 UV/VIS Detector (wavelength 210nM). Peak times and areas were recorded by the DP 800 Data Interface and ICI DP 800 Chromatography Data Station.

For the HPLC analysis of the Sephadex IEC samples, the dried fractions were reconstituted in 300µl 0.1% acetic acid and 100µl subsamples were injected onto the HPLC column. Peaks were recorded by the data station. The results have been presented as the percentage of the *total* peak area recorded for each peptide.

3.2.6 Further SP Trisacryl M Evaluations. A Resin Dependent Paradoxical Elution Shift.

During the course of the preliminary studies for this project SP Trisacryl M was chosen as the resin for further IEC procedures. However, it was noted over a period of time that the properties of the resin changed with each new batch of resin. The following study was performed to determine the elution profile of ^{125}I-SP for the different batches of SP Trisacryl M resin. [Note; the method for this study has differed slightly between the different batches, since it was conducted over a period of approximately two years. The difference in the methodology was not extensive and permitted a direct comparison between the different batches.]

3.2.6.i The IEC Elution Profile of ^{125}I-SP for the Different Batches of the SP Trisacryl M Resin.

Four batches of the SP Trisacryl M resin were studied. Their lot numbers were as follows; Batch 1 (not recorded), Batch 2 (9459), Batch 3 (1741) and Batch 4 (1741). A mixture of Batch 1 and Batch 2 (the exact proportions were unknown) resins was assessed, as well as the various batches alone. The basic properties of the method did not essentially differ between the determinations. An outline of the basic method is presented below. (Note: problems with the resin emerged when the Batch 2 resin was used. Lot numbers were recorded when the anomaly with the resin was first suspected).

1. The ^{125}I-SP was diluted to yield between 15,000 and 20,000 cpm/250 µl. The diluents were for Batch 1: 1% acetic acid, for Batch 2: 0.018M pyridine/0.1M formic acid, for a combination of Batches 1 and 2: 5:1 methanol/0.1M HCl, for Batch 3: 1% acetic acid (part a) and 0.018M pyridine/0.1M formic acid (part b) and for Batch 4: 0.018M pyridine/0.1M formic acid. (The diluents were chosen according to requirements pertaining to other experiments conducted at the same time).

2. 250 µl aliquots of the diluted ^{125}I-SP were loaded onto prepared IEC columns containing the specified batch of SP Trisacryl M resin, or set aside for Total Count (TC) determinations.

3. The IEC columns were run in the usual manner for Batch 1, the mixture of Batches 1 and 2, Batch 2 and Batch 3 (part a).

The IEC method used for Batch 3 (part b) and Batch 4 differed slightly from that outlined above. The first three IEC buffers were eluted in order but not collected (*i.e.*, the 0.018M, 0.1M and 0.35M fractions). Immediately after the 0.35M fraction had been eluted, one of the following IEC buffers was run through the IEC column and collected for subsequent determination of radioactivity; 0.8M, 1.5M, 1.7M, 1.9M or 2.1M pyridine/formic acid solutions. Thus, the elution of these collected buffers was non-consecutive. The preparation of the 1.7M, 1.9M and 2.1M buffers is shown in Table 3.5.

All collected fractions had their radioactive content determined by an LKB Wallac 1261 Multigamma γ-counter (Turku, Finland; counting efficiency 75%).

IEC Buffer	*Pyridine (ml)*	*88% Formic Acid (ml)*
1.7M	13.75	7.29
1.9M	15.37	8.15
2.1M	16.98	9.00

Table 3.5. Preparation of additional IEC buffers required for the current study. All IEC buffers were made up to a final volume of 100ml with GDW.

Results have been expressed as percent Total Counts (%TC; determined from cpm) recovered. The results have been represented graphically and as cumulative %TC for the IEC fractions for Batch 1, the mixture of Batches 1 and 2 and Batch 3 (part a). Batch 3 (part b) and Batch 4 results have been represented

graphically as %TC, but were not cumulative results over the range of IEC fractions.

3.2.7 Evaluation of the Shift of Substance P on SP Trisacryl M Resin.

It became apparent that there was a paradoxical shift in the elution profile of substance P (as indicated by the ^{125}I-SP determinations) when different batches of the SP Trisacryl M resin were used in the IEC procedure (Results section; *3.3.2*). It was therefore necessary to determine the circumstances which led to the alteration in the elution profile and to eliminate this factor (if possible).

Examination of the IEC procedure revealed various factors which may have caused this specific shift. These included possible interference in stock solutions, the water used in the preparation of the buffers, or in the IEC buffers themselves. Subtle differences in the pH of the IEC buffers may have also accounted for the discrepancies, as may contamination of the IEC column components such as the filters.

Although RIA measurements were not performed on samples used in the determination of the elution profile shift, the following study also examined factors with the potential to alter the quantification of SP by RIA. These include the possible contamination of RIA samples by the presence of pyridine, the effect of drying, reconstitution times and the composition of the test tubes used in the reconstitutions.

Other variables examined were the composition of the column itself (glass versus plastic), the volume of the resin bed, the volume of buffer used for each fraction, the effects of ascorbic acid, dithiothreitol (both antioxidants) and EDTA (a metal chelator) and the washing conditions of the resin prior to use. A final section of the study assessed the elution profile of SP Sephadex C-25 resin.

The radioactive content of IEC fractions collected in the following studies was determined in an LKB Wallac 1261 Multigamma γ-counter (Turku, Finland;

counting efficiency 75%), abbreviated in the following sections as the LKB counter. Original ^{125}I-SP samples were diluted to yield around 30,000 cpm/500µl.

The results of these determinations have been expressed as the %TC eluted per IEC fraction (mean ± SEM).

3.2.7.i Influence of the Resin Washing Conditions Prior to the IEC Procedure.

The Batch 4 SP Trisacryl M resin was washed in 10 volumes of 1M NaCl (as suggested by the IBF Biotechnics Instruction Sheet accompanying the resin) and left overnight before loading onto the IEC column in the usual manner. The NaCl solution was then eluted off and the columns were pre-equilibrated with either 5:1 MeOH/0.1M HCl (N=3 columns) or 0.018M pyridine/0.018M formic acid (N=3 columns).

Synthetic substance P was made up to a concentration of 100pg/ml in 5:1 MeOH/0.1M HCl and 1ml of this dilution was loaded on to the prepared IEC columns. The following 4ml fractions were collected (in consecutive order), vacuum dried and processed by RIA; 0.018M, 0.1M, 0.35M, 0.8M, 1.5M, 1.7M, 1.9M and 2.1M.

Results have been expressed as the mean ± the SEM of the recovery of added SP per fraction as determined by the RIA.

3.2.7.ii Influence of the IEC Buffers.

Two types of IEC buffers were assessed in the study; a) *Stored* buffers which had been used in previous determinations and then stored at ambient temperature until required again (sometimes for a period of two to three months) and b) *Fresh* buffers which had been freshly prepared from the stock solutions on the day prior to this study. An outline of the experimental method is presented in Table 3.6 (next page).

Resin Used	SP Trisacryl M (Batch 2)
Sample	^{125}I-SP (in 5:1 MeOH/0.1M HCl)
Sample Volume	250 µl
Number of IEC Columns	12
Variable Assessed	Elution profile comparing fresh IEC buffers with stored buffers
TC Determinations	5
IEC Fractions Collected	0.018M to 1.5M

Table 3.6. An outline of the experimental conditions for the determination of the influence of IEC buffers.

3.2.7.iii Influence of Pyridine.

The following study was performed to determine whether a contamination of the pyridine stock had occurred and subsequently altered the elution profile of the peptide. A new stock source of pyridine was used to prepare the new pyridine buffers, whilst the old pyridine buffers were those that had been used in the earlier elution profile determinations. An outline of the experimental method is presented in Table 3.7 (next page).

A problem with the pyridine stock (or the formic acid stock, which is discussed on the next page) would have been apparent with the use of either resin. The demonstrated shift in the elution profile was large. If the source of pyridine was the cause, this should have been easily detected, even with the relatively small sample size.

Resin Used	SP Sephadex C-25
Sample	^{125}I-SP (in 5:1 MeOH/0.1M HCl)
Sample Volume	500 µl
Number of IEC Columns	4
Variable Assessed	Elution profile comparing fresh and existing sources of pyridine
TC Determinations	10
IEC Fractions Collected	0.018M to 2.1M

Table 3.7. An outline of the experimental conditions for the determination of the influence of pyridine.

3.2.7.iv Influence of Formic Acid.

The following experiment assessed whether the source of formic acid used to prepare the IEC buffers was of significance. A new source of formic acid was used to prepare the new formic acid buffers, whilst the old formic acid buffers were those that had been used in the earlier elution profile determinations. An outline of the experimental method is presented in Table 3.8.

Resin Used	SP Sephadex C-25
Sample	^{125}I-SP (in 5:1 MeOH/0.1M HCl)
Sample Volume	500 µl
Number of IEC Columns	14
Variable Assessed	Elution profile comparing fresh and existing formic acid
TC Determinations	6
IEC Fractions Collected	0.018M to 2.1M

Table 3.8. An outline of the experimental conditions for the determination of the influence of formic acid.

3.2.7.v Influence of Purified Laboratory Water.

The IEC buffers prior to this study had all been prepared with GDW (glass distilled water). The study compared the elution profile obtained with buffers prepared with GDW with the profile obtained with buffers prepared from Milli-Q water. This was an effort to ascertain whether the source of water had affected the IEC elution profile. An outline of the experimental method has been presented in Table 3.9.

Resin Used	SP Trisacryl M (Batch 4)
Sample	^{125}I-SP (in 0.018M made with Milli-Q)
Sample Volume	500 µl
Number of IEC Columns	8
Variable Assessed	Elution profile comparing sources of water used in IEC buffers
TC Determinations	9
IEC Fractions Collected	0.018M to 2.1M

Table 3.9. An outline of the experimental conditions for the determination of the influence of water source.

3.2.7.vi Influence of IEC Column Material.

All of the IEC work contained in this thesis has used 2ml plastic disposable syringes as the IEC column. The following study briefly investigated whether replacement of the column support material (*i.e.,* glass for plastic) would return the elution of the peptide to its original profile.

Glass wool was inserted into the base of glass pipettes (N=3) so that the resin would be retained on the column. It was not possible to fit the usual paper filter to form an adequate retainer for the resin under these circumstances. The glass IEC columns were loaded with the prepared resin to a depth of

approximately 1.5-2.0cm of the column. An outline of the experimental method is presented in Table 3.10.

Resin Used	SP Trisacryl M (Batch 4)
Sample	^{125}I-SP (in 0.018M)
Sample Volume	500 μl
Number of IEC Columns	3
Variable Assessed	Use of glass instead of plastic for column support
TC Determinations	4
IEC Fractions Collected	0.018M to 2.1M

Table 3.10. An outline of the experimental conditions for the determination of the influence of composition of column material.

3.2.7.vii Influence of the pH of the 0.8M IEC Buffer.

The method of Bergstrom and colleagues (1983) stipulated that the pH of the IEC buffers should be 4.4, with the exception of the 0.018M buffer which should be at a pH of 3.2. The following examined the effect of manipulating the pH of the 0.8M and 1.5M buffers to be within the range of pH 4.20 to pH 4.60.

The prepared 0.8M and 1.5M IEC buffers were adjusted (with 3M formic acid or 1M NaOH) to the following pHs; 4.20, 4.40, 4.45, 4.50, 4.55 and 4.60.

All columns were run in the usual manner for the 0.018M, 0.1M and 0.35M IEC fractions (but none of these fractions were collected). Then, duplicate columns were run for each of the 0.8M buffers at each of the specified pHs. All of the 0.8M fractions were collected. The columns were then eluted with the 1.5M buffer. This buffer was of the same pH as the 0.8M buffer which had been used on the column previously (*i.e.*, the pH 4.40 0.8M buffer was followed by the pH 4.40 1.5M buffer on the same column). All of the 1.5M fractions were collected as well. An outline of the experimental method is presented in Table 3.11 (next page).

Resin Used	SP Trisacryl M (Batch 2)
Sample	^{125}I-SP (in 0.018M)
Sample Volume	250 µl
Number of IEC Columns	12
Variable Assessed	Effect of pH (of the 0.8M buffer) on the elution profile
TC Determinations	8
IEC Fractions Collected	0.8M and 1.5M only

Table 3.11. An outline of the experimental conditions for the determination of the influence of the pH of the 0.8M IEC buffer.

3.2.7.viii Influence of the Volume of the 0.8M IEC Buffer.

The following study was designed to determine whether the volume of buffer used to elute the peptide could affect the elution profile (*i.e.,* would an increase in elution buffer concomitantly increase recovery in the 0.8M fraction).

The IEC columns were run in the usual manner with one slight difference; *i.e.,* three columns were eluted with 2 x 2ml 0.8M, three columns were eluted with 3 x 2ml 0.8M and the last three columns were eluted with 4 x 2ml 0.8M.

All fractions (0.018M to 2.1M) were collected from the usual IEC procedure (*i.e.,* the one which used 2 x 2ml 0.8M). Only the 0.8M and higher fractions were collected for the 3 x 2ml and 4 x 2ml 0.8M procedures (the results would be the same as the earlier fractions for the 2 x 2ml 0.8M since the 0.018M, 0.1M and 0.35M elutions were all performed with the usual 2 x 2ml volume). An outline of the experimental method is presented in Table 3.12 (next page).

Resin Used	SP Trisacryl M (Batch 4)
Sample	^{125}I-SP (in 0.018M)
Sample Volume	500 µl
Number of IEC Columns	9
Variable Assessed	Effect of elution volume on the elution profile
TC Determinations	7
IEC Fractions Collected	0.018M to 2.1M (for 2 x 2ml) 0.8M to 2.1M (for 3 x 2ml and 4 x 2ml)

Table 3.12. An outline of the experimental conditions for the determination of the influence of IEC buffer volume.

3.2.7.ix Influence of the Resin Bed Volume.

The following study was designed to determine the effect of changing the volume of the resin bed on the elution profile of ^{125}I-SP. Four resin bed volumes were tested; 1ml, 0.5ml, 0.2ml and 0.1ml. An outline of the experimental method is presented in Table 3.13.

Resin Used	SP Trisacryl M (Batch 4)
Sample	^{125}I-SP (in 0.018M)
Sample Volume	500 µl
Number of IEC Columns	4
Variable Assessed	Effect of different resin bed volumes on the elution profile
TC Determinations	7
IEC Fractions Collected	0.018M to 2.1M

Table 3.13. An outline of the experimental conditions for the determination of the influence of resin bed volume.

3.2.7.x *Effect of Ascorbic Acid, Dithiothreitol and EDTA.*

^{125}I-SP was diluted in the usual 0.018M pyridine/0.1M formic acid, or ascorbic acid (100 µg/ml), or dithiothreitol (1mM), or EDTA (1mM) to determine whether the presence of any of these chemicals could influence the elution profile.

On the day of the study, 3µl of ^{125}I-SP were added to 10ml of each of the aforementioned solutions and mixed thoroughly (these solutions were kept on ice until required). Triplicate columns were run for each of the these dilutions of radiolabel. Thus, 1ml of each of these solutions was loaded onto three IEC columns. Aliquots of 1ml were then set aside for the determination of TC (N=6 for all except the 0.018M solution, N=7). An outline of the experimental method is presented in Table 3.14.

Resin Used	SP Trisacryl M (Batch 4)
Sample	^{125}I-SP
Sample Volume	1 ml
Number of IEC Columns	12
Variable Assessed	Effect of ascorbic acid, dithiothreitol and EDTA
TC Determinations	6 or 7
IEC Fractions Collected	0.018M to 2.1M

Table 3.14. An outline of the experimental conditions for the determination of the influence of various agents on the elution profile.

3.2.7.xi. Elution Profile of Synthetic Substance P versus ^{125}I-Substance P.

In the event that it was the ^{125}I-SP which had resulted in a different elution profile with each batch of radiolabel, the elution profile of non-labelled SP was determined. The elution profile was established for 50pg, 500pg, 5ng, 500ng and 1μg of substance P. (The following study was performed with Batch 4 SP Trisacryl M resin).

Substance P was diluted in 0.018M pyridine/0.1M formic acid to yield the following solutions; 10μg/ml, 100ng/ml, 10ng/ml and 1ng/ml. Ten IEC columns were loaded with 1ml of the prepared resin and the SP solutions were loaded onto the (duplicate) columns according to the manner outlined in Table 3.15.

Final Concentration of SP	***Volume of Solution***
50 pg	50 μl of 1ng/ml
500 pg	50 μl of 10ng/ml
5 ng	50 μl of 100ng/ml
500 ng	50 μl of 10μg/ml
1 μg	100 μl of 10μg/ml

Table 3.15. Summary of substance P dilutions and solution volumes used in the present study.

The columns were run in the usual manner (using the buffers 0.018M to 1.7M) and the 0.8M, 1.5M and 1.7M fractions collected and vacuum dried. The fractions were then processed by the usual RIA procedure (Chapter 2; *2.2.2 The Basic RIA Method*). However, selected samples required diluting prior to the RIA, as they (potentially) contained levels of SP above 1,000pg.

The 50pg and 500pg IEC samples were not diluted prior to the RIA. The 5ng sample was diluted by a factor of ten, the 500ng sample was diluted by a factor of 100 and the 1μg sample was diluted by a factor of 1,000 and 10,000 for the RIA. The dilutions were performed in the RIA diluent buffer. After the RIA determination of SP levels in the fractions, the values were corrected to take into account the various dilutions which had been performed.

The results have been presented as the % recovery of SP loaded onto the columns (a mean of two determinations) and for each of the dilutions (if performed).

3.2.8 Assessment of Post-IEC Variables.

After examining the possible influence of various IEC parameters on the elution profile of SP (which if unchecked would subsequently result in altered RIA SP determinations), it appeared prudent to examine the various post-IEC parameters. This would ensure that the most appropriate conditions for maximum recovery of SP during the drying-reconstitution process would be known and adhered to.

3.2.8.i Effect of Drying on the Measurement of Substance P.

^{125}I-SP was diluted in 0.018M pyridine/0.1M formic acid buffer to yield around 50,000 cpm/500 μl. Aliquots of 500 μl of the diluted ^{125}I-SP were set aside for the determination of TC (N=4) or 500 μl subsamples were placed into the usual 5ml fraction collection test tubes (N=4) and vacuum dried.

The dried samples were then reconstituted in 500 μl 1% acetic acid. Then, the reconstituted samples and the tubes in which they were reconstituted were counted separately, along with the TC determinations. The radioactive content was measured in the LKB counter. The results have been expressed as cpm (mean ± SEM).

3.2.8.ii Effect of Test Tube Composition Used in the Reconstitution for the Measurement of Substance P.

This was performed to determine the most suitable test tubes for the reconstitution of dried samples. The following types of test tubes were assessed; *i.e.,* the usual IEC collection tubes (polypropylene), the usual RIA tubes (polystyrene) and glass test tubes.

^{125}I-SP was diluted in 0.018M pyridine/0.1M formic acid to yield approximately 10,000 cpm/500 µl. Aliquots of 500 µl were then set aside for the determination of TC (N=7) or placed in polypropylene (N=4), or polystyrene (N=3), or glass test tubes (N=4). The samples were then vacuum dried and reconstituted. The reconstitutions were performed by the addition of 500 µl of 1% acetic acid to each tube followed by thorough vortexing. The samples were left for 2 hours and then removed for counting. Four subsequent washes (of 500 µl 1% acetic acid) were then immediately added to the tubes, mixed and removed for determination of cpm as well. The tubes were also retrieved for determination of cpm.

The radioactive content was measured in the LKB counter. The results have been expressed as % TC recovered (mean $\pm$ SEM).

3.2.8.iii Influence of Reconstitution Volume for the Measurement of Substance P.

The following study was performed to determine which reconstitution volume would retain the maximum sample (in the smallest volume). Reconstitution volumes assessed were either 250 µl or 500 µl (the usual reconstitution volume) of 1% acetic acid.

^{125}I-SP was diluted in 0.018M pyridine/0.1M formic acid to yield between 15,000 and 20,000 cpm/500 µl. Aliquots of 500 µl were then set aside for the determination of TC (N=6) or placed in the usual IEC collection tubes (N=10). These samples (N=10) were then vacuum dried and reconstituted with either 250 µl (N=5) or 500 µl (N=5) 1% acetic acid. Immediately after the reconstitution, four

more washes (with the same volume of 1% acetic acid) were added to the tubes and retained for determination of counts.

The radioactive content of the reconstituted samples, the washes, the TC samples and the tubes were measured in the LKB counter. The results have been expressed as % TC recovered (mean $\pm$ SEM).

3.2.8.iv Influence of the Acetic Acid Concentration on the Reconstitution and Subsequent Measurement of Substance P.

The effect of increasing the concentration of acetic acid used in the reconstitution of dried samples was examined. Concentrations of acetic acid used were 1% (the usual concentration), 5%, 10% and also 5% formic acid.

^{125}I-SP was diluted in 0.018M pyridine/0.1M formic acid to yield between 15,000 and 20,000 cpm/500 µl. Aliquots of 500 µl were then set aside for the determination of TC (N=9) or placed in the usual IEC collection tubes (N=20) and vacuum dried.

The dried samples were split into four groups of five tubes and reconstituted with 500 µl of (i) 1% acetic acid, (ii) 5% acetic acid, (iii) 10% acetic acid, or (iv) 5% formic acid. Immediately after the reconstitution, four more washes (with 500 µl of the same acid) were added to the tubes and retained for determination of counts.

The radioactive content of the reconstituted samples, the washes, the TC samples and the tubes were measured in the LKB counter. The results have been expressed as % TC recovered (mean $\pm$ SEM).

3.2.8.v Influence of Reconstitution Time on the Subsequent Measurement of Substance P.

The following study examined the effect of immediate reconstitution and reconstitution over 0.5 hours (the usual reconstitution time) on the recovery of ^{125}I-SP.

^{125}I-SP was diluted in 0.018M pyridine/0.1M formic acid to yield between 15,000 and 20,000 cpm/500 µl. Aliquots of 500 µl were then set aside for the determination of TC (N=6) or placed in the usual IEC collection tubes (N=10) and vacuum dried.

Half of the dried samples were reconstituted in 250 µl of 1% acetic acid and four more washes of 250 µl 1% acetic acid were *immediately* added and retained for determination of counts. The remaining samples were reconstituted in the same manner, but left for 30 minutes (after vortexing) before taking the sample for analysis. These samples then had four more subsequent washes (as above) which were retained for determination of TC.

The radioactive content of the reconstituted samples, the washes, the TC samples and the tubes were measured in the LKB counter. The results have been expressed as % TC recovered (mean $\pm$ SEM).

3.2.9 Elution Profile of SP Sephadex C-25.

As the elution profile did not appear to improve with any of the modifications assessed on the SP Trisacryl M resin, the elution profile of SP Sephadex C-25 was examined. An outline of the experimental method is presented in Table 3.16 (next page).

Resin Used	SP Sephadex C-25
Sample	^{125}I-SP (in 0.018M)
Sample Volume	500 µl
Number of IEC Columns	6
Variable Assessed	New resin
TC Determinations	4
IEC Fractions Collected	0.018M to 2.1M

Table 3.16. An outline of the experimental conditions for the determination of the influence of a new resin on the elution profile.

3.2.9.i Influence of Pyridine/Formic Acid Residues in RIA Samples.

A final consideration was whether the IEC buffers themselves may add to (or even detract from) the quantification of substance P by the RIA, *i.e.*, do the IEC buffers, once they have passed through the column, register in the RIA in any way (either adding to the measurement of SP or reducing it). The following study examined the "level of substance P" in the IEC buffers after passing through a column which was composed of the SP Sephadex C-25 resin. An outline of the experimental method is presented in Table 3.17.

Resin Used	SP Sephadex C-25
Sample	None (Blank run)
Number of IEC Columns	8
Variable Assessed	Background substance P levels eluted from IEC columns (as determined by RIA)
IEC Fractions Collected	0.018M to 1.7M

Table 3.17. An outline of the experimental conditions for the determination of background substance P levels from the IEC column.

The results have been expressed as "pg of SP" per IEC fraction (mean ± SEM).

3.3 RESULTS

3.3.1 The IEC Elution Profile of SP Trisacryl M versus SP Sephadex C-25; HPLC Analysis of the IEC Fractions.

3.3.1.i Results for SP Trisacryl M

Retention times for the Trisacryl HPLC standards have been presented in Table 3.18.

Peptide	*Retention Time (mins)*	*N*
SP 4-11/5-11	29.13 ± 0.13	5
SP 3-11	29.91 ± 0.24	3
Tyr^8-SP	31.02 ± 0.14	3
Substance P	31.36 ± 0.10	4
SP 6-11	31.67 ± 0.06	5

Table 3.18. The retention times (mean ± SEM) for the (Trisacryl) HPLC standards of substance P and related peptides.

The fragments SP 4-11 and SP 5-11 were found to elute so closely together that resolution was not possible. Minor modifications to flow rates and the gradient program around the time of elution of these fragments did not improve resolution. In view of this co-elution, SP 4-11 and SP 5-11 have been considered a single entity for the remainder of the Trisacryl HPLC study.

Chronological elution from the HPLC column saw the emergence of SP 4-11/5-11 first, followed by SP 3-11, Tyr^8-SP, SP, and finally SP 6-11. HPLC elution of SP 4-11/5-11 and SP 3-11 preceded the other peptides by over one minute. However, SP 3-11 was separated from SP 4-11/5-11 by almost another minute

again. SP, Tyr^8-SP and SP 6-11 had very similar retention times (within one minute of each other). Nevertheless, the retention times of these three peptides were separated by approximately 20 seconds, allowing clear resolution from each other. Thus, all peptides were able to be resolved from each other (with the exception of SP 4-11 and SP 5-11) and to be identified by their retention time.

The elution profile of substance P alone from the Trisacryl IEC column may be seen in Figure 3.1. No SP was detected in the 0.018M, 0.1M and 1.5M IEC fractions. The major proportion of SP was detected in the 0.8M fraction with 99.5% of the total peak area accounted for. The remainder of the SP was found to elute into the 0.35M IEC fraction (a negligible 0.5%).

The Trisacryl IEC elution profile of the peptide mix can be seen in Figure 3.2. Loading SP with the similar peptides did not greatly affect the elution profile of this peptide. The majority of SP was detected in the 0.8M fraction (99.4% of the total peak area). No SP was detected in the 0.018M, 0.35M, 1.5M and 1.7M fractions and only a negligible 0.6% was detected in the 0.1M IEC fraction.

Tyr^8-SP completely eluted into a single IEC fraction, *i.e.*, the 0.8M fraction (Fig. 3.2). No Tyr^8-SP was detected in the other fractions (*viz* 0.018M, 0.1M, 0.35M, 1.5M and 1.7M).

The remaining peptides did not elute "cleanly" into one discrete IEC fraction. Instead, they were detected in significant levels in two or three consecutive IEC fractions (Fig. 3.2). SP 3-11 was detected in the 0.35M (78.3%) and 0.8M IEC fractions (21.6%), with little or no SP 3-11 detected in the 0.018M, 0.1M, 1.5M and 1.7M fractions. The SP 4-11/5-11 combination was found to elute into almost all of the IEC fractions in varying proportions as follows; 0.018M (15.2%), 0.1M (27.4%), 0.35M (53.5%), 0.8M (2.7%), 1.5M (1.2%) and 1.7M (negligible). SP 6-11 was found to elute mainly in the first three IEC fractions. The SP 6-11 results were; 0.018M (23.8%), 0.1M (11.5%) and 0.35M (63.2%). Very little SP 6-11 was detected in the remaining fractions (*i.e.*, the 0.8M, 1.5M and 1.7M fractions).

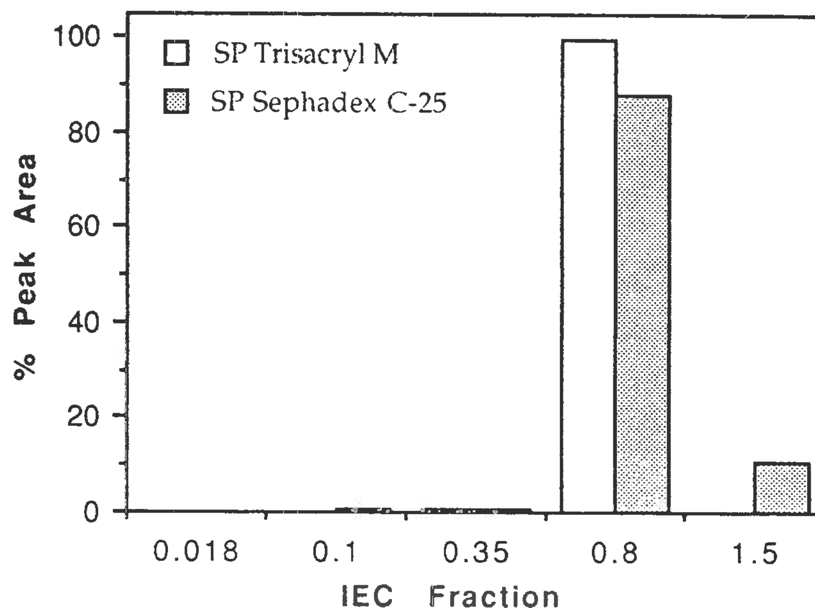


Fig. 3.1. HPLC analysis of the ion exchange chromatography (IEC) elution profile of substance P (SP) on SP Trisacryl M resin and SP Sephadex C-25 resin.

IEC columns were prepared with either SP Trisacryl M resin or SP Sephadex C-25 resin. SP samples (20µg in 500µl 0.1% acetic acid) were loaded onto the IEC columns and the IEC method was performed in the usual manner. All resulting IEC fractions were collected and lyophilized. The dried fractions were reconstituted and analysed by the HPLC methods outlined in section 3.2.5.i and 3.2.5.ii. The figure shows the elution of SP (as % peak area) for each IEC fraction when using either SP Trisacryl M resin or SP Sephadex C-25 resin (N=2).

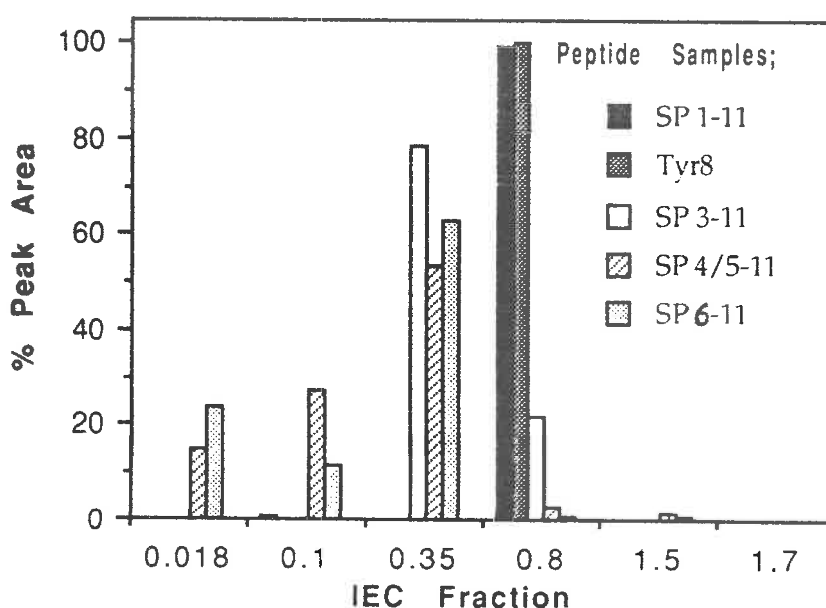


Fig. 3.2. HPLC analysis of the ion exchange chromatography (IEC) elution profile of substance P (SP) and related peptides when using SP Trisacryl M resin.

IEC columns were prepared with SP Trisacryl M resin. Samples containing 20µg each of substance P (SP 1-11), tyrosine^{8}-SP (Tyr^{8}-SP), nona-substance P (SP 3-11), octa-substance P (SP 4-11), hepta-substance P (SP 5-11) and hexa-substance P (SP 6-11) (in 500µl of 0.1% acetic acid) were loaded onto the IEC columns and the IEC method was performed in the usual manner. All resulting IEC fractions were collected and lyophilized. The samples were reconstituted and analysed by the HPLC method outlined in section 3.2.5.i. The figure shows the elution of each of the peptides (as % peak area; see legend in figure) for each IEC fraction (N=2).

3.3.1.ii Results for SP Sephadex C-25.

Retention times for the Sephadex HPLC standards have been presented in Table 3.19.

Peptide	***Retention Time (mins)***	***N***
Tyr^8-SP	21.38 ± 0.14	10
Substance P	23.24 ± 0.19	8
SP 3-11	23.85 ± 0.09	5
SP 4-11/5-11	24.61 ± 0.03	8
SP 6-11	25.78 ± 0.06	9

Table 3.19. The retention times (mean ± SEM) for the (Sephadex) HPLC standards of substance P and related peptides.

As fragments SP 4-11 and SP 5-11 again eluted so closely together, isolation between the two was not possible. Modifications to the HPLC elution properties did not improve their resolution. Thus, SP 4-11 and SP 5-11 have been recognized as a single entity for the remainder of the Sephadex HPLC study.

Chronological elution from the HPLC column saw the emergence of Tyr^8-SP first, followed by SP, SP 3-11, SP 4-11/5-11 and finally SP 6-11. Tyr^8-SP preceded SP by almost two minutes, whilst SP and SP 3-11 had very similar retention times (within one minute of each other), but could be clearly resolved. Clear resolution was also obtained with SP 4-11/5-11 and also SP 6-11 from all the other peptides. Thus, in general, all peptides were able to be resolved from each other (with the exception of SP 4-11 and SP 5-11) and identified by their retention time.

The elution profile of substance P alone from the Sephadex IEC column may be seen in Figure 3.1. Negligible levels of SP were detected in the 0.018M,

0.1M and 0.35M IEC fractions. The major proportion of SP was detected in the 0.8M fraction with 87.8% of the total peak area accounted for. The remainder of the SP was found to elute into the 1.5M IEC fraction (10.5%).

The Sephadex IEC elution profile of the peptide mixture is shown in Figure 3.3. The elution of SP was spread over a number of the fractions. The fractions with a significant level of SP were; 0.35M (34.4%), 0.8M (57.7%) and 1.5M (7.4%). The remaining fractions (0.18M, 0.1M and 1.7M) had negligible levels of SP.

Most of the Tyr^{8}-SP eluted into a single Sephadex IEC fraction (Fig. 3.3). Notable Tyr^{8}-SP levels were detected in the 0.8M (85.9%) and 1.5M (13.4%) fractions. Very little Tyr^{8}-SP was detected in the 0.018M, 0.1M, 0.35M and 1.7M IEC fractions.

A major proportion of SP 3-11 did elute into the 0.8M Sephadex fraction (97.1%) (Fig.3.3). A noticeable amount was detected in the 0.018M fraction (2.5%) whilst very little was detected in the 0.1M, 0.35M, 1.5M and 1.7M IEC fractions.

A spread into various fractions was observed in the Sephadex IEC elution profiles for the SP 4-11/5-11 combination and SP 6-11 (Fig.3.3). The major proportion of SP 4-11/5-11 was detected in the 0.1M (63.3%) and 0.35M (32.1%) IEC fractions. Small levels were also detected in the 0.018M (2.4%) and 0.8M (1.5%) fractions, but also with very little of these fragments detected in the 1.5M and 1.7M IEC fractions.

The SP 6-11 fragment eluted from the Sephadex IEC column in the early fractions; 0.18M (33.8%), 0.1M (34.7%) and 0.35M (29.1%) (Fig.3.3). A small amount was detected in the 1.5M fraction (1.5%), with negligible levels detected in the 0.8M and 1.7M IEC fractions.

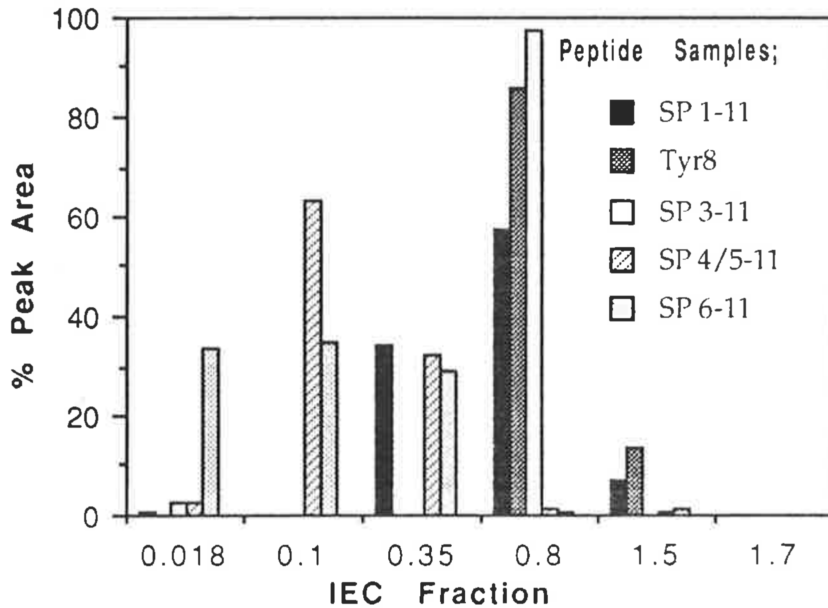


Fig. 3.3. HPLC analysis of the ion exchange chromatography (IEC) elution profile of substance P (SP) and related peptides when using SP Sephadex C-25 resin.

IEC columns were prepared with SP Sephadex C-25 resin. Samples containing 20µg each of substance P (SP 1-11), tyrosine^{8}-SP (Tyr^{8}-SP), nona-substance P (SP 3-11), octa-substance P (SP 4-11), hepta-substance P (SP 5-11) and hexa-substance P (SP 6-11) (in 500µl of 0.1% acetic acid) were loaded onto the IEC columns and the IEC method was performed in the usual manner. All resulting IEC fractions were collected and lyophilized. The samples were reconstituted and analysed by the HPLC method outlined in section 3.2.5.ii. The figure shows the elution of each of the peptides (as % peak area; see legend in figure) for each IEC fraction (N=2).

3.3.2. Further SP-Trisacryl M Evaluations. A Resin Dependent Paradoxical Elution Shift.

The mean TC determination from the six resin profile studies was 18,091.5 ± 1,287.8 cpm. Figure 3.4. depicts the cumulative elution profile of ^{125}I-SP on the different batches of the SP Trisacryl M resin. Over 98% of TC was recovered in IEC fractions up to and including the 0.8M fraction for the initial batch of the SP Trisacryl M resin (Batch 1, N=2). A major proportion of the ^{125}I-SP was recovered in the 0.8M fraction alone (95.0%). The remainder of the TC was recovered in the 1.5M fraction for the Batch 1 resin.

The combination of the two batches (Batch 1 and 2, N=6) displayed a slightly altered elution profile (Fig. 3.4). Only a low level of TC was recovered in the first three IEC fractions; up to 6.0% of TC were found in the 0.35M fraction. The majority of the TC were recovered in the 0.8M IEC fraction (70.3% in this fraction alone; 76.3% cumulative). The remainder of the TC was found to elute into the 1.5M IEC fraction.

Batch 2 SP Trisacryl M resin exhibited a different ^{125}I-SP elution profile (Fig. 3.4, N=6). A slightly higher proportion of the TC were recovered in the first three IEC fractions; up to 11% of the TC (cumulative) had been eluted in the 0.35M fraction. The 0.8M fraction was found to elute 24% of the TC (35.4% cumulative). However, the majority of the TC were recovered in the 1.5M IEC fraction, resulting in total cumulative recovery of TC over the range of IEC fractions tested.

The Batch 3 resin was assessed on two different occasions over two different IEC elution ranges (Fig. 3.4). The first (part a) was conducted over the usual IEC range (0.018M to 1.5M), whilst part b extended the IEC range to a 2.1M IEC buffer. Very little of the TC were recovered from the Batch 3 resin even up to the 0.8M IEC buffer (3.7% cumulative). Only a little over half of the TC were recovered by the 1.5M fraction (56.4% cumulative).

The second Batch 3 determination (part b) revealed a similar elution profile (Fig 3.4). Low levels of TC were recovered in the 0.8M (13.6%) and 1.5M fractions

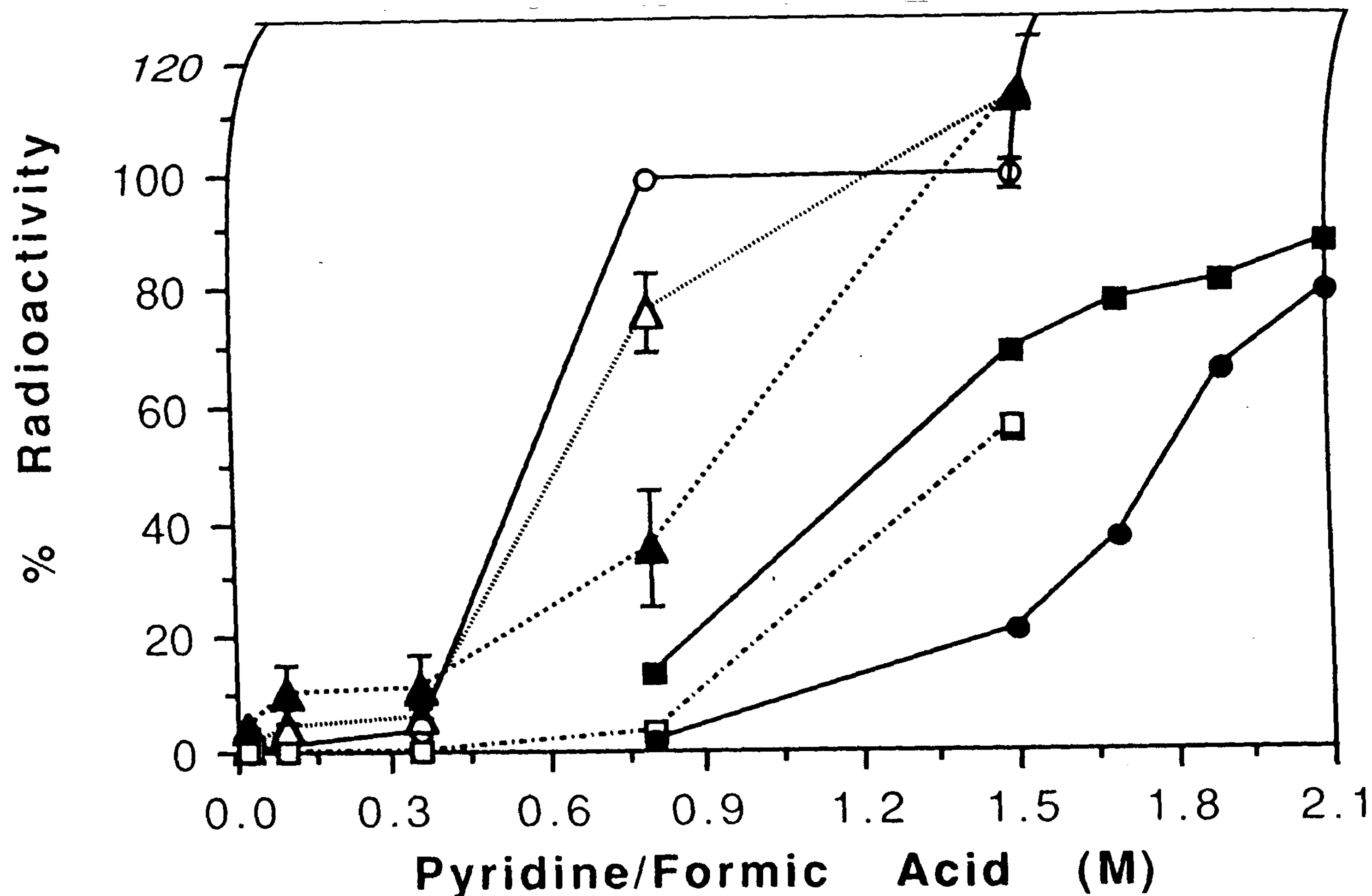


Fig. 3.4. The ion exchange chromatography (IEC) elution profile of ^{125}I-SP with different batches of SP Trisacryl M resin.

The elution profile of ^{125}I-SP was determined for four batches (i.e., separate containers) of SP Trisacryl M resin. The ^{125}I-SP was diluted to yield between 15,000 and 20,000 cpm/250µl. 250µl samples of ^{125}I-SP were then loaded onto prepared IEC columns containing either Batch 1, a mixture of Batch 1&2, Batch 2, Batch 3 or Batch 4 of the resin. The IEC was run in the usual manner (although more concentrated pyridine/formic acid solutions were used for Batch 3b and Batch 4) and the resulting fractions were collected and the radioactive content determined. This figure shows the elution of ^{125}I-SP (as % radioactivity [mean ± SEM, where applicable]) in relation to each of the IEC fractions (expressed as pyridine/formic acid [M]). (N=2 to 5). Legend; Batch 1 ——o——, Batch 1&2 ········△········, Batch 2 ·····▲·····, Batch 3a ·-·-□-·-·-, Batch 3b ——■——, Batch 4 ——●——.

(69.0%). Increasing the buffer concentration did consequently increase the recovery of TC, but even at the 2.1M buffer only 87.6% of TC were recovered.

The Batch 4 SP Trisacryl M resin was the same lot number as the Batch 3 resin, but these two batches were ordered and delivered on separate occasions. Despite originating from the same lot, Batch 3 and Batch 4 did reveal distinct ^{125}I-SP elution profiles (Fig. 3.4). A very low proportion of TC was recovered in the 0.8M IEC fraction (1.5%). The recovery of TC in the other IEC fractions was as follows; 1.5M (20.8%), 1.7M (37.1%), 1.9M (65.5%) and 2.1M (79.6%).

3.3.3 Evaluation of the Shift of Substance P on SP-Trisacryl M Resin.

3.3.3.i Influence of the Resin Washing Conditions Prior to the IEC Procedure.

The results have been presented in Figure 3.5 for the MeOH/HCl equilibration and Figure 3.6 for the 0.018M equilibration (100pg of SP was loaded onto the columns). When the MeOH/HCl was used for equilibration, substantial levels of SP appeared in the 0.018M and 0.1M fractions by the RIA (23.7 ± 1.4 pg and 31.9 ± 8.9 pg respectively). Slightly lower levels of SP were detected by the RIA in the subsequent three fractions; 0.35M (10.9 ± 7.3 pg), 0.8M (13.4 ± 5.8 pg) and 1.5M (13.4 ± 3.9 pg). A peak in the elution of SP was detected in the 1.7M IEC fraction (61.6 ± 4.8 pg). The level of SP detected in the final two fractions was found to decline; 1.9M (14.3 ± 2.1 pg) and 2.1M (2.1 ± 1.1 pg). However, the total SP detected by the RIA from all of the IEC fractions was 171.3 ± 35.3 pg.

The results for the 0.018M equilibration have been presented in Figure 3.6. Substantial levels of SP were detected in the 0.018M and 0.1M IEC fractions by the RIA (36.5 ± 17.5 pg and 30.5 ± 6.9 pg respectively). Lower levels of SP were detected in the 0.35M (3.9 ± 0.8 pg), 0.8M (6.2 ± 3.8 pg) and 1.5M IEC fractions (15.4 ± 1.6 pg), before a peak was detected in the 1.7M IEC fraction (68.1 ± 4.4 pg). Levels of the peptide were found to decline in the last two IEC fractions; 1.9M (5.4

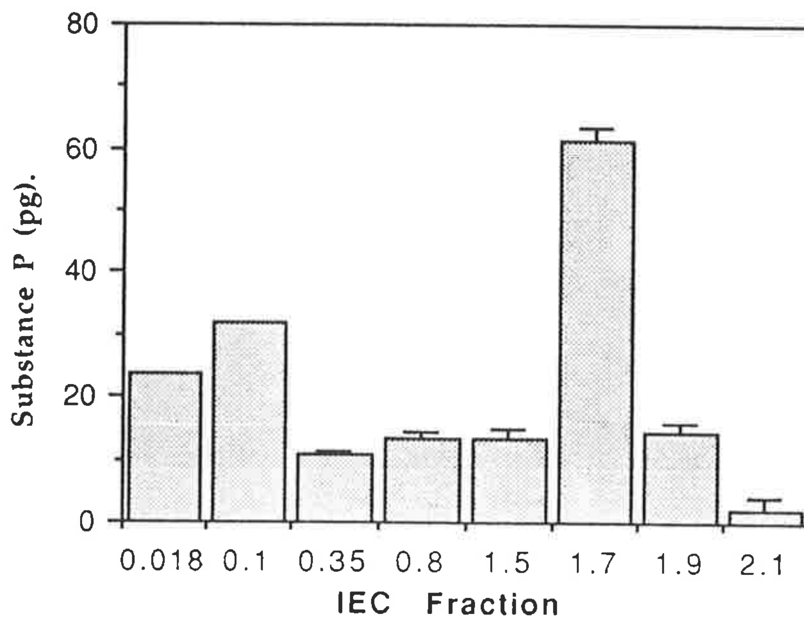


Fig. 3.5. Influence of resin washing conditions prior to ion exchange chromatography (IEC).

Batch 4 SP Trisacryl M resin was prewashed in 1M NaCl before loading onto the IEC columns (N=3). Columns were pre-equilibrated with 5:1 methanol/0.1M HCl. [Note; see text for the usual prewash conditions]. Substance P (100pg) was loaded onto the columns and the IEC method performed in the usual manner. All IEC fractions were collected and lyophilized and the SP content determined by RIA. The figure shows the recovery of SP (mean ± SEM pg) for each IEC fraction.

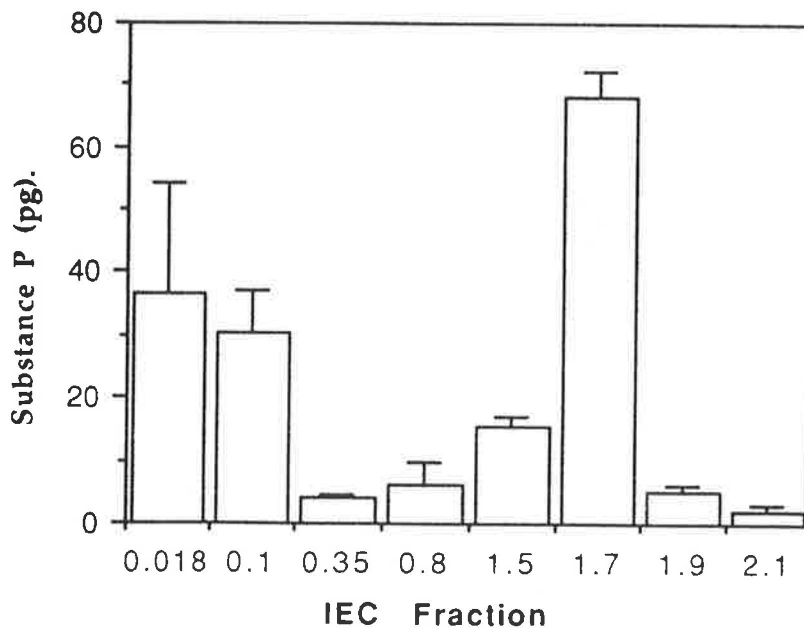


Fig. 3.6. Influence of resin washing conditions prior to ion exchange chromatography (IEC).

Batch 4 SP Trisacryl M resin was prewashed in 1M NaCl before loading onto the IEC columns (N=3). Columns were pre-equilibrated with 0.018M pyridine/0.018M formic acid (the first IEC buffer). Substance P (100pg) was loaded onto the columns and the IEC method performed in the usual manner. All IEC fractions were collected and lyophilized and the SP content determined by RIA. The figure shows the recovery of SP (mean ± SEM pg) for each IEC fraction.

$\pm$ 1.0 pg) and 2.1M (2.1 $\pm$ 1.0 pg). Total SP detected by the RIA from all of the IEC fractions was 168.1 $\pm$ 37.0 pg.

Overall, as can be seen in Figures 3.5 and 3.6, the resin washing conditions made very little difference to the elution profile.

3.3.3.ii Influence of the IEC Buffers.

Mean and SEM for the TC determinations was 23,772.8 $\pm$ 248.3 cpm. The stored buffer elution profile of ^{125}I-SP and the fresh buffer profile have been presented in Figure 3.7. Negligible levels of ^{125}I-SP were detected in the 0.018M, 0.1M and 0.35M IEC fractions for both the stored and fresh buffers.

Most of the ^{125}I-SP was eluted into the final two IEC fractions. Stored buffers resulted in recovery of 22.3 $\pm$ 1.7 % of TC in the 0.8M fraction and 68.7 $\pm$ 1.9 % in the 1.5M fraction. Elution with the fresh buffers yielded 9.0 $\pm$ 1.6% of TC in the 0.8M IEC fraction and 82.8 $\pm$ 1.8% in the 1.5M fraction.

Overall total recovery of added ^{125}I-SP from all of the IEC fractions was 93.6 $\pm$ 5.0% with stored buffers and 92.3 $\pm$ 3.9% with fresh buffers. Thus, the elution profile was similar in both conditions.

3.3.3.iii Influence of Pyridine.

Determination of TC revealed 31,299.5 $\pm$ 82.4 cpm. Figure 3.8 shows the elution profile of ^{125}I-SP with the old pyridine buffers. Negligible levels of TC were recovered in the 0.018M, 0.1M and 0.35M IEC fractions. Most of the TC were in the 0.8M (24.5%) and 1.5M (43.6%) IEC fractions. A small amount of TC were detected in the 1.7M fraction (3.5%), whilst very low levels were in the 1.9M and 2.1M fractions. Overall, 72.3% of the TC loaded onto the IEC columns were recovered.

The elution profile of ^{125}I-SP with the new pyridine buffers has been presented in Figure 3.8. Only traces of TC were detected in the 0.018M, 0.1M and 0.35M IEC fractions. The majority of the TC were in the 0.8M (28.3%) and 1.5M (39.3%) IEC fractions, with a small proportion in the 1.7M (2.4%) fraction. A small

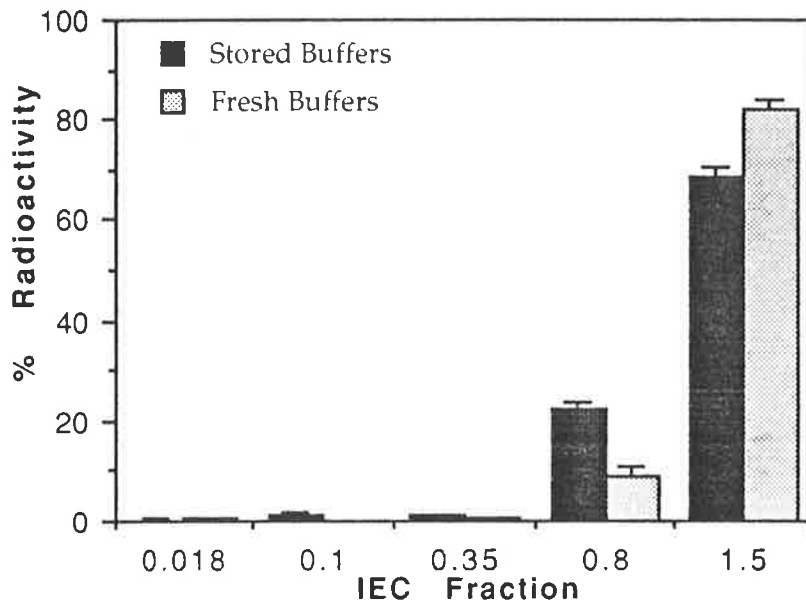


Fig. 3.7. Influence of fresh and stored buffer on the recovery of ^{125}I-SP.

IEC columns were prepared with SP Trisacryl M resin (Batch 2). Diluted samples of ^{125}I-SP were loaded onto the columns which were eluted with either fresh or stored IEC buffers (N=12). All IEC fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as % total radioactivity mean ± SEM) for each IEC fraction using either fresh or stored buffers.

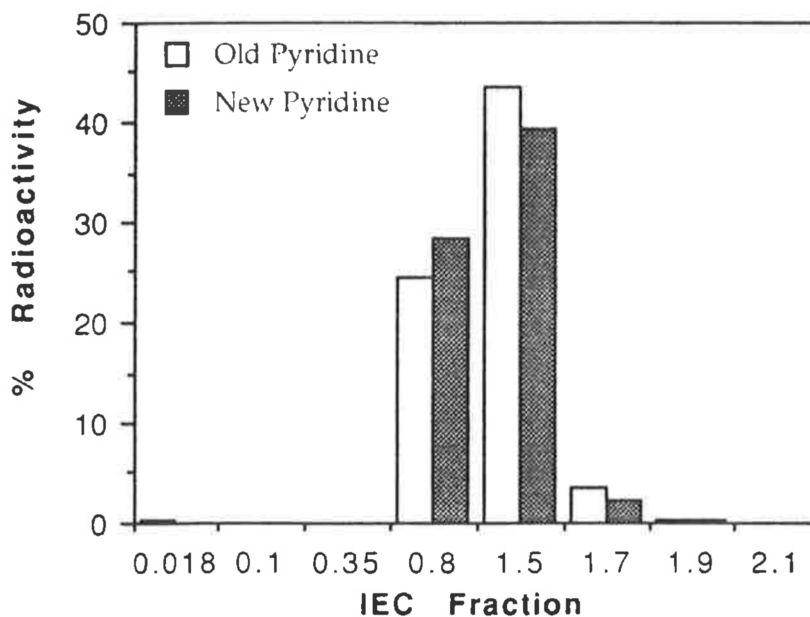


Fig. 3.8. Influence of old and new pyridine on the recovery of ^{125}I-SP.

IEC columns were prepared with SP Sephadex C-25 resin. Diluted samples of ^{125}I-SP were loaded onto the columns which were eluted with buffers made up with old or new pyridine stock (N=4). All IEC fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as mean % total radioactivity) for each IEC fraction using either old or new pyridine stock buffers.

amount of TC was in the 1.9M and 2.1M IEC fractions. Total TC recovered in the IEC fractions was 70.4%. Thus, the source of pyridine had little influence on the elution profile of ^{125}I-SP.

3.3.3.iv *Influence of Formic Acid.*

Determination of the TC revealed 30,292.9 ± 102.0 cpm. The elution profile of ^{125}I-SP with the old formic acid buffers revealed low levels of the peptide detected in the 0.018M, 0.1M and 0.35M IEC fractions (Fig. 3.9). Most TC were recovered in the 0.8M (21.8%) and 1.5M (40.9%) fractions, with a little detected in the 1.7M fraction (1.9%). Negligible counts were detected in the 1.9M and 2.1M IEC fractions. The total recovery of added ^{125}I-SP was 65.3%

A similar elution profile was observed with the new formic acid buffers (Fig. 3.9). The majority of the TC were recovered in the 0.8M and 1.5M IEC fractions (29.3 and 30.4% respectively). Negligible levels were detected in the remaining fractions; *i.e.*, 0.018M, 0.1M, 0.35M, 1.7M, 1.9M and 2.1M. Overall recovery of TC in the fractions was 60.5%. Thus, the source of formic acid had little influence on the elution profile of ^{125}I-SP.

3.3.3.v *Influence of Purified Laboratory Water.*

Determination of TC revealed 26,925 ± 873.4 cpm. The elution profile of ^{125}I-SP with the GDW buffers revealed low levels of the label in the 0.018M, 0.1M, 0.35M, 1.9M and 2.1M IEC fractions (Fig. 3.10). Most TC were recovered in the 1.5M (53.3 ± 2.4%) and 1.7M (39.3 ± 3.1%) fractions, with a little in the 0.8M fraction (1.1 ± 0.2%). The total TC recovered from the sum of all of the IEC fractions was 94.9 ± 6.1%.

The elution profile with the Milli-Q buffers was similar to that seen with the GDW buffers (Fig. 3.10). The majority of the TC were recovered in the 1.5M (56.5 ± 2.2%) and 1.7M (37.4 ± 2.9%) IEC fractions. The remaining fractions contained less than 1% of the TC each (*i.e.*, 0.018M, 0.1M, 0.35M, 0.8M, 1.9M and 2.1M) with the 0.1M and 0.35M fractions revealing no radioactive content at all.

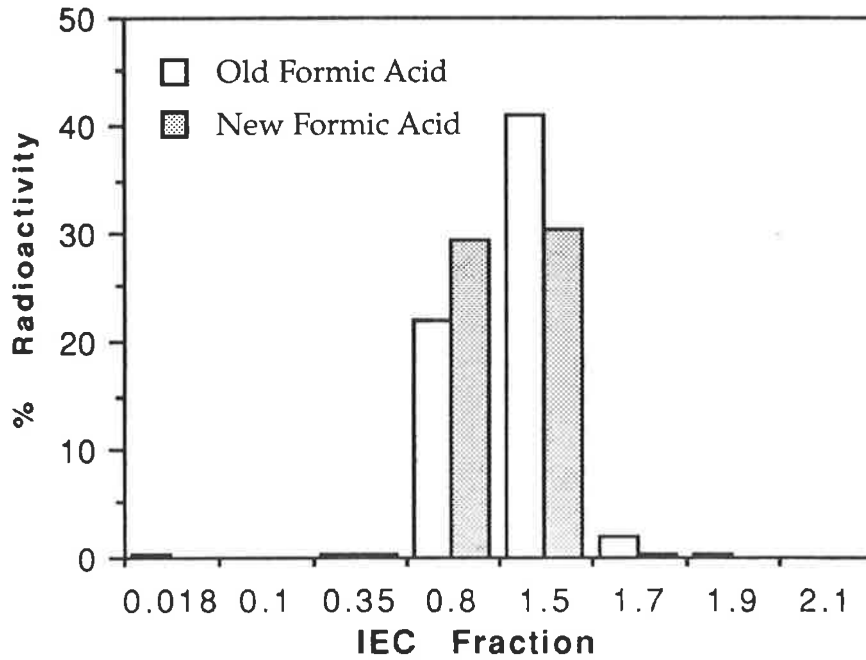


Fig. 3.9. Influence of old and new formic acid on the recovery of ^{125}I-SP.

IEC columns were prepared with SP Sephadex C-25 resin. Diluted samples of ^{125}I-SP were loaded onto the columns which were eluted with buffers made up with old or new formic acid stock (N=14). All IEC fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as mean % total radioactivity) for each IEC fraction using either old or new formic acid buffers.

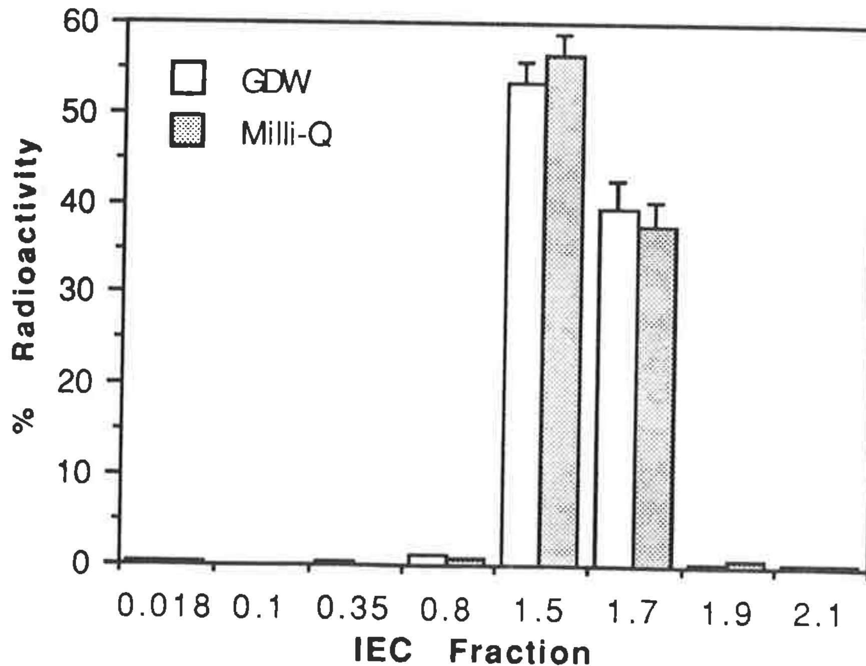


Fig. 3.10. Influence of water source on the recovery of ^{125}I-SP.

IEC columns were prepared with SP Trisacryl M (Batch 4) resin. Diluted samples of ^{125}I-SP were loaded onto the columns which were eluted with buffers prepared from either glass distilled water (GDW) or MilliQ water (N=8). All IEC fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as mean % total radioactivity ± SEM) for each IEC fraction using buffers prepared from either glass distilled water (GDW) or MilliQ water.

The total TC recovered from the sum of all of the IEC fractions was 95.7 $\pm$ 5.6%. Thus, the source of water had little influence on the elution profile of ^{125}I-SP.

3.3.3.vi Influence of IEC Column Material.

Determination of TC revealed 39,876 $\pm$ 346.7 cpm. The elution profile of ^{125}I-SP using glass IEC columns may be seen in Figure 3.11. The bulk of TC were recovered in the 1.5M IEC fraction (71.0 $\pm$ 1.3%), with a lesser proportion in the 0.8M (8.8 $\pm$ 0.4%) and 1.7M (13.4 $\pm$ 1.2%) fractions. No counts were detected in the 0.1M, 0.35M and 2.1M fractions, with very little in the 0.018M (0.3 $\pm$ 0.1%) and 1.9M (0.2 $\pm$ 0.0%) fractions. The overall recovery of TC from the column was 93.7 $\pm$ 3.0%. Thus, substitution of the column material did not significantly alter the elution profile of ^{125}I-SP.

3.3.3.vii Influence of the pH of the 0.8M IEC Buffer.

Determination of TC revealed 17,643 $\pm$ 135.3 cpm. The elution profile of ^{125}I-SP at the different pHs for the 0.8M IEC buffer (and 1.5M buffer) may be seen in Figure 3.12. The recovery of TC in the 0.8M fraction ranged from 24.7% to 33.6% depending upon the pH, whilst the 1.5M fraction TC recovery ranged from 55.4% to 73.6%. The exact TC recoveries have been summarized in Table 3.20 (next page). It is apparent that changes in pH had little effect on recovery in these two fractions.

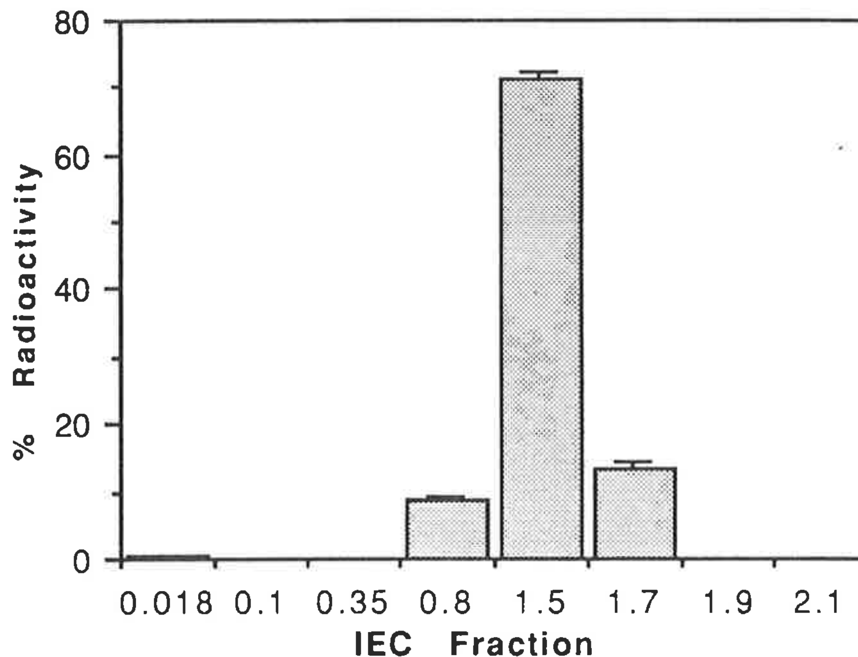


Fig. 3.11. Influence of IEC column material on the recovery of ^{125}I-SP.

The usual IEC columns composed of plastic syringes were substituted for glass columns containing glass wool in the base (N=3). SP Trisacryl M (Batch 4) resin was loaded onto the columns followed by the diluted ^{125}I-SP samples. The IEC method was performed in the usual manner and all fractions were collected and their radioactive content determined. The figure shows the recovery of ^{125}I-SP (as % radioactivity mean ± SEM) for each IEC fraction.

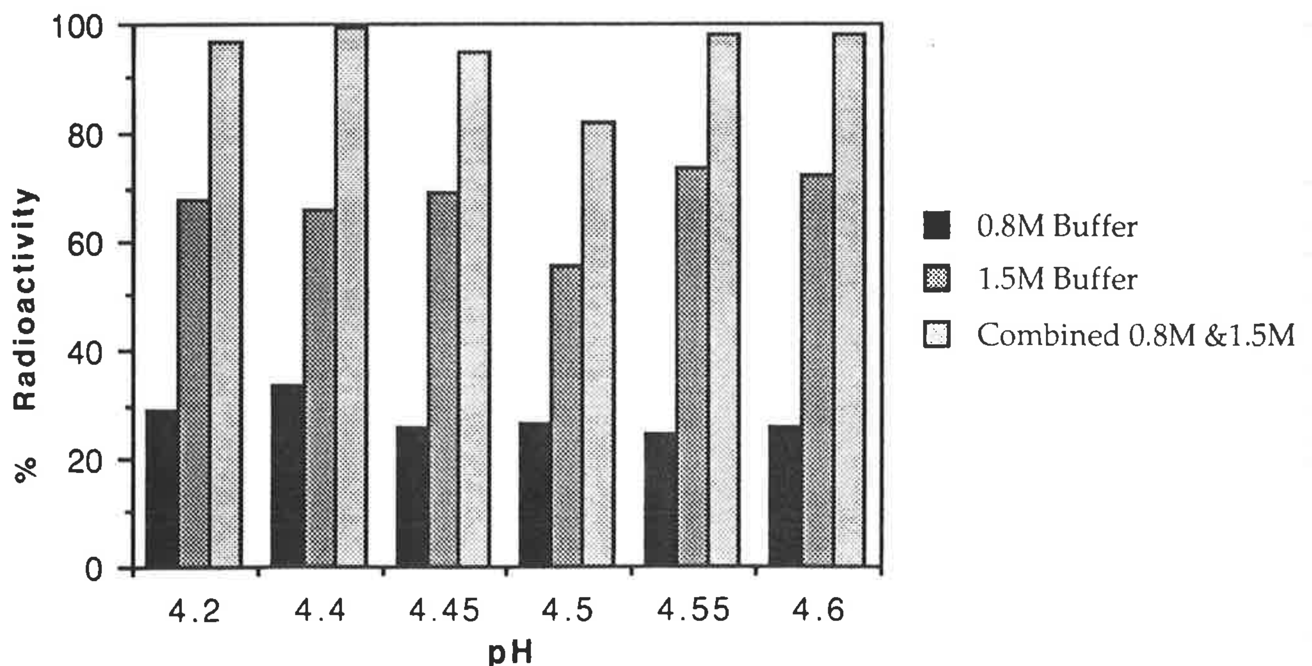


Fig. 3.12. Influence of the pH of the 0.8M IEC buffer on the recovery of ^{125}I-SP.

The IEC columns were loaded with SP Trisacryl M (Batch 2) resin followed by diluted ^{125}I-SP samples. The IEC method was performed in the usual manner up to the 0.8M IEC fraction. The columns were then eluted with a 0.8M buffer which had been adjusted to a pH of 4.2, 4.4, 4.45, 4.5, 4.55 or 4.6. The 0.8M and 1.5M IEC fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as % mean radioactivity; N=12) for the 0.8M, 1.5M and combined 0.8M & 1.5M IEC fractions at each of the pHs tested.

pH	*%TC in 0.8M*	*%TC in 1.5M*	*%TC in 0.8M+1.5M*
4.20	29.1	67.7	96.8
4.40	33.6	65.8	99.4
4.45	25.7	69.0	94.8
4.50	26.3	55.4	81.7
4.55	24.7	73.6	98.2
4.60	25.6	72.5	98.1

Table 3.20. Recovery of ^{125}I-SP in the 0.8M IEC fraction and the 1.5M IEC fraction at selected pHs.

3.3.3.viii Influence of the Volume of the 0.8M IEC Buffer.

Determination of TC revealed 31,093 ± 867.0 cpm. The elution profile for ^{125}I-SP with the different volumes of the 0.8M IEC buffer are shown in Figure 3.13. The elution of the radiolabel into the 0.018M, 0.1M and 0.35M fractions was negligible. When the usual elution volume of the 0.8M buffer was used (a total of 4ml), the recovery of the TC were as follows; 0.8M (1.7 ± 0.0%), 1.5M (49.7 ± 0.5%), 1.7M (38.9 ± 0.5%), 1.9M (0.5 ± 0.1%) and 2.1M (0.1 ± 0.0%).

Using a total volume of 6ml 0.8M IEC buffer resulted in a similar elution profile. The bulk of the TC were recovered in the 1.5M (51.8 ± 3.2%) fraction, with 33.6 ± 3.0% of TC found in the 1.7M fraction and 5.3 ± 0.4% of TC detected in the 0.8M fraction. Very little of the TC were recovered in the 1.9M (0.5 ± 0.2%) and 2.1M (0.1 ± 0.1%) fractions.

A similar profile was also obtained when double the usual volume of 0.8M buffer was used (*i.e.*, a total of 8ml: Fig.3.13). The majority of TC was recovered in the 1.5M IEC fraction (57.1 ± 3.3%). Radioactivity was also detected in the 1.7M (26.4 ± 1.5%), 0.8M (6.9 ± 0.1%), 1.9M (0.2 ± 0.1%) and 2.1M (0.1 ± 0.0%) fractions.

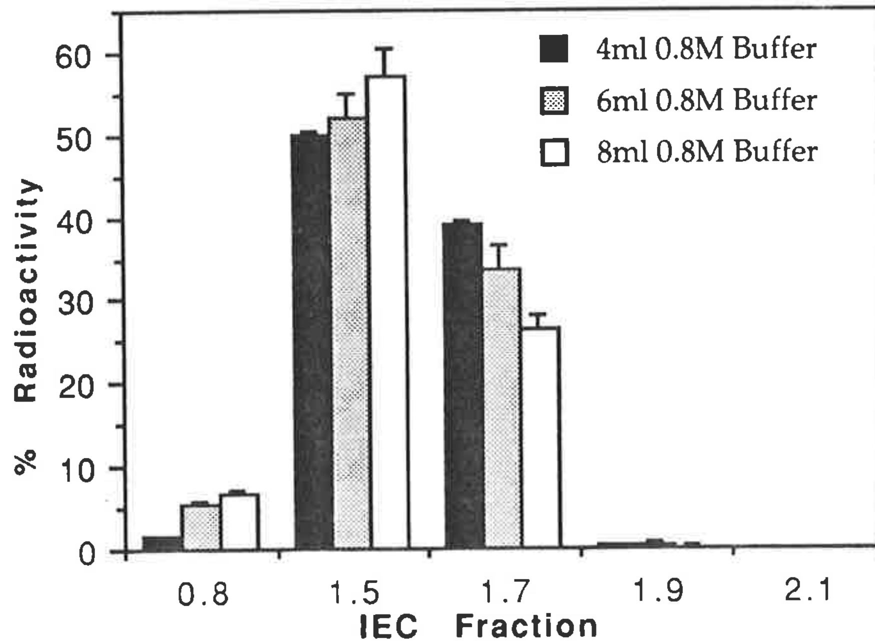


Fig. 3.13. Influence of the volume of the 0.8M IEC buffer on the recovery of ^{125}I-SP.

The IEC columns were loaded with SP Trisacryl M (Batch 4) resin followed by diluted ^{125}I-SP samples. The IEC method was performed in the usual manner up to the 0.8M IEC fraction. At this point 2ml, 4ml or 8ml of the IEC buffer was used for the elution (compared with the standard 4ml) and the elution method then continued in the usual manner. All fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as % mean radioactivity ± SEM; N=9) for each IEC fraction at each volume of 0.8M IEC buffer.

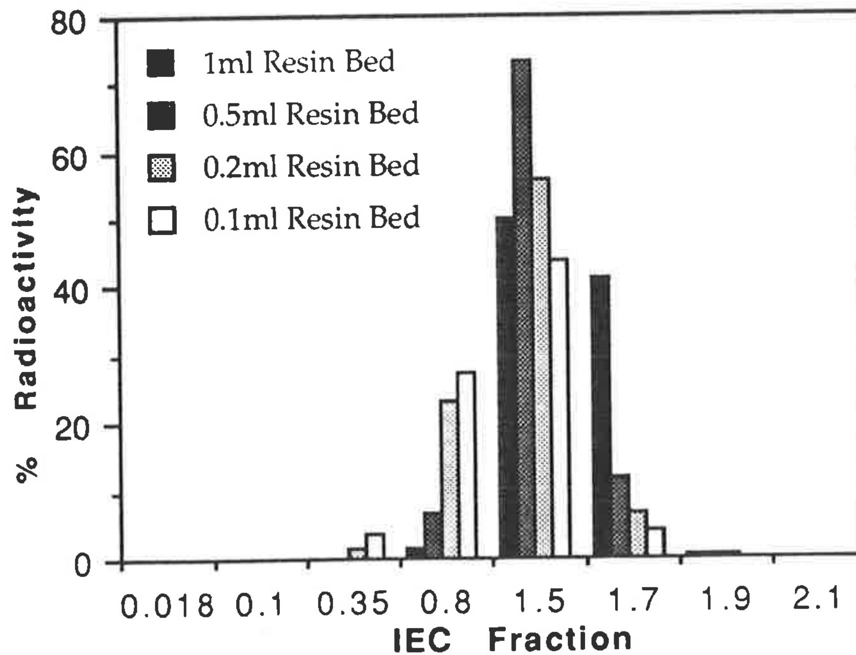


Fig. 3.14. Influence of the resin bed volume on the recovery of ^{125}I-SP.

The IEC columns were loaded with either 0.1ml, 0.2ml, 0.5ml or 1.0ml of SP Trisacryl M (Batch 4) resin (N=4 each) followed by diluted ^{125}I-SP samples. The IEC method was performed in the usual manner and all fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as % mean radioactivity ± SEM) for each IEC fraction at each resin bed volume.

Thus, increasing the volume of the 0.8M IEC buffer did not significantly improve the recovery of ^{125}I-SP in this fraction.

3.3.3.ix Influence of the Resin Bed Volume.

Determination of TC revealed 30,421 ± 512.3 cpm. The bulk of the TC was recovered in the 1.5M IEC fraction (50.0%) with a 1.0ml bed volume (Fig. 3.14). Two other fractions contained a proportion of the TC; 0.8M (1.3%) and 1.7M (41.2%). Negligible counts were detected in the 0.018M, 0.1M, 0.35M, 1.9M and 2.1M IEC fractions when the bed volume was 1.0ml.

The bulk of the recovery of TC was in the 1.5M fraction for all bed volumes; 0.5ml (73.2%), 0.2ml (56.0%) and 0.1ml (43.7%) (Fig.3.14). Other fractions with the radiolabel were the 1.7M fraction (0.5ml [11.8%], 0.2ml, [6.7%] and 0.1ml [4.1%]) and the 0.8M fraction (0.5ml [6.5%], 0.2ml [23.1%] and 0.1ml [27.3%]), with a small amount in the 0.35M fraction obtained from the 0.1ml bed volume (3.8%). Negligible levels of TC were recovered in the 0.018M, 0.1M, 0.35M, 1.9M and 2.1M fractions for all resin bed volumes used.

3.3.3.x Effect of Ascorbic Acid, Dithiothreitol and EDTA.

Determination of TC revealed 56,461.3 ± 131.9 cpm for the ^{125}I-SP dilutions in the 0.018M buffer, 38,653.9 ± 494.0 cpm for the ascorbic acid TC, 30,919.4 ± 151.4 cpm for the dithiothreitol TC and 28,594.3 ± 172.1 cpm for the EDTA TC.

The IEC elution profile of the radiolabel in the various diluents may be seen in Figure 3.15. In general, the profiles for the 0.018M dilution buffer, ascorbic acid and EDTA were almost identical. The 1.5M fraction had the bulk of the TC (58.0 ± 1.6% [0.018M dilution buffer], 59.6 ± 1.8% [ascorbic acid] and 56.3 ± 0.8% [EDTA]), with around a further third of the TC in the 1.7M fraction (32.3 ± 1.3% [0.018M dilution buffer], 33.3 ± 1.3% [ascorbic acid] and 32.0 ± 1.3% [EDTA]). The 0.8M fraction had low levels of TC; for the 0.018M dilution buffer it was 1.4 ± 0.0%, 1.5 ± 0.1% for ascorbic acid and 2.5 ± 0.2% for EDTA. The remaining 0.018M dilution buffer fractions had negligible levels of radioactivity. Similar low TC

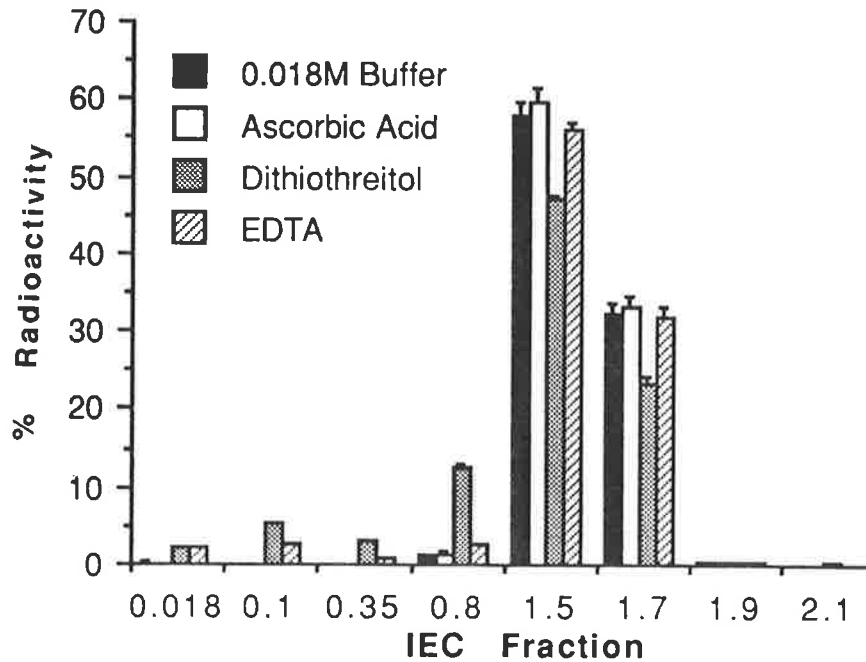


Fig. 3.15. Influence of ascorbic acid, EDTA and dithiothreitol on the recovery of ^{125}I-SP.

IEC columns were loaded with SP Trisacryl M (Batch 4) resin followed by diluted ^{125}I-SP which had been pre-mixed with either ascorbic acid (100µg/ml), EDTA (1mM) or dithiothreitol (1mM) (N=3 each). The IEC method was performed in the usual manner and all fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (as % mean radioactivity ± SEM) in the presence of either ascorbic acid, EDTA or dithiothreitol for each IEC fraction.

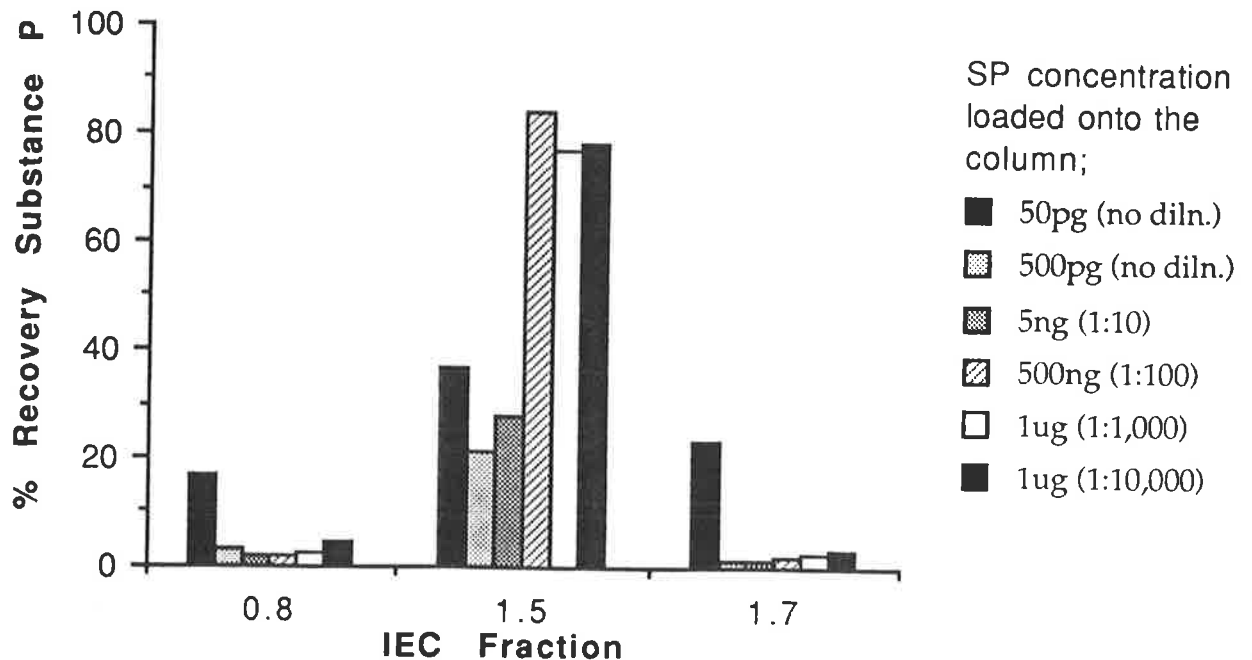


Fig. 3.16. Recovery of synthetic substance P (SP).

IEC columns were prepared with SP Trisacryl M (batch 4) resin. Synthetic SP was diluted (in the first IEC buffer) to yield samples containing 50pg, 500pg, 5ng, 500ng and 1µg of SP, which were loaded onto the columns. The IEC method was performed in the usual manner and the 0.8M, 1.5M and 1.7M IEC fractions were collected and lyophilized. The SP content was determined by RIA (samples > 1,000pg required dilution prior to RIA processing). The figure shows the recovery of SP (mean % ± SEM; N=12) for each concentration of SP in each of the IEC fractions.

recovery levels were in the 0.018M, 0.1M, 0.35M, 1.9M and 2.1M fractions for ascorbic acid and EDTA.

Dithiothreitol produced a roughly similar elution profile to the other agents (Fig. 3.15). The same general trend of the majority of the TC eluting into the 1.5M fraction was retained (47.3 ± 0.2%), but with slightly lower TC recovery in the 1.7M fraction (23.5 ± 0.6%). However, generally low recovery of TC was observed for the remaining fractions; 0.018M (2.4 ± 0.0%), 0.1M (5.6 ± 0.0%), 0.35M (3.1 ± 0.1%), 0.8M (12.4 ± 0.5%), 1.9M (0.4 ± 0.1%) and 2.1M (0.4 ± 0.0%).

Total recovery of TC from the IEC columns was as follows; for the 0.018M dilution buffer 92.5 ± 3.0%, the ascorbic acid columns were 95.2 ± 3.2%, 95.1 ± 1.5% for dithiothreitol and 96.8 ± 2.6% for EDTA. Thus, addition of any of these agents did not result in greater than 80% recovery of ^{125}I-SP in the 0.8M IEC fraction.

3.3.3.xi. Elution Profile of Synthetic Substance P.

In general, low recoveries were obtained for all concentrations of SP in the 0.8M IEC fraction (Fig. 3.16). They were 16.8% for 50pg, 3.3% for 500pg, 1.8% for 5ng (1:10 dilution), 2.2% for 500ng (1:100 dilution), 2.9% for 1µg (1:1,000 dilution) and 4.3% for 1µg (1:10,000 dilution).

The bulk of the peptide was recovered in the 1.5M IEC fraction (Fig. 3.16). The recoveries were 36.8% for 50pg, 21.5% for 500pg, 27.8% for 5ng (1:10 dilution), 83.9% for 500ng (1:100 dilution), 77.0% for 1µg (1:1,000 dilution) and 78.0% for 1µg (1:10,000 dilution).

Low levels of the peptide were found in the 1.7M IEC fraction, with the exception of the 50pg sample where 23.2% of the peptide was recovered (Fig. 3.16). Other values were 1.6% for 500pg, 1.3% for 5ng (1:10 dilution), 2.2% for 500ng (1:100 dilution), 2.3% for 1µg (1:1,000 dilution) and 3.0% for 1µg (1:10,000 dilution).

The overall recoveries of added SP to the IEC columns in the three assessed fractions (0.8M, 1.5M and 1.7M) were 76.8% for 50pg, 26.4% for 500pg, 30.9% for

5ng (1:10 dilution), 88.3% for 500ng (1:100 dilution), 82.2% for 1μg (1:1,000 dilution) and 85.3% for 1μg (1:10,000 dilution). Thus, poor recoveries were seen in the 0.8M IEC fraction regardless of whether radiolabelled or synthetic forms of the peptide were used.

3.3.4 Assessment of Post-IEC Variables.

3.3.4.i Effect of Drying on the Measurement of Substance P.

The results for this experiment may be seen in Figure 3.17. Determination of TC revealed 49,069.8 ± 466.5 cpm (*i.e.*, 100 ± 1.0%). Recovery of counts in the dried and reconstituted samples was 34,860.1 ± 2,119.2 cpm (71.0 ± 4.3%) and for the test tubes alone was 13,766.9 ± 2119.2 cpm (28.1 ± 4.3%). Therefore, the reconstituted samples taken together with the test tubes that the samples were reconstituted in accounted for 99.1 ± 8.6% of the TC. It appears that the bulk of the ^{125}I-SP was recovered in the reconstituted sample.

3.3.4.ii Effect of Test Tube Composition Used in the Reconstitution for the Measurement of Substance P.

Determination of TC revealed 8,930.6 ± 322.5 cpm. The results may be viewed graphically in Fig 3.18, or in Table 3.21 (next page).

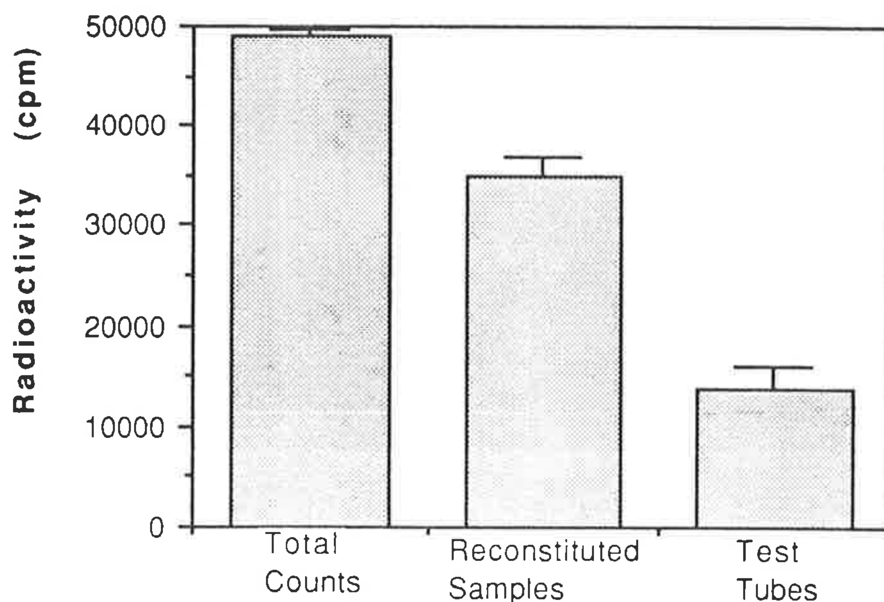


Fig. 3.17. Effect of vacuum drying on the recovery of ^{125}I-SP.

^{125}I-SP was diluted and 500µl aliquots were set aside for determination of total radioactive content (total counts; N=4), or placed in collection tubes and vacuum dried (N=4). Dried samples were reconstituted (500µl 1% acetic acid) and the radioactive content of the test tubes, reconstituted samples and total counts were determined. The figure shows the recovery of ^{125}I-SP (mean cpm + SEM) in reconstituted samples and the test tubes and in the total counts.

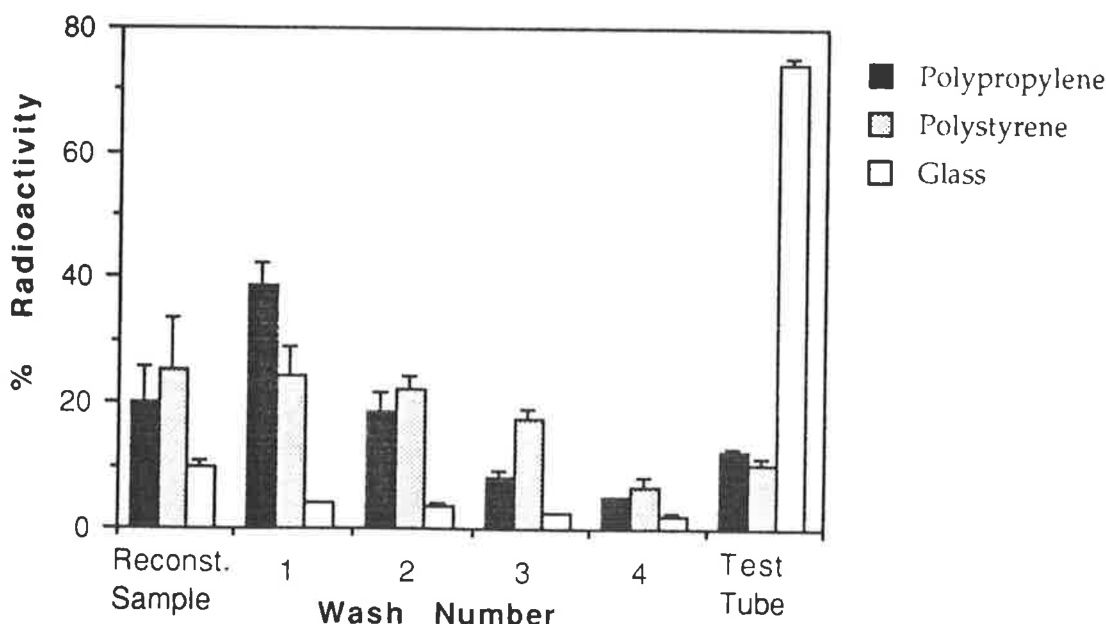


Fig. 3.18. Effect of test tube composition on the reconstitution recovery of ^{125}I-SP.

^{125}I-SP was diluted and 500µl aliquots were set aside for determination of total radioactive content (total counts;N=7) or placed in polypropylene (N=4), polystyrene (N=3) or glass test tubes (N=4). The samples were dried and reconstituted (see text for method) and this was followed by four subsequent washes which were also retained for determination of radioactive content. The figure shows the recovery of ^{125}I-SP (as % mean radioactivity ± SEM) for each type of tube for the reconstituted samples, subsequent washes and the test tubes.

Wash	*Polypropylene*	*Polystyrene*	*Glass*
Reconst.	20.3 $\pm$ 5.7	25.5 $\pm$ 7.9	10.0 $\pm$ 0.9
Wash 1	38.7 $\pm$ 3.4	24.4 $\pm$ 4.4	3.9 $\pm$ 0.4
Wash 2	18.4 $\pm$ 3.1	22.0 $\pm$ 2.1	3.6 $\pm$ 0.5
Wash 3	8.3 $\pm$ 1.1	17.8 $\pm$ 1.4	2.5 $\pm$ 0.1
Wash 4	5.1 $\pm$ 0.3	6.9 $\pm$ 1.1	2.2 $\pm$ 0.3
Test Tube	12.6 $\pm$ 0.4	10.3 $\pm$ 1.3	74.2 $\pm$ 1.3

Table 3.21. A summary of the TC recovered from the reconstitution and subsequent washings of dried ^{125}I-SP samples. TC remaining in the test tubes after the washes were also determined. (N=4, N=3 for polystyrene). Note Reconst. = reconstitution.

The glass test tubes retained a large proportion of the TC, even after the four washes (almost 75%; Fig. 3.18). Recovery of TC in the reconstitution sample was poor for the polypropylene or polystyrene tubes (roughly 20% and 25% respectively). Only a small proportion of TC were retained by the tubes themselves (compared with the glass test tubes). The polypropylene tubes retained 12.6% of the TC and the polystyrene tubes retained 10.3%.

Overall recovery of the TC (for all reconstitutions, washes and test tubes) was 103.4 $\pm$ 14.0% for the polypropylene samples, 106.9 $\pm$ 18.2% for polystyrene samples and 96.4 $\pm$ 3.5% for the glass samples. Thus, altering the test tubes used in the reconstitution did not improve recovery of the sample.

3.3.4.iii Influence of Reconstitution Volume for the Measurement of Substance P.

Determination of TC revealed 17,401.5 ± 283.5 cpm. The bulk of the radiolabel was in the reconstitution sample, regardless of the volume (Fig.3.19). 77.2 ± 2.0 % of TC were in the 250 µl volume and 76.0 ± 2.4 % in the 500 µl volume. A reasonable number of counts were in the subsequent initial wash (around 11-12% for both reconstitution volumes), with very few counts in the later washes. The tubes in which the samples were dried and reconstituted had less than 5.0% of the TC for both reconstitution volumes.

Overall recovery of TC was 97.4 ± 3.9% for the 250 µl reconstitution volume and 98.3 ± 4.7% for the 500 µl reconstitution volume. It appears that the reconstitution volume does not have a significant bearing on sample recovery.

3.3.4.iv Influence of the Acetic Acid Concentration on the Reconstitution and Subsequent Measurement of Substance P.

Determination of TC revealed 18,369.8 ± 230.8 cpm. The majority of the TC was in the reconstitution sample (Fig. 3.20). Exact values were; 1% acetic acid (85.6 ± 1.2%), 5% acetic acid (77.8 ± 3.2%), 10% acetic acid (87.1 ± 2.1%) and 5% formic acid (78.0 ± 1.5%). Most of the remaining counts were in the first wash; 1% acetic acid (9.0 ± 0.7%), 5% acetic acid (14.7 ± 2.5%), 10% acetic acid (5.0 ± 0.5%) and 5% formic acid (8.5 ± 0.8%). The later washes and the test tubes all contained less than 4% of the TC.

Overall TC recoveries were; 1% acetic acid (98.7 ± 2.1%), 5% acetic acid (100.6 ± 7.1%), 10% acetic acid (96.6 ± 3.3%) and 5% formic acid (92.1 ± 3.2%). Thus, acetic acid concentration had little effect on recovery.

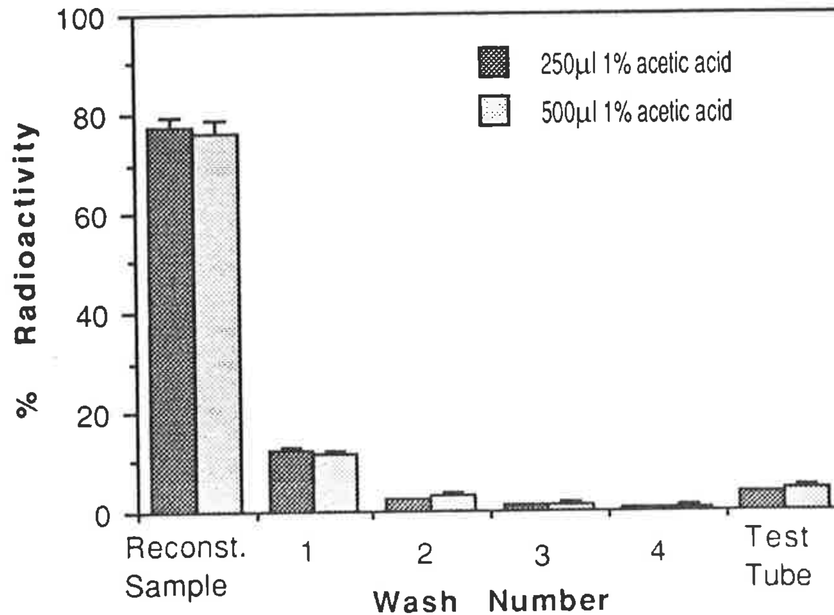


Fig. 3.19. Effect of reconstitution volume on the recovery of ^{125}I-SP.

^{125}I-SP was diluted and 500µl aliquots were set aside for determination of total radioactive content (total counts;N=6) or placed in collection tubes and vacuum dried. Samples were reconstituted with either 250µl (N=5) or 500µl (N=5) of 1% acetic acid. Following reconstitution, four subsequent washes were performed (with the same volume of 1% acetic acid) and retained. The figure shows the recovery of ^{125}I-SP (as % mean radioactivity ± SEM) for the reconstituted samples, subsequent washes and the test tubes when using a reconstitution volume of 250µl or 500µl 1% acetic acid.

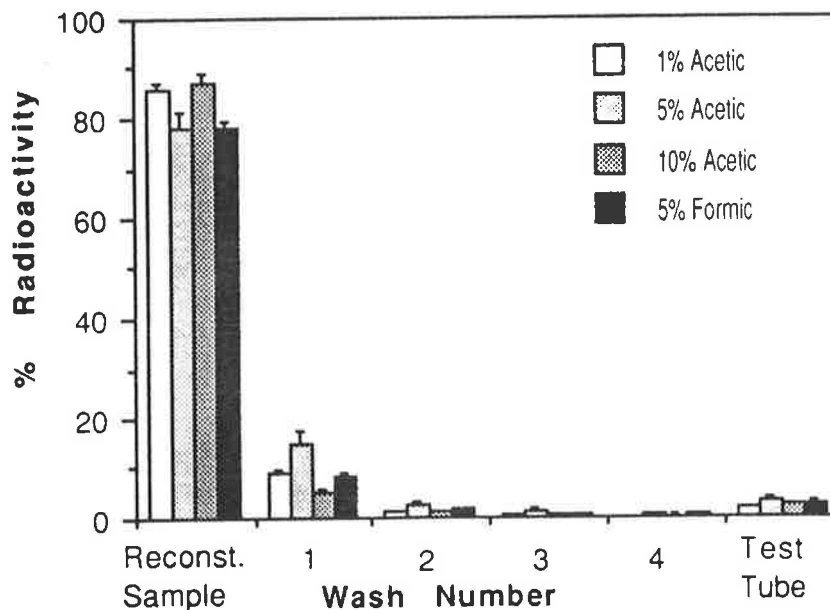


Fig. 3.20. Effect of acetic acid (HAc) concentration on the recovery of ^{125}I-SP.

^{125}I-SP was diluted and 500µl aliquots were set aside for determination of total radioactive content (total counts;N=9) or placed in collection tubes and vacuum dried. Samples were reconstituted with 500µl of either 1% HAc,. 5% HAc, 10% HAc or 5% formic acid (N=5 each), followed by four subsequent washes of 500µl of the same concentration of the same acid. The figure shows the recovery of ^{125}I-SP (as % mean radioactivity ± SEM) for the reconstituted samples, subsequent washes and the test tubes when using 500µl of either 1% HAc,. 5% HAc, 10% HAc or 5% formic acid.

3.3.4.v Influence of Reconstitution Time on the Subsequent Measurement of Substance P.

Determination of TC revealed 19,389.9 ± 150.8 cpm. The bulk of the TC was in the reconstituted samples (Fig.3.21). The immediate reconstitution had 85.4 ± 2.0% of the TC, whilst the 0.5 hour reconstitution had 89.0 ± 3.1% of the TC. Very similar retention values of the TC were seen in the first wash for both reconstitutions; 7.4 ± 0.8% for the immediate and 7.5 ± 2.4% for the 0.5 hour reconstitution. Low counts were in the remaining washes and the tubes themselves for both reconstitution conditions.

Overall recovery of TC was 100.2 ± 3.9% for the immediate reconstitution and 102.1 ± 6.5% for the 0.5 hour reconstitution. Thus, reconstitution time does not appear to influence the recovery of the sample.

3.3.5 Elution Profile of SP Sephadex C-25.

Determination of TC revealed 24,301.2 ± 536.2 cpm. The elution profile of ^{125}I-SP on SP Sephadex C-25 resin may be seen in Figure 3.22. The bulk of the TC eluted into two main IEC fractions. The 0.8M fraction had 51.6 ± 2.6% of the TC and the 1.5M fraction had 34.8 ± 2.4% of the TC. The 1.7M IEC fraction contained just under 2% of the TC (1.6 ± 0.2%), with the remaining fractions containing negligible levels of the radiolabel. The overall recovery of TC from the IEC column was 88.9 ± 5.2%. Thus, the majority of the ^{125}I-SP was recovered in the 0.8M IEC fraction with the remainder eluted in the 1.5M fraction.

3.3.5.i Influence of Pyridine/Formic Acid Residues in RIA Samples.

It was apparent that the RIA detected some "substance P" in all of the tested blank IEC fractions, even though no substance P was present (Fig.3.23). Nevertheless, the highest reading of SP in the blank IEC fractions was only 3.52 ± 0.55 pg SP in the 0.8M fraction. The results for each IEC fraction have been summarized in Table 3.22. It appears that the RIA does register some "substance P" in the blank IEC fractions, although readings were less than 5pg SP.

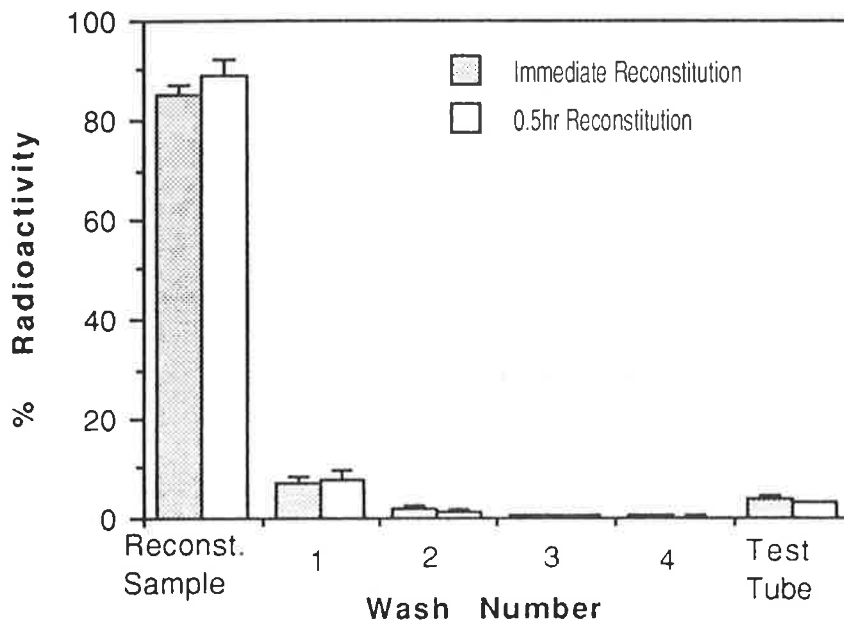


Fig. 3.21. Influence of reconstitution time on the recovery of ^{125}I-SP.

^{125}I-SP was diluted and 500µl aliquots were set aside for determination of total radioactive content (total counts;N=6) or placed in collection tubes and vacuum dried. Samples were reconstituted immediately with 250µl of 1% acetic acid and followed by the four subsequent washes (N=5), or left (after vortexing) for 0.5 hr before commencing the four subsequent washes (which were commenced immediately). The figure shows the recovery of ^{125}I-SP (as % mean radioactivity ± SEM) for the reconstituted samples, subsequent washes and the test tubes for the immediate and 0.5hr reconstitution times.

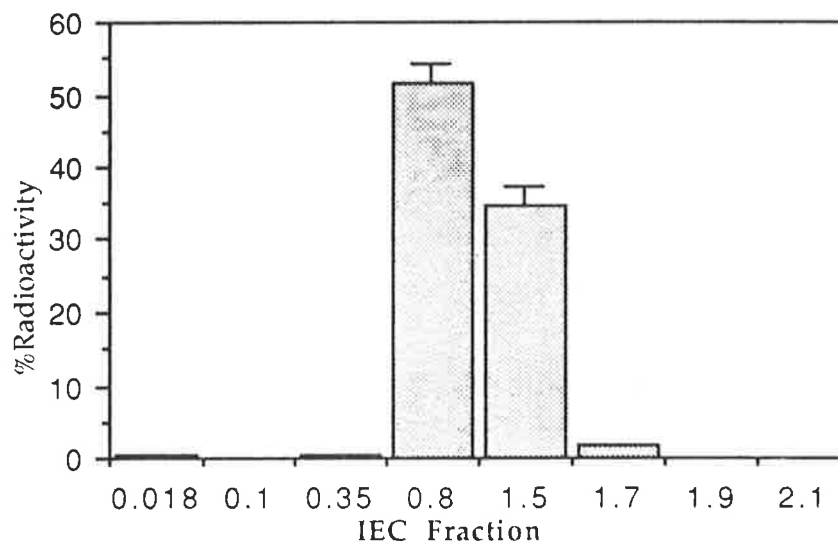


Fig. 3.22. Recovery of ^{125}I-SP from SP Sephadex C-25 resin.

IEC columns were prepared with SP Sephadex C-25 resin. Diluted samples of ^{125}I-SP were loaded onto the columns (or set aside for determination of radioactive content) and the IEC method was performed in the usual manner. All fractions were collected and the radioactive content determined. The figure shows the recovery of ^{125}I-SP (% mean radioactivity ± SEM) in each of the IEC fractions.

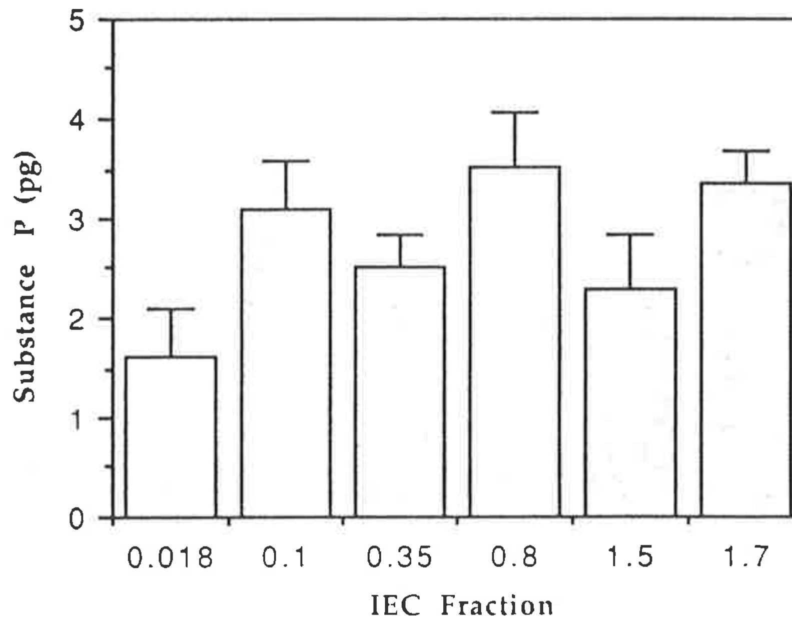


Fig. 3.23. Influence of pyridine/formic acid residues in the radioimmunoassay (RIA).

IEC columns were prepared with SP Sephadex C-25 resin. No samples were loaded onto the columns. The IEC method was performed in the usual manner and all fractions were collected and lyophilized. The "substance P-like" content was then determined. The figure shows the "substance P-like" content (mean pg ± SEM) of the blank run for each of the IEC fractions.

IEC Fraction	*SP Detected by the RIA (pg)*
0.018M	1.61 $\pm$ 0.50
0.1M	3.10 $\pm$ 0.49
0.35M	2.52 $\pm$ 0.55
0.8M	3.52 $\pm$ 0.55
1.5M	2.28 $\pm$ 0.56
1.7M	3.34 $\pm$ 0.33

Table 3.22. A summary of the "substance P" content of blank IEC fractions as detected by the RIA method from SP Sephadex C-25 columns (N=8).

3.4 DISCUSSION

The determination of the retention times for the HPLC techniques was necessary in order to identify the peaks obtained from the HPLC traces, since the RIA antibody could not discriminate between substance P, Tyr^8-SP and the fragments SP 3-11, SP 4-11, SP 5-11 and SP 6-11 (Chapter 2; 2.4 *Discussion*).

The IEC elution profile of SP alone on the SP Trisacryl M resin, in early experiments as determined by the HPLC techniques, revealed the majority of the peptide was in the 0.8M IEC fraction (99.5%; Fig.3.1). This was consistent with the results obtained by Bergstrom and colleagues (1983). The current SP Sephadex C-25 IEC elution profile of SP (as determined by HPLC) revealed a recovery of 87.8% in the 0.8M fraction, with the remainder of the peptide eluting into the 1.5M fraction (Fig.3.1). This resin was the same as that used by Bergstrom *et al.* (1983). Whether the recovery was similar for Bergstrom *et al.* is unknown as they did not quantify the recoveries of SP alone using the IEC technique in their paper.

However, the recovery of substance P which had been added to tissue samples and processed by IEC and RIA was in the vicinity of 70 to 80%

(Bergstrom *et al,* 1983). Whether this reduced recovery was due to tissue enzymatic degradation, the IEC separation procedure or even a limitation of the RIA (or perhaps a combination of these factors) was not discussed. Thus, the value of 87.8% recovery of SP in the 0.8M IEC fraction for SP Sephadex C-25 is consistent with the results of Bergstrom *et al.* (1983).

The most important finding from these HPLC analyses was that the elution of substance P was found to occur predominantly in one IEC fraction (*i.e.*, the 0.8M IEC fraction) for both resins. Nevertheless, SP Trisacryl M was superior to SP Sephadex C-25 in relation to recovery of SP, yielding 99.5% and 87.8% recoveries respectively. This result, in conjunction with the manufacturer's claims of high resolution and high flow rate in particular (IBF Product Catalogue, 1987), led to the selection of the SP Trisacryl M resin for the IEC procedure.

Furthermore, the IEC elution profile of SP in the presence of the related peptides was similar to that of SP alone for SP Trisacryl M (Fig.3.2 and Fig.3.1 respectively). The IEC profile for SP and related peptides on the SP Sephadex C-25 resin resulted in a spread of SP into three IEC fractions (Fig.3.3) with only 57.7% recovery in the 0.8M IEC fraction. These results further supported the selection of SP Trisacryl M as the resin for the IEC procedure.

Rather than assess the elution profile for each of the fragments of SP and Tyr^8-SP (which is not an endogenous peptide), it was of greater importance to ascertain the degree of elution of these fragments into the 0.8M IEC fraction. It was known that the antibody in the RIA could not discriminate between SP and these fragments. Therefore, if they all eluted into the 0.8M IEC fraction the RIA would be a measuring SP and related peptides, rather than SP alone.

The elution of the various peptides into the 0.8M IEC fraction for SP Trisacryl M was SP (99.4%), Tyr^8-SP (100%), SP 3-11 (21.6%), SP 4/5-11 (2.7%) and SP 6-11 (0.7%). For SP Sephadex C-25 it was SP (57.7%), Tyr^8-SP (85.9%), SP 3-11 (97.1%), SP 4/5-11 (1.5%) and SP 6-11 (0.7%). The elution of Tyr^8-SP was not a consideration since it is not a naturally occurring peptide. The elution of SP 3-11 was considerably lower in the SP Trisacryl M resin, but there were generally low

levels of SP 4/5-11 and SP 6-11 eluted from both resins. These results also suggested SP Trisacryl M to be the better resin for use in the current study.

Thus, SP Trisacryl M resin was selected and used for the IEC procedure, but it later became apparent that somewhat unusual data was being generated from the IEC technique. It was noted that results became more anomalous with each new "batch" of the SP Trisacryl M resin.

Comparisons of the elution profile with the different batches of resin revealed startling differences (Fig.3.4). The elution profile with the original batch of resin revealed recovery of 95.5% in the 0.8M fraction. This was consistent with the HPLC results presented and with Bergstrom *et al.* (1983). With the next batch of resin (Batch 2), only 24.2% of the radiolabel was eluted into the 0.8M fraction. Batch 3 resin retained between 3 and 13% and Batch 4 resin retained less than 2%. (It must be noted that the change in the elution profile of the resin was first observed with the Batch 2 resin; the Batch 3 and Batch 4 resins were subsequently ordered specifically to obtain these IEC profiles).

It was obvious that the elution profile had changed significantly with each new batch of resin. It was no longer possible to isolate SP into a single *consistent* IEC fraction, or know if any of the fragments were also eluting into this fraction (and in what proportion). Slightly different IEC starting conditions would have occurred with the different diluents used, but the first 2ml application of the 0.018M IEC buffer would equilibrate all columns to the same conditions. As the 0.8M-1.7M buffer range of the elution profile was of most concern, these different diluents would not affect this range. It was also unclear as to why the Batch 3 and Batch 4 resins (from the same lot number) had such different IEC elution profiles (Fig.3.4). The only difference between these two batches was that Batch 4 was ordered and delivered 3 months after Batch 3.

Before the resin could be held responsible for the paradoxical shift of the IEC elution profile of SP, other factors which may have affected the IEC profile had to be excluded. A major portion of the work presented in this chapter was therefore devoted to this task.

The first component examined was the preparation of the resin. Early experiments followed the pre-wash method of Bergstrom *et al.* (1983) (see Table 3.1). Later experiments used the pre-wash conditions recommended by the manufacturer of the resin (IBF Biotechnics) on the Batch 4 resin (summarized in section *3.2.7.i).*

Neither of these resin preparation methods succeeded in recovering the major proportion of the SP in the 0.8M IEC fraction (Fig.3.5 and Fig.3.6). The recovery in this fraction was only 13.4 $\pm$ 5.8pg and 6.2 $\pm$ 3.8pg for the MeOH and 0.018M pre-equilibrations respectively. The IEC fraction with the best recovery was the 1.7M fraction for both pre-equilibrations (between 60% and 70%; Fig.3.5 and Fig.3.6). Thus, changing the pre-wash conditions of the resin did not change the IEC elution profile back to the original (almost total) elution of SP into the 0.8M IEC fraction.

It was also suspected that the stored IEC buffers may have become contaminated with bacteria and that this may have altered the IEC elution profile. Considering that the original IEC elution profile yielded greater than 95% recovery in the 0.8M IEC fraction, recoveries of 22.3% and 9.0% in this fraction using the stored and fresh buffers respectively did not improve the profile. Almost total recovery was obtained with the 1.5M IEC buffer (Fig.3.7). This was to be expected from the IEC elution profile generated previously on the Batch 2 resin (Fig.3.4). Thus, preparing fresh IEC buffers for each IEC procedure would not return the IEC elution profile of the peptide to its original form.

The individual components of the IEC buffers (i.e., pyridine and formic acid) were also examined for a possible contamination of stock solutions, which would subsequently be carried through to both stored and freshly prepared IEC buffers. Essentially, the greater proportion of the peptide was recovered in the 1.5M IEC fraction, regardless of whether fresh or old stock of pyridine or formic acid was used to prepare the IEC buffers (Fig.3.8 and Fig.3.9). Thus, the aim of total recovery in the 0.8M IEC fraction was not achieved by the introduction of fresh sources of stock solutions.

A study comparing the IEC elution profiles of buffers prepared with GDW with buffers prepared from Milli-Q water revealed less than 2.0% recovery in the 0.8M IEC fraction, regardless of the water source used (Fig.3.10). Nearly all of the peptide was recovered in the 1.5M and 1.7M fractions (Fig.3.10). Thus, changing the water source for the preparation of the IEC buffers did not revert the IEC elution profile of the peptide back the original profile (maximum yield of the peptide in the 0.8M fraction).

The next variable examined was the composition of the IEC column itself. The usual columns were composed of plastic (2ml disposable syringes). Glass was substituted for plastic to determine the effect on the IEC elution profile. Recovery of the radiolabel in the 0.8M IEC fraction was still only a low 8.8% (Fig.3.11). The major recovery was in the later 1.5M (71.0%) and 1.7M (13.4%) IEC fractions (Fig.3.11). Hence, substitution of glass for plastic as the support material for the column did not result in a return to the original IEC elution profile.

Although Bergstrom *et al.* (1983) specified that the pH of the 0.8M IEC buffer should be pH 4.4, the effect of altering the pH on the IEC elution profile was examined. The chosen pH range was pH 4.20 to 4.60. The IEC elution profile for the 0.8M IEC fraction did not alter over this pH range (Fig.3.12). Recoveries ranged from 24.7% to 33.6% (with no specific trend) in the 0.8M fraction. Better recoveries were obtained in the 1.5M IEC fraction (Fig.3.12). Most of the radiolabel was recovered within the 0.8M and 1.5M IEC fractions when combined (the 1.5M fraction had around 60%). Nevertheless, manipulating the pH did not alter the IEC elution profile to obtain maximum revovery of the peptide in the 0.8M IEC fraction.

The usual volume of the 0.8M buffer used in the IEC procedure was 4ml (2 x 2ml), but the effect of increasing this volume to 6ml and 8ml was also examined. The recovery of the radiolabel in the 0.8M IEC buffer did increase with increasing volume of the elution buffer, but the increases were only relatively small (Fig.3.13). Extrapolating from these improvements with increasing volume, over 100ml of the IEC buffer may be expected to fully elute the peptide into the 0.8M

IEC buffer. However, there were no guarantees that further increases in the volume of buffer used would result in further increases in the recovery of the peptide. Essentially, doubling the volume of the 0.8M IEC buffer did not improve the recovery of the peptide in the 0.8M IEC fraction anywhere close to the initial recovery of 95% or greater.

The resin bed volume was decreased to determine whether the capacity of the resin to bind the peptide was a reflection of the binding sites available. (This was more readily determined by reducing the resin bed volume rather than increasing it). In general, the trend observed was an increase in the recovery of the peptide in the 0.8M IEC fraction, with a decrease in the resin bed volume (Fig.3.14). However, the sample size for each volume was only one, so whether this trend represented a real effect was unknown. In any case, the maximum recovery of the radiolabel (27.3%) remained well short of the expected 95% for the 0.8M IEC fraction. Nevertheless, this study demonstrated that reducing the resin bed volume did not adversely affect the recovery of the peptide in the 0.8M IEC fraction. This suggested that the capacity of the resin to bind the peptide at the 1.0ml bed volume used for the IEC procedure would not be exceeded under experimental conditions and would thus be sufficient for the proposed experiments.

The IEC elution profile was also determined in the presence of various antioxidants (ascorbic acid and dithiothreitol) and a heavy metal chelator (EDTA) to ascertain whether they would help the elution of the peptide into the 0.8M IEC fraction. The recoveries obtained in the presence of these chemicals in the 0.8M IEC fraction were low under all conditions (Fig.3.15). The majority of the radiolabel was recovered in the 1.5M IEC fraction, (recoveries ranged from 47.3% to 59.6%), while approximately one third of the label was recovered in the 1.7M IEC fraction (Fig.3.15). Thus, the addition of any of these agents to the samples loaded onto the IEC column did not effectively improve the recovery of the peptide to the original 95% or give greater recovery in the 0.8M IEC fraction.

In the event that it was a difference between the batches of radiolabel which were affecting the outcome of the IEC profiles, the profile of non-endogenous, non-radiolabelled SP was determined. Concentrations of the peptide used were 50pg, 500pg, 5ng and 1μg.

At all concentrations, the IEC elution profile revealed low recoveries in the 0.8M IEC fraction, high recoveries in the 1.5M fraction, and low recoveries in the 1.7M IEC fraction (Fig.3.16). This result was really no different from the IEC elution profiles obtained with ^{125}I-SP (*i.e.*, Figs.3.10, 3.11, 3.12, 3.13, 3.14). Therefore, it did not matter whether the IEC elution profile was determined with the synthetic form of the peptide or the radiolabelled form of the peptide. However, in both cases, the IEC elution profile of the peptide still demonstrated a distinct shift to the right from its initial counterpart.

During the course of the study, various post-IEC variables were also examined; *i.e.*, the effect of drying on the samples, the test tube composition used during reconstitutions, the reconstitution volume, the acetic acid concentration used in the reconstitution and the time allowed for the reconstitution.

After vacuum drying, around 70% of the original sample was retained in the resulting liquid following reconstitution (Fig.3.17). The remaining 30% of the sample remained in (or bound to) the test tube. In an effort to maximize the recovery of the dried sample in reconstituted form, the other post-IEC variables were examined further.

The recovery of a reconstituted sample was determined for polypropylene (the usual reconstitution test tubes), polystyrene and glass test tubes. Glass test tubes retained a high proportion of the sample even after four washes (around 75%; Fig.3.18). The reconstitution recovery was similar for both polypropylene and polystyrene test tubes (Fig.3.18). However, the polypropylene tubes held a 4ml volume easily, whilst the polystyrene tubes could not. Furthermore, the polypropylene tubes were more pliable and less prone to damage during the vacuum drying process. Therefore, the polypropylene tubes continued to be used to perform the reconstitutions.

An assessment of the reconstitution volume used compared a 250 µl and 500 µl aliquots (Fig.3.19). Just over 75% of the dried sample was retained in the reconstituted sample for both volumes. Nevertheless, the 500 µl reconstitution volume was retained for reconstitutions since the preparation of the RIA standards was based upon assaying 200 µl samples. Changing the reconstitution volume to 250 µl would have also meant reducing the RIA assay sample size and therefore reducing the overall volume of the RIA standards.

Increasing the concentration of acetic acid used in the reconstitution did not change the recovery of the dried sample in the reconstitution. Values ranged from 77.8% to 87.1% (Fig.3.20). Thus, the 1% acetic acid was retained for the reconstitution of dried samples prior to the RIA. The timing of reconstitution did not alter recovery (Fig. 3.21) and a standard time of 0.5 hours after the addition of 1% acetic acid was used for all experiments.

Finally, the IEC elution profile of SP Sephadex C-25 was determined, since the shift of the elution profile on the SP Trisacryl M resin could not be explained. It was found that 51.6% of the radiolabel appeared in the 0.8M IEC fraction, with a further 34.8% eluting into the 1.5M IEC fraction (Fig.3.22). The earlier HPLC determinations had revealed a recovery of 87.8% in the 0.8M IEC fraction and 10.5% in the 1.5M fraction (Fig.3.1). The later 0.8M recovery values were lower than expected but, the overall recovery in the 0.8M and 1.5M fractions together was high (over 85%). As the SP Sephadex C-25 IEC elution profile remained relatively unchanged and since the Bergstrom method (1983) upon which this procedure has been based utilized this particular resin, the SP Sephadex C-25 resin was selected to replace the SP Trisacryl M resin.

The subsequent SP Sephadex C-25 resin IEC procedure did contain one modification; *i.e.*, the sequence of elution buffers was 0.018M, 0.1M, 0.35M and 1.5M. This was to remove the fragments in the earlier IEC buffers and to recover the substance P in one whole IEC fraction. With the SP Sephadex C-25 resin, SP eluted into the 0.8M and 1.5M IEC fractions. Therefore omitting the 0.8M buffer

eluted all of the SP into the 1.5M fraction. Thus, the 0.8M buffer was not used on the SP Sephadex C-25 IEC columns.

The final study assessed the "inherent pseudo-substance P" elutions from the SP Sephadex C-25 columns. Blank SP Sephadex C-25 columns were processed in the usual manner and the fractions assayed for SP content by the RIA. This gave an indication of interference in the assay due to the IEC buffers which may be obtained from non-SP-containing samples.

Low levels of SP were detected in all IEC fractions (Fig.3.23). Nevertheless, these values were all below 4pg. The background level for the RIA has therefore been set at 5pg. Thus, any reading obtained by the RIA of 5pg or less was acknowledged as a zero reading. For the animal studies in Chapter 8, the background value for the RIA was set at 10pg.

In summary, the HPLC determinations of the IEC profiles of substance P and related peptides on SP Trisacryl M and SP Sephadex C-25 IEC columns indicated that the SP Trisacryl M resin was better suited for the current body of work. However, continued use of the SP Trisacryl M resin resulted in inconsistent sets of data. Examination of those data in relation to the dates of delivery of new batches of the resin suggested a connection. Determination of the IEC elution profile for different batches of the SP Trisacryl M resin revealed a paradoxical shift of the elution profile. Variables examined as a possible cause of this shift were;

resin washing conditions
contamination of the IEC buffers
contamination of pyridine or formic acid stock
contamination of water
composition of the IEC column
manipulation of the pH of the 0.8M buffer
elution volume of the 0.8M buffer
resin bed volume

effect of ascorbic acid, dithiothreitol and EDTA

contamination of ^{125}I-SP.

None of these factors appeared to be the cause of the shift in the IEC elution profile of the peptide. This suggested that there may have been a change in the inherent characteristics of the resin itself to account for the shift. Whatever the reason, it was no longer possible to elute SP into one consistent IEC fraction when the SP Trisacryl M resin was used in the IEC columns.

Thus, the IEC elution profile was determined for the SP Sephadex C-25 resin. Although the profile obtained with this resin was not perfect, it was a better option than the SP Trisacryl M resin. Hence, the SP Trisacryl M resin was replaced with the SP Sephadex C-25 resin in the later IEC procedures.

Assessment of the post-IEC procedures determined that the reconstitution conditions proposed for the dried samples prior to the RIA were well suited to obtain maximum recovery in the reconstituted sample. However, total recovery from the dried sample was not possible, with only around 85% recovery in the reconstituted sample being attained.

CHAPTER 4

THE METABOLISM OF SUBSTANCE P.

4.1 BACKGROUND AND AIMS

Bearing in mind that the primary aim of this project was to determine the level of substance P in tissues taken from SHRs and WKYs (Chapter 1; 1.7.5 *Aims of the Present Study*), it was wise to ascertain the stability of substance P in physiological conditions. Ultimately, the half life of the compound would be determined by the degradation rate of the peptide in tissues and plasma.

Synthetic substance P is degraded in both animal and human blood or plasma in a time- and temperature-dependent manner (Boileau *et al.*, 1970; Bury and Mashford, 1977a; Lembeck *et al.*, 1978; Berger *et al.*, 1979; Couture and Regoli, 1981; Conlon and Sheehan, 1983; Conlon and Goke, 1984; Theodorsson-Norheim *et al.*, 1987). Pernow (1983) has noted that endogenous substance P may be degraded in plasma in a manner which is different to that observed with the exogenous peptide.

Substance P is a substrate for a number of enzymes which have been noted in Chapter 1 (1.6 *Metabolism of Substance P*), although the relative contribution of these enzymes in the metabolism of substance P *in vivo* has not been fully elucidated.

The following experiments were designed to characterize the metabolism of substance P, the kinetics of metabolism and the nature of the metabolic products utilizing radiolabelled, exogenous and endogenous forms of the peptide. Human plasma was chosen as a representative physiological source of enzymes due to its easy availability and uncomplicated incubations and procedures. It was assumed animal plasma would contain similar degradative enzymes and kinetics for substance P as the human plasma.

4.2 MATERIALS AND METHODS

4.2.1 Materials and Chemicals.

10ml heparinized polyethylene tubes (Labsupply, Adelaide, Australia). Reagents required as per the IEC procedure (Chapter 3; *3.2.1.i Materials and Chemicals for the Basic IEC.*) and RIA method (Chapter 2; *2.2.1.i Materials and Chemicals for the Basic RIA.*). Substance-P,[2-L-Prolyl-3,4-^{3}H(N)]- (NEN, DuPont, C.A., U.S.A; ^{3}H-SP), scintillant (see *Appendix III*).

4.2.2 Source of Plasma Samples.

The following set of experiments were performed as three discrete subsections; using (a) radiolabelled substance P (^{3}H-SP and ^{125}I-SP), (b) synthetic substance P and (c) endogenous substance P. [(a), (b) and (c) will be used to denote the different sub-sections hereafter.]

Section (a): All plasma samples were obtained from the same subject; a healthy, normotensive, 39 year old male.

Section (b): Plasma samples were collected from 4 healthy, normotensive individuals (2 male, 2 female; mean age 32.3 $\pm$ 5.1 years).

Section (c): Plasma samples were collected from 5 healthy, normotensive individuals (3 male, 2 female; mean age 33.6 $\pm$ 4.6 years).

All plasma samples were collected between 8.50 and 10.30 in the morning. The four subjects in (b) and (c) were the same individuals with an extra male recruited into (c).

4.2.3 Protocol.

A flow chart of the basic protocol has been presented below.

TIME (MIN).	PROTOCOL.
0	Collect blood (50ml) in heparinized tubes. Centrifuge and separate plasma samples (400µl). Place on ice (4°C) or keep at room temperature (21°C). Add (a) ^{3}H-SP or ^{125}I-SP (b) synthetic substance P or (c) no addition.
20 40 60 90 120 180 240	Add 1.2ml ice-cold 5:1 MeOH/0.1M HCl and vortex. Centrifuge (at 13G for 20 mins at 4°C) and collect the supernatant. Add 1.6ml 0.018M pyridine buffer. Perform IEC method (Chapter 3; *3.2.3 The Basic IEC Method*) and collect all of the fractions (0.018M to 1.5M). Measure substance P content by; (a) determining the radioactivity present and in (b) and (c) by RIA (Chapter 2; *2.2.2 The Basic RIA Method.*)

Note: the IEC procedure for this chapter utilized Batch 1 Trisacryl M resin.

4.2.4 Methodology.

1. Prior to the collection of blood, ^{125}I-SP was diluted 1:100 in the 0.018M IEC buffer. ^{3}H-SP was not diluted. Synthetic substance P was diluted in physiological saline to yield a 10ng/ml solution.

2. Blood (50ml) was collected from the subjects in the morning and transferred to heparinized tubes. The samples were centrifuged immediately (2.4G for 10min at 4°C) and approximately 20ml of plasma collected.

3(a). For ^{125}I-SP samples incubated at room temperature, 60µl of 1:100 ^{125}I-SP was added immediately to 4.5ml plasma, vortexed and separated into samples of 400µl.

For samples incubated at 4°C, 150µl of 1:100 ^{125}I-SP was immediately added to 4ml plasma (more label was required due to the natural decrease in the specific activity of the label with time after purchase). The plasma was vortexed and separated into 400µl samples.

Similarly, 400µl of ^{3}H-SP was added immediately to 4ml of plasma, vortexed and separated into 400µl samples. ^{3}H-SP samples were not incubated at 4°C.

3(b). 2ml of 10ng/ml substance P was added to 8ml of plasma and vortexed. Samples of 400µl were then taken for incubation at 4°C or room temperature.

3(c). Plasma samples were separated into 400µl samples for incubation at 4°C or room temperature.

4. Following incubation (at 4°C or room temperature) for 20, 40, 60, 90, 120, 180 or 240 minutes, incubations were stopped and large proteins precipitated by the addition of 1.2ml ice-cold 5:1 methanol/0.1M hydrochloric acid. Samples were vortexed, centrifuged (13G for 20min at 4°C) and the supernatant decanted into clean tubes.

5. 1.6ml 0.018M pyridine/0.1M formic acid buffer was added to the supernatants, vortexed and the resultant solution loaded onto pre-pepared 2ml ion exchange columns with a 1ml bed volume of SP Trisacryl M. The ion exchange chromatography was performed as described in Chapter 3 (*3.2.3 The Basic IEC Method*).

6. All ion exchange chromatography fractions were collected (*i.e.*, the 0.018M, 0.1M, 0.35M, 0.8M and 1.5M fractions.)

7(a). The radioactivity in each of the ion exchange buffer fractions was determined in a γ-counter (LKB Wallac 1261 Multigamma γ-counter [Turku, Finland; counting efficiency 75%]) or β-counter (LKB Wallac 1218 Rackbeta Liquid Scintillation Counter [β-radiation counting efficiency 30%]) for ^{125}I-SP incubated samples and ^{3}H-SP incubated samples respectively. Tritiated samples (500µl) were mixed with 4.5ml scintillant prior to counting.

Approximate total counts were determined for ^{125}I-SP and ^{3}H-SP after calculating the volume of the label in a 400µl plasma sample and measuring the radioactivity in this volume of the diluted ^{125}I-SP or ^{3}H-SP.

7(b),(c). The ion exchange fractions were lyophilized on a Savant Speedivac and the SP content determined by RIA. The RIA method has been described earlier in Chapter 2 (*2.2.2 The Basic RIA Method*).

8(a). Results have been expressed as cpm per IEC fraction.

8(b),(c) Results have been expressed as the mean ($\pm$ SEM) immunoreactive SP in 1ml of plasma (pg/ml). Differences in SP concentration over time, between the different temperatures and between the different ion exchange chromatographic fractions were determined by two-way ANOVA (analysis of variance) with post Tukey-Kramer Multiple Comparisons Test (*i.e.*, the post test; if required). The level of significance was $p < 0.05$.

4.3 RESULTS

4.3.1 Degradation of Radiolabelled Forms of Substance P in Human Plasma.

Incubating either ^{3}H-SP or ^{125}I-SP in plasma at room temperature resulted in a steady decline in radioactivity measured in the 0.8M fraction (the SP-containing fraction) over a 4 hour period (Fig. 4.1a and 4.1b respectively). Concurrently, the radioactivity detected in the other ion exchange fractions steadily increased in a manner proportional to the decline of the radiolabelled peptide in the 0.8M fraction (Fig. 4.2a and 4.2b). However, it was the 0.018M

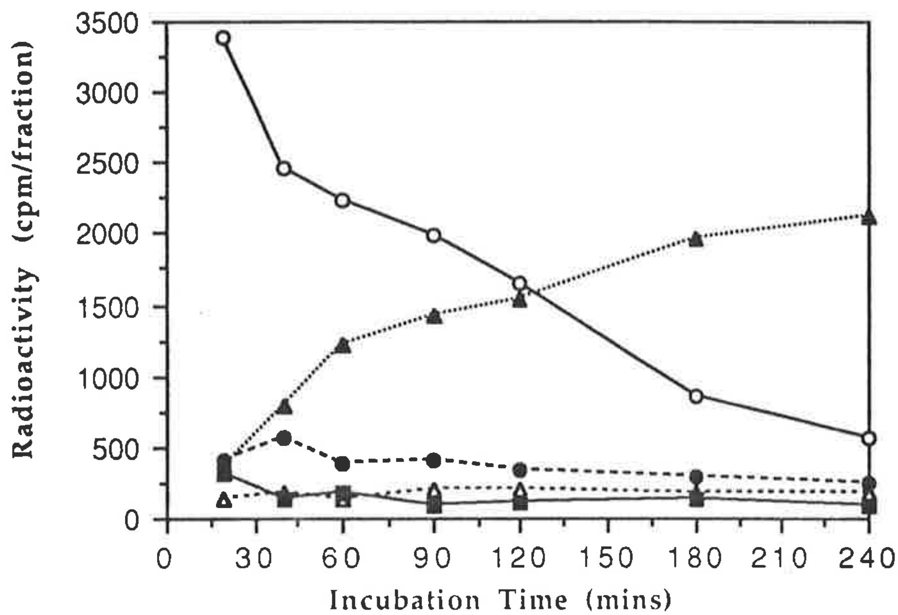


Fig. 4.1.a. Incubation of ^{3}H-substance P (^{3}H-SP) in human plasma at room temperature.

400µl of ^{3}H-SP was mixed with 4ml human plasma and separated into 400µl samples. The samples were incubated for 20 to 240 minutes at room temperature and the incubation stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all IEC fractions were collected and the radioactive content determined. The figure shows the level of radioactivity (cpm/fraction) at each incubation time point for each of the IEC fractions. Legend; 0.018M ....▲........ , 0.1M ------△----, 0.35M ----●----- , 0.8M ——○—— and 1.5M——■——.

[Note: the 0.8M IEC fraction was the substance P-containing fraction].

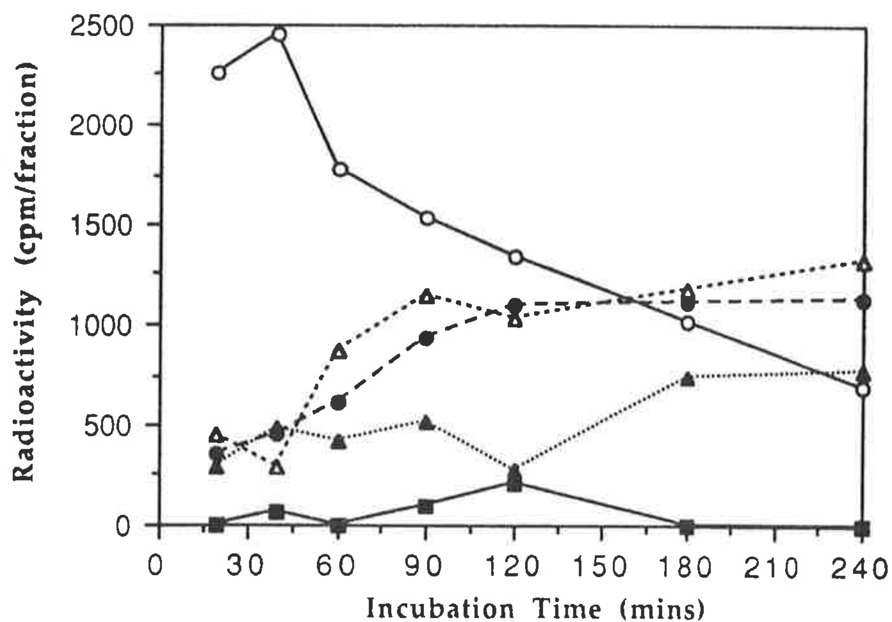


Fig. 4.1.b. Incubation of ^{125}I-substance P (^{125}I-SP) in human plasma at room temperature.

60µl of 1:100 (pre-diluted) ^{125}I-SP was mixed with 4.5ml human plasma and separated into 400µl samples. The samples were incubated for 20 to 240 minutes at room temperature and the incubation stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all IEC fractions were collected and the radioactive content determined. The figure shows the level of radioactivity (cpm/fraction) at each incubation time point for each of the IEC fractions. Legend; 0.018M ....▲........ , 0.1M ------△---, 0.35M ---●--- , 0.8M——○——and 1.5M ——■——.

[Note: the 0.8M IEC fraction was the substance P-containing fraction].

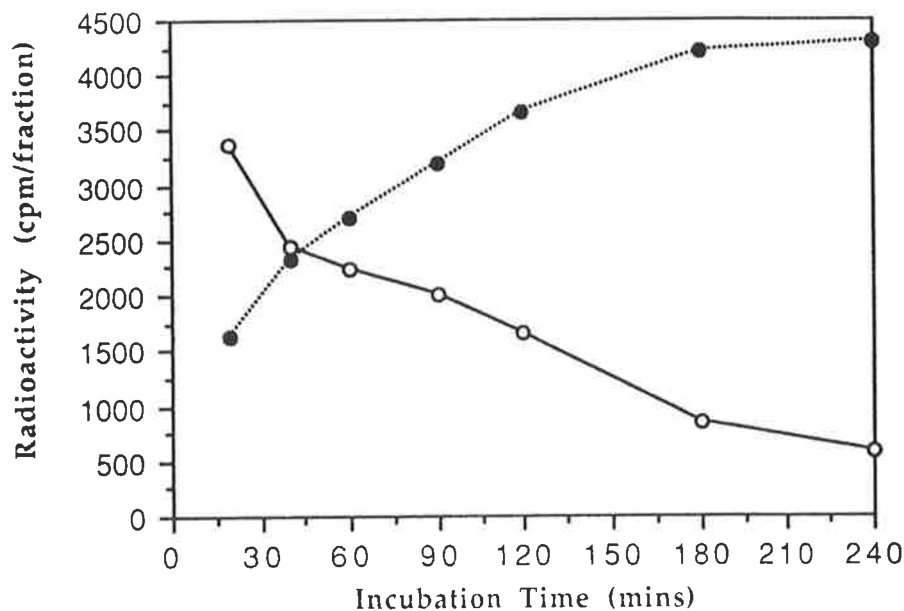


Fig. 4.2.a. Incubation of ^{3}H-substance P (^{3}H-SP) in human plasma at room temperature.

400µl of ^{3}H-SP was mixed with 4ml human plasma and separated into 400µl samples. The samples were incubated for 20 to 240 minutes at room temperature and the incubation stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all IEC fractions were collected and the radioactive content determined. The figure shows the level of radioactivity (cpm/fraction) found in the 0.8M IEC fraction (the substance P-containing fraction) compared with that found in all of the other IEC fractions at each incubation time point. Legend; 0.8M ——o—— and all other fractions ......●......

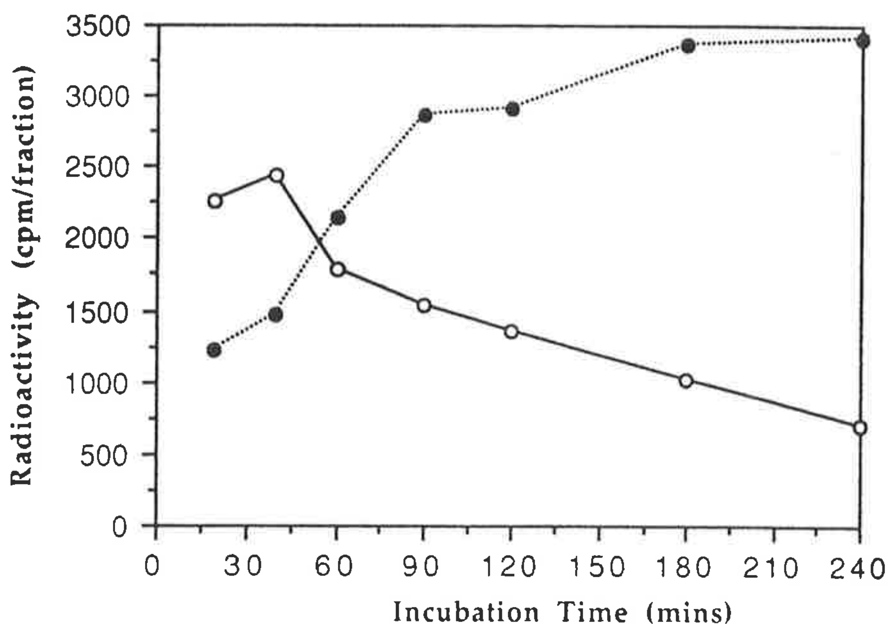


Fig. 4.2.b. Incubation of ^{125}I-substance P (^{125}I-SP) in human plasma at room temperature.

60µl of 1:100 (pre-diluted) ^{125}I-SP was mixed with 4.5ml human plasma and separated into 400µl samples. The samples were incubated for 20 to 240 minutes at room temperature and the incubation stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all IEC fractions were collected and the radioactive content determined. The figure shows the level of radioactivity (cpm/fraction) found in the 0.8M IEC fraction (the substance P-containing fraction) compared with that found in all of the other IEC fractions at each incubation time point. Legend; 0.8M ——o—— and all other fractions ......●...... .

fraction which increased in radioactivity following incubation of ^{3}H-SP in plasma (Fig. 4.1a), whilst the 0.1M fraction, 0.35M fraction and (later on) the 0.018M fraction increased in radioactivity following the incubation of ^{125}I-SP in plasma (Fig.4.1b).

Total counts for plasma samples incubated at room temperature were 12,068.8 cpm for ^{3}H-SP and 9,727.3 cpm for ^{125}I-SP. Although there was a steady decline in radioactivity over 4 hours in the 0.8M fractions, only around 25 percent of the original radioactivity was present after the 20 minute room temperature incubation (Table 4.1, below).

Conversely, there was little degradation of labelled peptide following 20 minutes of incubation of ^{125}I-SP in plasma at 4°C (TC were 14,413.2; Table 4.1). The degradation of the radiolabelled peptide was inhibited by the reduction of incubation temperature for 4 hours (Fig. 4.3). Consequently, the radioactivity in the remaining IEC fractions was at a constant low level.

Incubation Time (mins).	*^{3}H-SP Room Temp.*	*^{125}I-SP Room Temp.*	*^{125}I-SP 4°C.*
20	28.0	23.3	98.6
40	20.3	25.2	87.5
60	18.6	18.3	85.4
90	16.5	15.8	78.6
120	13.6	13.8	86.7
180	7.0	10.5	75.7
240	2.1	7.1	85.7

Table 4.1. The percentage of radioactivity present (calculated from the respective total counts) in the substance P-containing IEC fraction (0.8M fraction) following incubation in human plasma at room temperature (Room Temp.) or 4°C.

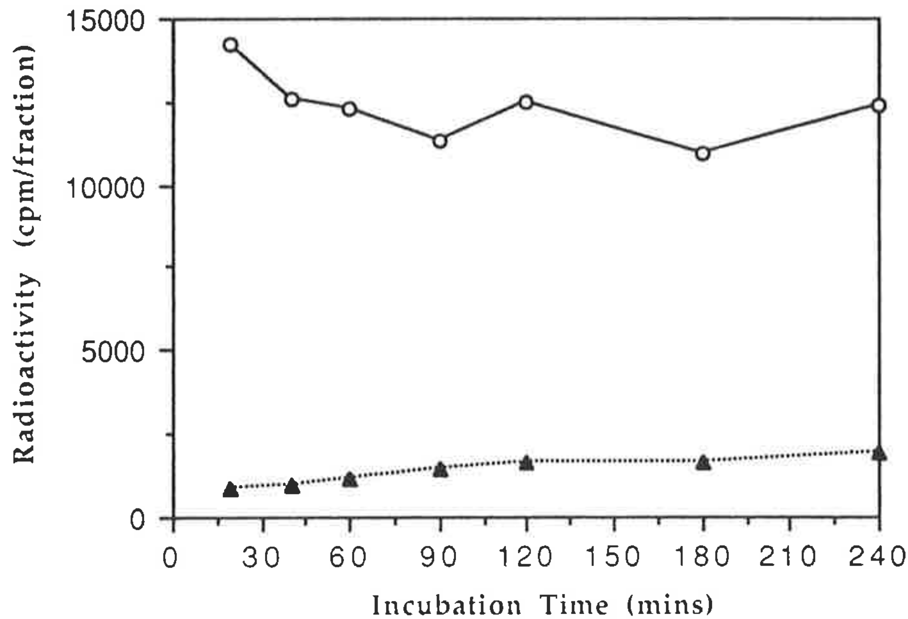


Fig. 4.3. Incubation of ^{125}I-substance P (^{125}I-SP) in human plasma at 4°C.

150µl of 1:100 (pre-diluted) ^{125}I-SP was mixed with 4ml human plasma and separated into 400µl samples. The samples were incubated for 20 to 240 minutes at 4°C and the incubation stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all IEC fractions were collected and the radioactive content determined. The figure shows the level of radioactivity (cpm/fraction) found in the 0.8M IEC fraction (the substance P-containing fraction) compared with that found in all of the other IEC fractions at each incubation time point. Legend; 0.8M ——o—— and all other fractions ·········▲········ .

4.3.2 Degradation of Synthetic Substance P in Human Plasma.

The effect of altering incubation temperature on the degradation of synthetic substance P (800pg) in human plasma has been shown in Figure 4.4. For incubations conducted at room temperature there was a steady decline in SP over the 4 hour incubation period (Fig. 4.4). Significantly lower levels of SP were found after 90, 120, 180 and 240 minute room temperature incubations when compared with the SP levels found in the 20 minute incubation (two-way ANOVA and post test, $p < 0.01$). Conversely, the SP levels in plasma incubated at $4^{O}C$ did not differ significantly at any of the incubation times tested (Fig.4.4; two-way ANOVA and post test, $p > 0.05$).

Significantly lower SP levels were in incubations conducted at 120, 180 and 240 minutes for plasma incubated at room temperature when compared with the plasma samples incubated at $4^{O}C$ at the same time point (two-way ANOVA and post test, $p < 0.01$; Figure 4.4).

The apparent immunoreactive substance P content of the other IEC fractions (as measured by RIA) for the plasma samples containing exogenous (800pg) SP and incubated at room temperature has been shown in Figure 4.5a. The major factor to note was that as the level of true SP declined with time (*i.e.*, in the 0.8M IEC fraction), there was an initial increase in immunoreactive "SP levels" in the 0.1M IEC fraction which later levelled off (at around the 120 minute incubation period; Fig. 4.5a). There was a small increase in the "SP content" of the 0.35M IEC fraction around the 40, 60 and 90 minute incubation periods (Fig. 4.5a). No real increases in the "SP levels" (or even significant levels of the peptide) were observed for the other IEC fractions.

For plasma samples spiked with 800pg SP and incubated at $4^{O}C$, no great difference in "SP content" was observed over the 4 hour incubation period for the other IEC fractions (Fig. 4.5b). There were reasonably high levels of immunoreactive "SP" in the 0.1M fraction, which tended to increase slightly at the later time points. Small time-related increases in the "SP content" were also observed for the 0.35M IEC fraction (Fig.4.5b).

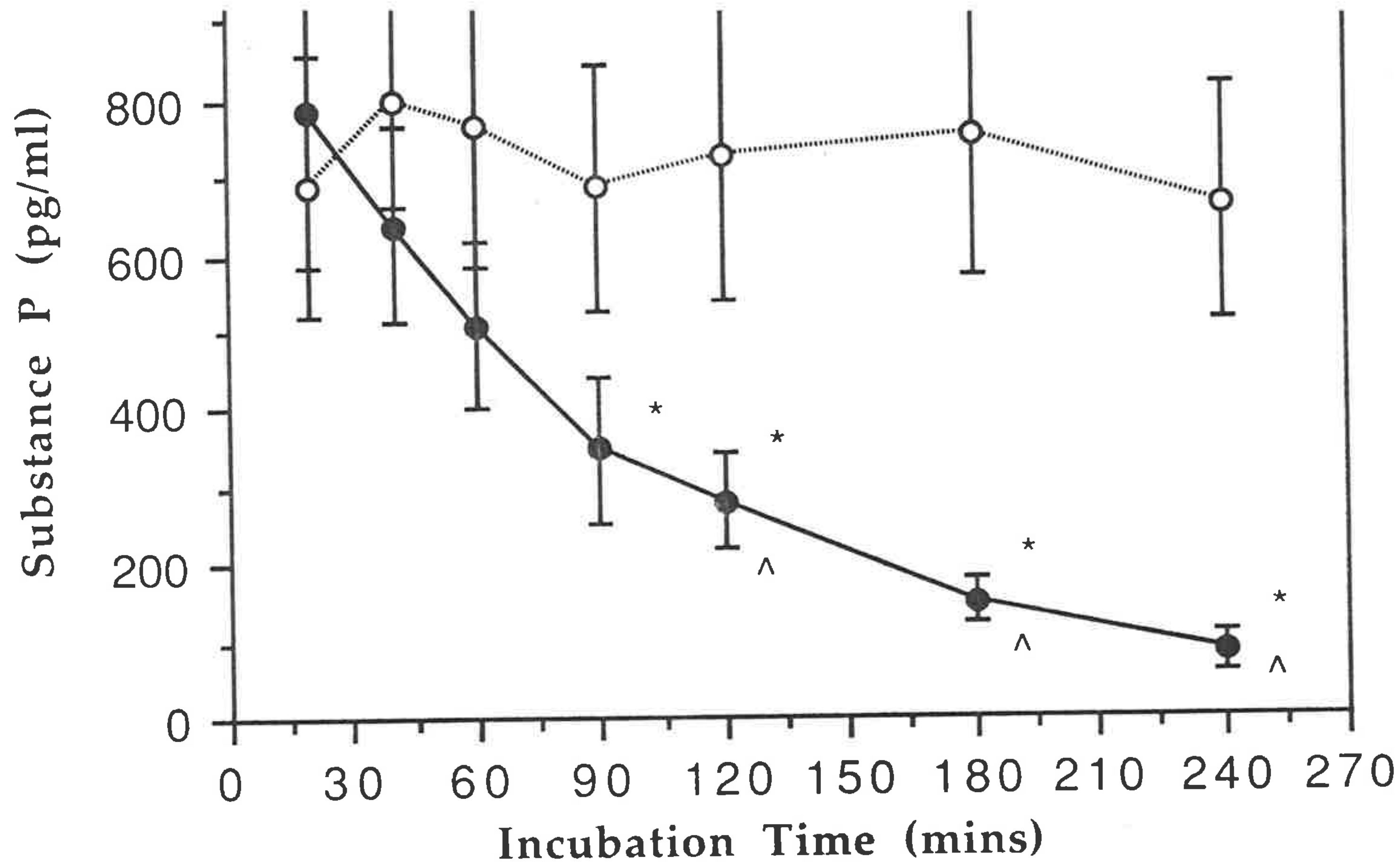


Fig. 4.4. Incubation of synthetic substance P (SP; 800pg/ml) in human plasma at room temperature and 4°C.

Synthetic SP was diluted in human plasma to yield 800pg/ml and separated into 400μl samples. The samples were incubated for between 20 to 240 minutes at either room temperature or 4°C (N=5 each). Then the incubation was stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and the 0.8M IEC fractions (i.e., SP-containing fraction) were collected and dried. The SP content was determined by RIA. The figure shows the SP content (mean pg/ml $\pm$ SEM) at each time point for incubations at room temperature ——●—— and at 4°C ·······○······· .

* *$p < 0.05$ for room temperature 20 minute incubation versus other incubation times.*

^ *$p < 0.01$ for room temperature incubation versus 4°C incubation at the same time point. [Two-way ANOVA with post-test].*

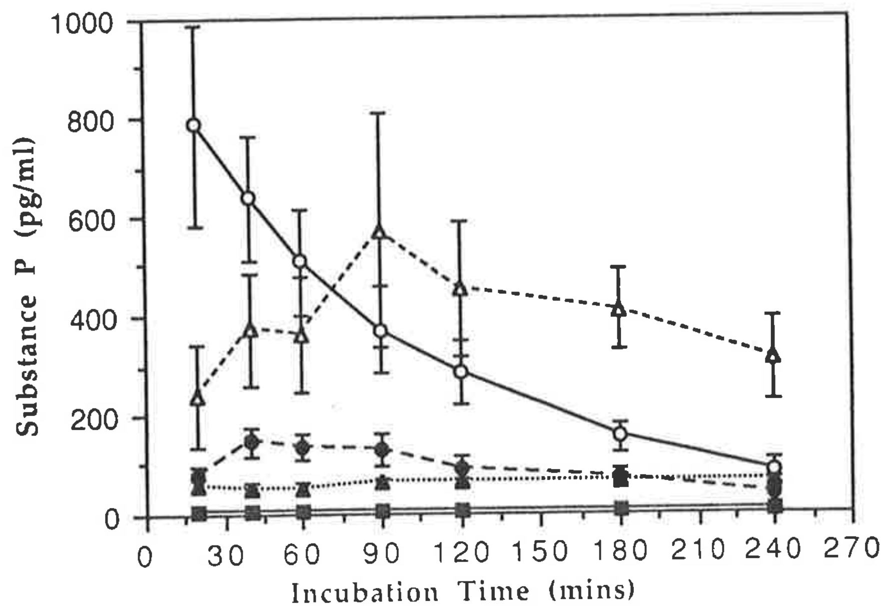


Fig. 4.5.a. Incubation of synthetic substance P (SP; 800pg/ml) in human plasma at room temperature.

Synthetic SP was diluted in human plasma to yield 800pg/ml and separated into 400µl samples. The samples were incubated for between 20 to 240 minutes at room temperature (N=5) and the incubation was stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all of the IEC fractions were collected and dried. The SP content was determined by RIA. The figure shows the SP content (mean pg/ml ± SEM) at each incubation time point for each IEC fraction.
Legend: 0.018M ·····▲·····, 0.1M----△----, 0.35M --●---, 0.8M ——○—— and 1.5M ——■——.
[Note: the 0.8M IEC fraction was the substance P-containing fraction].

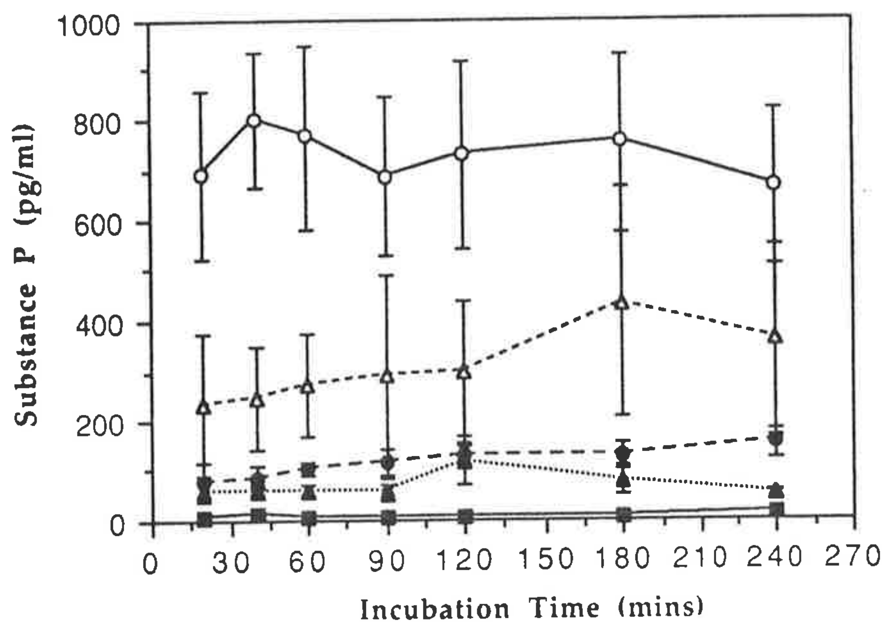


Fig. 4.5.b. Incubation of synthetic substance P (SP; 800pg/ml) in human plasma at 4°C.

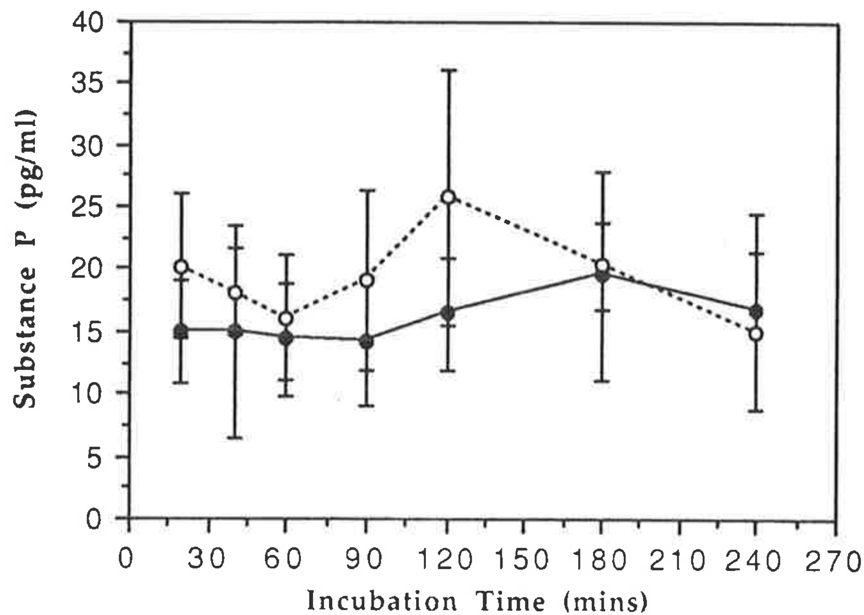
Synthetic SP was diluted in human plasma to yield 800pg/ml and separated into 400µl samples. The samples were incubated for between 20 to 240 minutes at 4°C (N=5) and the incubation was stopped (with a methanol/HCl solution). The samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all of the IEC fractions were collected and dried. The SP content was determined by RIA. The figure shows the SP content (mean pg/ml ± SEM) at each incubation time point for each IEC fraction.
Legend; 0.018M ·····▲·····, 0.1M----△----, 0.35M --●---, 0.8M ——○—— and 1.5M ——■——.
[Note: the 0.8M IEC fraction was the substance P-containing fraction].

4.3.3 Degradation of Endogenous Substance P in Human Plasma.

Incubating plasma at either $4^{o}C$ or room temperature for up to 4 hours did not significantly alter endogenous substance P levels (Fig. 4.6a). In fact, there was no significant difference in the observed endogenous SP content in plasma samples incubated at room temperature compared with samples incubated for the same length of time at $4^{o}C$ (two-way ANOVA, post test not required, $p > 0.05$; Fig.4.6a).

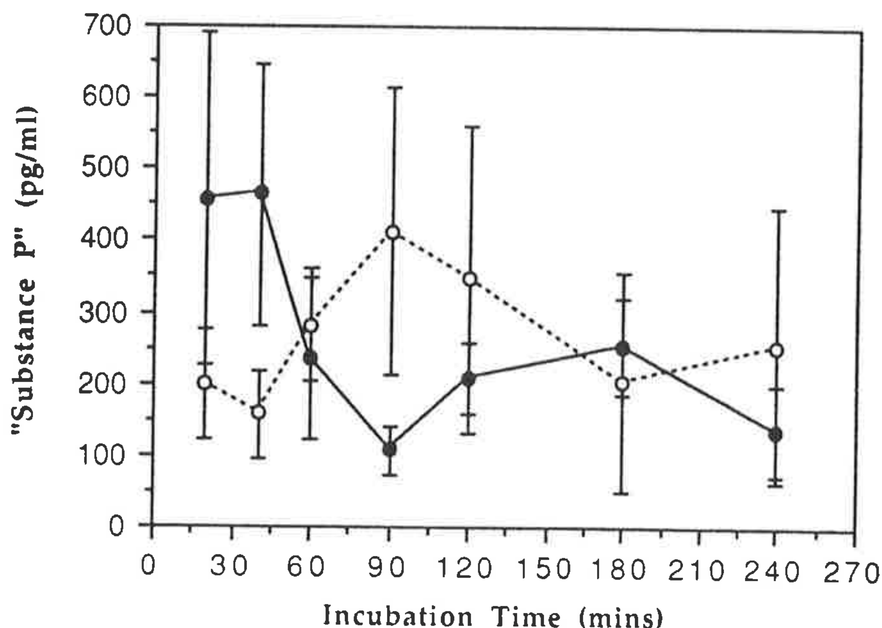
There were unusually high levels of immunoreactive "substance P" in the 0.1M fraction (Fig. 4.6b). Statistically, no significant differences were observed in plasma "substance P" content between samples incubated at room temperature or $4^{o}C$ for any of the time periods studied (two-way ANOVA and post test, $p > 0.05$).

However, the relative endogenous "substance P" plasma levels for each IEC fraction and the effect of temperature (after a 20 minute incubation) on each of these has been better represented in Figure 4.7. The endogenous plasma SP in the 0.8M IEC fraction was dwarfed by the immunoreactive "substance P" in the 0.1M IEC fraction (Fig.4.7). The 0.018M fraction also contained substantial levels of immunoreactive "SP" when compared with the 0.8M fraction (the true SP-containing fraction; Fig.4.7). Changing the plasma incubation temperature from room temperature to $4^{o}C$ did not significantly alter the "SP" content of any of the IEC fractions at any of the time periods studied (two-way ANOVA and post test, $p > 0.05$; Fig. 4.7).



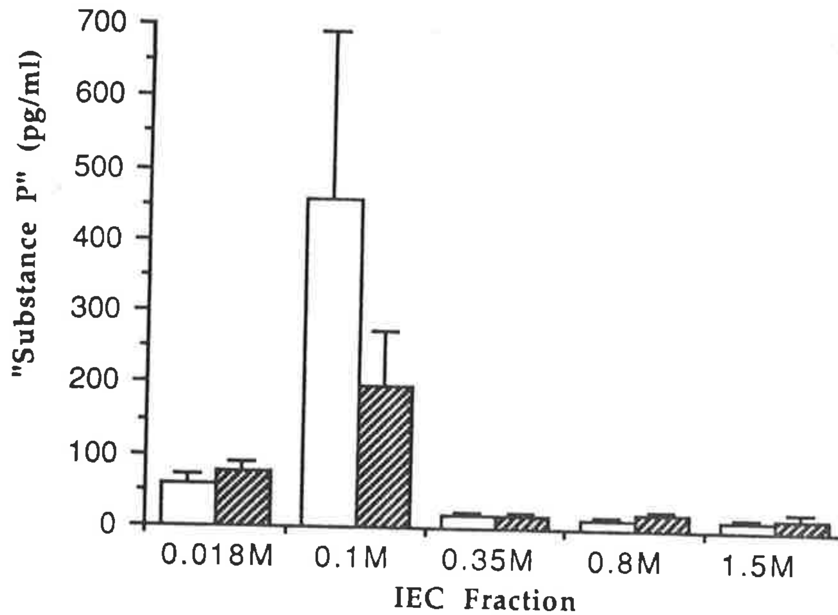
4.6.a. Endogenous substance P (SP) levels in human plasma.

400µl samples of human plasma were incubated for between 20 to 240 minutes at either room temperature (N=5) or at 4°C (N=5). The incubation was stopped (with a methanol/HCl solution) and the samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and the 0.8M IEC fractions (i.e., SP-containing fraction) were collected and dried. The SP content was determined by RIA. The figure shows the endogenous SP content (mean pg/ml ± SEM) at each time point for human plasma incubated at either room temperature ——●——, or at 4°C -----○------ . No significant difference was observed between samples incubated at room temperature to those incubated at 4°C [Two-way ANOVA and post-test].



4.6.b. Endogenous levels of a substance P-like peptide in human plasma.

400µl samples of human plasma were incubated for between 20 to 240 minutes at either room temperature (N=5) or at 4°C (N=5). The incubation was stopped (with a methanol/HCl solution) and the samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and the 0.1M IEC fractions were collected and dried. The SP content was determined by RIA. The figure shows the endogenous SP-like content (mean pg/ml ± SEM) of the 0.1M IEC fraction of human plasma incubated at room temperature ——●——, or at 4°C -----○------ for between 20 and 240 minutes. No significant difference was observed between samples incubated at room temperature to those incubated at 4°C [Two-way ANOVA and post-test].



4.7. Endogenous substance P-like content of human plasma.

400µl samples of human plasma were incubated for between 20 to 240 minutes at either room temperature (N=5) or at 4°C (N=5). The incubation was stopped (with a methanol/HCl solution) and the samples were centrifuged and the supernatant retained for ion exchange chromatography (IEC). The IEC was performed in the usual manner and all of the IEC fractions were collected and dried. The SP content was determined by RIA. The figure shows the endogenous SP-like content (mean pg/ml ± SEM) of each IEC fraction of human plasma incubated at room temperature ☐ , or at 4°C ▨ for between 20 and 240 minutes. Note; the 0.8M IEC fraction was the SP-containing fraction. No significant difference was observed between samples incubated at room temperature to those incubated at 4°C [Two-way ANOVA and post-test].

4.4 DISCUSSION

The degradation of the radiolabelled forms of the peptide (^{3}H-SP and ^{125}I-SP) were both time- and temperature-dependent (Figs. 4.1a, 4.1b and 4.3). At room temperature (21°C) both ^{3}H-SP and ^{125}I-SP were rapidly degraded with only 28.0% and 23.3% of the total respective radioactivity present after the 20 minute incubation (Table 4.1). After 4 hours of incubation this was reduced to 2.1% and 7.1% of the original total radioactivity respectively (Table 4.1).

Degradation of the ^{125}I-SP was effectively inhibited by reducing the incubation temperature of the plasma samples to 4°C; 98.6% and 85.7% of the total radioactivity was present after 20 and 240 minutes incubation respectively (Table 4.1).

As the ^{3}H-SP and ^{125}I-SP were degraded in plasma at room temperature the level of radioactivity in the other IEC fractions increased concurrently (Figs. 4.2a and b). However, the increase in radioactivity did not occur in the same IEC fractions when comparisons were made between the two radiolabels (Figs. 4.1a and b). Incubation with ^{3}H-SP led to an increase of radioactivity within the 0.018M fraction (Fig.4.1a), whilst incubation with ^{125}I-SP led to an increase in radioactivity in the 0.1M and 0.35M fractions first, followed by an increase in the 0.018M fraction. As the two labels are positioned at opposite ends of the peptide (Figs. 4.8a and b; next page) it is feasible that the peptide was cleaved between the two radiolabels, *i.e.* at the 2-3, 3-4, 4-5, 5-6, 6-7 or 7-8 amino acid bond.

Cathepsin G cleaves SP at the Phe^{7}-Phe^{8} bond (Skidgel *et al.*, 1991) and angiotensin converting enzyme acts primarily on the Phe^{8}-Gly^{9} bond (Cascieri *et al.*, 1984; Yokosawa *et al.*, 1985). However, ACE is also known to cleave the Phe^{8}-Gly^{9} and Gly^{9}-Leu^{10} bond (Mussap *et al.*, 1993). Rouissi and colleagues, (1990b) believe that neutral endopeptidase acts on the Gln^{6}-Phe^{7}, Phe^{7}-Phe^{8} or Gly^{9}-Leu^{10} bonds. Dipeptidyl aminopeptidase IV has also been shown to cleave SP at the Pro^{2}-Lys^{3} and Pro^{4}-Gln^{5} amino acid bonds (Chapter 1; 1.6.3 *Dipeptidyl aminopeptidase IV*).

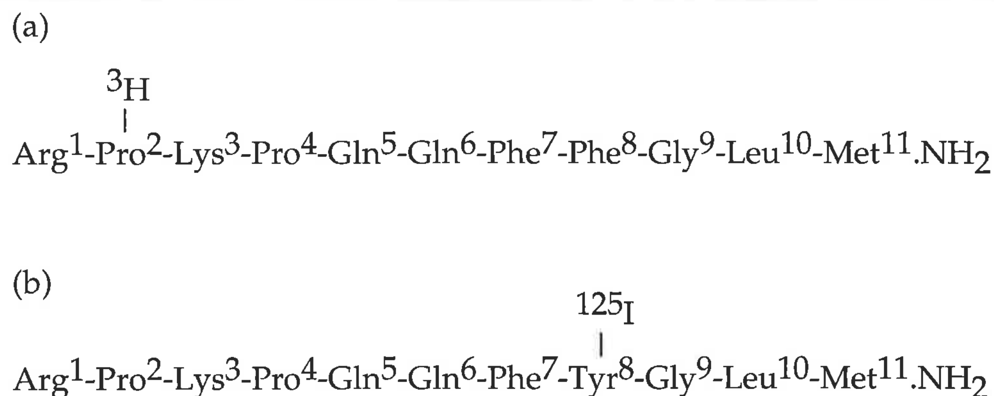


Fig. 4.8. The amino acid sequence and radiolabel position for (a) ^{3}H-SP and (b) ^{125}I-SP.

According to these inactivation sites for the enzymes on substance P, most of these enzymes (cathepsin G, NEP or DPP IV) may cleave the peptide between amino acids two and eight (with the exception of ACE). The most likely candidate appears to be DPP IV. Teichberg and Blumberg (1980) found that SP was cleaved at the Pro^{4}-Gln^{5} bond by synaptosomal fractions of rat brain resulting in the formation of the fragments SP 5-11 and SP 1-4. Later, substance P was found to be metabolised rapidly in human plasma producing the C-terminal fragments SP 3-11 and SP 5-11 (Conlon and Sheehan, 1983; Conlon and Goke, 1984 and it was suggested that DPP IV was the enzyme responsible. The action of DPP IV on substance P has been further substantiated by Palmieri and Ward (1983).

It was interesting to note in a study comparing the inactivation of substance P and its fragments in rat plasma (Couture and Regoli, 1981) that the fragment SP 4-11 was metabolised at approximately the same rate as the parent peptide. As the length of the fragment decreased however, the inactivation time was found to increase. Also, a study by Rogerson and associates (1989) has shown that the N-terminal fragment SP 1-3 may actually act to inhibit the action of brain ACE.

The studies with synthetic substance P in human plasma displayed similar results. Incubation of the peptide (800pg) at room temperature in human plasma resulted in a consistent decline in substance P levels (Fig. 4.4). There was only 11.0% of the peptide after 4 hours when compared with the level of SP after 20

minutes incubation. In contrast, incubating plasma at 4°C with the exogenous peptide for up to 4 hours resulted in no significant loss of substance P (two-way ANOVA and post test, $p > 0.05$; Fig.4.4). A significant difference in substance P content between plasma samples incubated at 4°C and room temperature was observed for incubation times of 120 minutes or longer (two-way ANOVA and post test, $p < 0.01$).

RIA of most of the other IEC fractions revealed relatively low levels of immunoreactive "substance P" present. As outlined in Chapter 2 (2.4 *Discussion*), the antibody used in the RIA does possess affinity for the C-terminal fragments SP 3-11, 4-11 and 5-11 as well as substance P. However, substance P has a higher affinity for the antibody compared to these C-terminal fragments (Chapter 2). HPLC studies (Chapter 3) have also shown that these C-terminal fragments of substance P elute into the earlier IEC fractions (*i.e.,* before the substance P-containing fraction). These studies (using SP Trisacryl M Batch 1 resin) revealed that the majority of the SP 3-11, 4-11 and 5-11 fragments eluted into the 0.35M and 0.1M IEC fractions (Chapter 3; 3.4 *Discussion*). Thus, the immunoreactive material in these non-substance-P fractions probably comprises some of the metabolic fragments of substance P. This would be true for the samples incubated with both exogenous substance P and the radiolabelled forms of the peptide.

Taking this into consideration, it appears that there were quite substantial levels of a fragment or fragments of substance P in the 0.1M fraction (Figure 4.5a and b). From data obtained in Chapter 3 (the IEC studies), these fragments are most likely SP 4-11, 5-11 or SP 6-11 (3.4 *Discussion*). In contrast to the synthetic substance P levels (in the 0.8M fraction), the levels of these fragments remained relatively constant over the 4 hour incubation period at room temperature and 4°C (Fig. 4.5a and b). In fact, there was no significant difference in the measured fragment levels at any of the incubation times when comparing the room temperature or 4°C incubations (two-way ANOVA and post test, $p > 0.05$).

The other IEC fractions contained only low levels of immunoreactive "substance P" (Fig.4.5a and b). Two explanations are possible for example (i) that

only low levels of substance P metabolites were present, or (ii) high levels of metabolites were present, but the affinity of these fragments for the antibody used in the RIA was low and therefore the RIA registered very little of these peptides. HPLC data (from Chapter 3) suggest that these SP Trisacryl M Batch 1 IEC fractions do contain fragments of substance P, thus (ii) may be the more likely explanation. However, as the antibody is C-terminally directed (for substance P), only C-terminal fragments could possibly be detected; N-terminal fragments, no matter how much was present, would not be detected.

The results of the incubation studies with endogenous plasma substance P contrast considerably with those involving exogenous and radiolabelled SP. Incubations of plasma conducted at $4^{O}C$ revealed no significant difference in endogenous substance P levels over the 4 hour period (Fig. 4.6a). This result was not unexpected when comparing the data obtained with exogenous and radiolabelled substance P (Figs. 4.3 and 4.4). However, in direct contrast, endogenous SP levels remained constant over the 4 hour room temperature incubation period (Figure 4.6a). In fact, there was no significant difference in endogenous SP levels in plasma samples incubated at room temperature when compared with samples incubated at $4^{O}C$ (two-way ANOVA, post test not required, $p > 0.05$; Fig. 4.6a).

Thus, exogenous substance P was rapidly metabolised in human plasma kept at room temperature (Figs. 4.1a and b and Fig. 4.4), yet endogenous substance P appears quite stable under the same conditions (Fig. 4.6a). Pernow (1983) has suggested that a possible explanation for this may be the presence of a protective endogenous binding protein. Another possibility may be that a homeostatic mechanism is operating, whereby substance P may be regulated within a set physiological range by increasing synthesis or inactivation of the peptide. Although a binding protein may be the most plausible explanation, there is little published research in this area.

Despite the difference in substance P stability in plasma in its various forms, the 0.1M IEC fraction contained elevated levels of immunoreactive

"substance P" (Fig. 4.6b), which most likely were the fragments SP 4-11 and SP 5-11. These fragments remained at a constant level for the duration of the 4 hour incubation and for both the room temperature and 4°C incubations (two-way ANOVA and post test, $p > 0.05$). RIA determinations of endogenous plasma levels of these fragments were approximately 10 times greater than those of substance P (compare Figs. 4.6a and b). This can be better seen in Figure 4.7. Note that for all of the IEC fractions there was no significant difference in immunoreactive "substance P" content between incubations conducted at room temperature compared to 4°C (Fig. 4.7). The level of endogenous immunoreactive "substance P" (*i.e.*, SP 4-11 and SP 5-11) isolated in the 0.1M fraction exceeded that present in the 0.8M (SP-containing) fraction. There were reasonable levels of endogenous immunoreactive "substance P" in the other IEC fractions. Taking this into account, in conjunction with the HPLC data (Chapter 3), it is possible that other metabolic fragments, such as SP 3-11 or the N-terminal fragments, were present in plasma.

In conclusion, it is possible that dipeptidylpeptidase IV is the enzyme responsible for the metabolism of substance P in human plasma; however, other enzymes cannot be discounted. The metabolism of radiolabelled (^{3}H-SP and ^{125}I-SP) and exogenous substance P was extremely rapid at room temperature. Decreasing the incubation temperature inhibited metabolism. Endogenous substance P plasma levels did not decline in incubations at room temperature, suggesting the presence of a protective binding protein or homeostatic process. Elevated levels of metabolic fragments of substance P were found to be present in plasma, perhaps suggesting a physiological role for these metabolic by-products. Furthermore, although endogenous substance P appeared stable in human plasma at room temperature, it would be prudent to assay tissue/plasma samples immediately after collection, or to place samples on ice immediately and assay for substance P as soon as possible.

CHAPTER 5

EVIDENCE FOR THE EXISTENCE OF A BINDING PROTEIN IN HUMAN PLASMA FOR SUBSTANCE P.

5.1 BACKGROUND AND AIMS

Data presented in the previous chapter (Chapter 4; *The Metabolism of Substance P*), indicated that endogenous SP was not metabolised in human plasma at the same rate as the radiolabelled or exogenous forms of the peptide. Whilst the latter two forms are extensively metabolised after four hours incubation in human plasma at room temperature (21°C), endogenous SP levels do not change.

It is possible that the addition of the radiolabels (in this case tritium and iodine) to the peptide may have affected the affinity of the peptide for the enzymes responsible for the metabolism of SP. This may be important for the iodinated form of the peptide where tyrosine has been substituted for phenylalanine (in position eight) to enable labelling to be possible. However, this seems unlikely for the rates of metabolism of both ^{3}H-SP and ^{125}I-SP in human plasma were comparable to that seen with the unlabelled exogenous form (Chapter 4; 4.3.1 *Degradation of Radiolabelled Forms of Substance P in Human Plasma* and 4.3.2 *Degradation of Synthetic Substance P in Human Plasma*). In contrast, endogenous SP appears to be stable in human plasma in conditions which allow the metabolism of the more labile forms of the peptide (*i.e.*, the radiolabelled and exogenous substance P). Therefore, it is possible that there may be a plasma binding protein for endogenous SP which protects against enzymatic degradation.

This possibility was raised by Pernow (1983) who suggested that endogenous circulating SP may become associated with large proteins in the blood and subsequently is protected from degradation. Literature reports released since the completion of the experiments described in this chapter lend credence to this possibility. Corbally and colleagues (1990) have demonstrated that SP in blood is associated with a high molecular weight complex, although the identity of the

complex is unknown. Another group (Oblas *et al.*, 1990) have shown that SP can bind to a member of the heat shock protein family (GRP 78), but the significance of this phenomenon *in vivo* is unknown.

The following experiments utilizing SDS-PAGE (polyacrylamide gel electrophoresis, also known as Western Ligand Blot analysis) and dialysis techniques explored the possibility of the existence of such a binding protein for SP in human plasma.

5.2 MATERIALS AND CHEMICALS

5.2.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

5.2.1.i. Plasma Samples.

For the first analysis, six plasma samples were used. They comprised four human (two female, two male) and two rat plasma samples (one Wistar Kyoto rat and one Stroke-prone Spontaneously Hypertensive rat).

On the second gel, four human plasma samples were analysed (three female, one male). For both gels sample volumes of 5µl and 10µl were used.

5.2.1.ii Materials and Chemicals for the SDS-PAGE.

LKB 2050 Midget Electrophoresis Unit, clamps, LKB 2051 Midget Multiblot Electrophoretic Transfer Unit, aluminium oxide plates, glass plates and 1.5mm spacers (LKB, Sweden).

Electrophoresis Power Supply, EPS 500/400 (Pharmacia, Australia), waxpaper (Hoefer Scientific Instruments, CA, USA) and 16 lane comb, 1.5mm (CSIRO workshop). Cellulosenitrate, 0.45µm, cut to size (Schleicher and Schuell, W.Germany), Whatman Chromatography Paper, 3MM Chr, cut to size, (Whatman, England) and Konica X-ray film (medical), 18x24cm (Konica Corporation, Japan). Rainbow protein molecular weight (MW) markers, ^{14}C-labelled (Amersham, England) and [^{125}I-Tyr^{8}]-Substance P (NEN Dupont, Australia). For preparation of gels and buffers see *Appendix IV*.

5.2.2 SDS-PAGE Protocol.

The gels were prepared as outlined in *Appendix IV*. The 12.5% separating gel was poured, to within 4-5cm of the top plates. Once set, the 4% stacking gel was poured on top, to a level approximately 5mm from the top of the plates. The comb was inserted and the gel allowed to set. The comb was then removed, the wells flushed with water and filled with running buffer before storing in the refrigerator (sandwiched by waxpaper) until required.

5µl of loading buffer was added to the plasma samples (5µl and 10µl) and mixed. The samples were heated at 65°C for 15 minutes in a water bath before loading onto the gel. The MW marker (for preparation see *Appendix IV*) was loaded into the first well as a standard.

The electrophoresis unit was set up and filled with running buffer. The gel was run at 15mA (300V) until the smallest MW marker had almost reached the end of the gel (approximately 2 hours). Then, the gel was removed and a piece of nitrocellulose paper (N/C; pre-soaked in Towbin buffer) fitted. The gel and N/C were sandwiched between two Whatman filter papers and placed in the electrotransfer apparatus (previously filled with Towbin buffer). The transfer was carried out at 300mA (300V) for approximately 1 hour. The N/C was removed and air dried overnight.

The next day, the N/C was first washed (with shaking) in 1% Triton at 4°C for 30 minutes. This was followed by washing (with shaking) with 1% BSA at 4°C for 2 hours and then a final wash (with shaking) with 0.1% Tween at 4°C for 10 minutes.

The N/C was then transferred to a probing box which contained the 1% BSA buffer and approximately 500,000cpm of the probe ([^{125}I-Tyr^{8}]-Substance P). The N/C was left in the probing box overnight (with shaking) at 4°C.

On the next morning, the 1% BSA/probe buffer was removed and the N/C washed with 0.1% Tween (with shaking) at 4°C for 30 minutes. The N/C was then washed for a further 90 minutes with fresh 0.1% Tween (with shaking) at 4°C. Following this, the N/C was then air dried for 5-6 hours.

When the N/C was dry, it was placed inside an autoradiograph cassette with X-ray film and stored at -80°C for about one week. After this time, the X-ray film was developed on an automatic developing machine. Photographs of the developed SDS-PAGE X-rays may be seen in the results section (5.3.1 *SDS-PAGE*; Figs. 5.1.a and b).

5.2.3 Plasma Dialysis.

5.2.3.i Plasma Samples.

Blood samples were obtained from four polycythaemic subjects who were visiting the Royal Adelaide Hospital for routine venesection. These blood packs were delivered to the laboratory immediately (in ice-filled containers) for instant dialysis.

The dialysis procedure for plasma was performed on two occasions. The first procedure involved plasma obtained from one individual which was dialysed in triplicate. The second procedure involved plasma obtained from the other three polycythaemics. Only one plasma sample was dialysed from each subject in this instance. (Note: the blood packs were available only when the polycythaemics were due for venesection. As it happened only one polycythaemic was treated on the first occassion, whereas three were seen on the same morning on the second occassion).

5.2.3.ii Materials and Chemicals for Plasma Dialysis.

10ml heparinized blood collection tubes (Labsupply, Adelaide, Australia), physiological saline (0.9% sodium chloride w/v, Ajax Chemicals, Sydney, Australia), nitrogen (CIG, Adelaide, Australia), Spectra/Por 2 Membrane (MWCO 12-14,000, width 10mm; Spectrum Medical Industries Inc., Texas, USA), [^{125}I-Tyr^{8}]-Substance P (^{125}I-SP; NEN Dupont, Australia), substance P (triacetate) (Sigma, Mo., USA), LKB Wallac 1261 Multigamma γ-counter (the LKB counter, Turku, Finland; counting efficiency 75%).

5.2.4 Plasma Dialysis Theory.

Dialysis is a technique which utilizes a semi-permeable membrane that allows free passage of low molecular weight (MW) complexes but does not permit the passage of high MW complexes. In the following protocol, plasma was mixed with a known concentration of radiolabelled SP and placed into dialysis membranes, which were then put into physiological saline. Any unbound radiolabel was free to pass between the plasma and saline. However, if the radiolabel was to bind to a plasma protein, this complex would be too large to pass through the membrane and would remain trapped in the plasma membrane compartment.

After equilibration, the concentration of unbound radiolabel would be the same in the saline as in the plasma. However, in the presence of a binding protein, an extra component of bound radiolabel would be trapped in the plasma compartment. Thus, under these circumstances, it would be expected that the total level of radioactivity in the plasma would be greater than that found in the saline.

As a further addition to the dialysis study, displacement of bound radiolabel from the potential binding protein was attempted by the addition of unlabelled SP to the plasma after equilibrium had been achieved. It was envisaged that the unlabelled SP would compete with the radiolabelled SP for the binding sites, and thus displace some of the radiolabel from the binding protein. This unbound radiolabel could diffuse through to the saline to maintain the equal concentration of free radiolabel on both sides of the membrane. The final result would be a decrease in radioactivity in the plasma with a concomitant increase in the radioactive content of the saline.

5.2.5 Plasma Dialysis Protocol.

The heparinized blood packs obtained from the Royal Adelaide Hospital (see 5.2.3.i *Plasma Samples*) were kept on ice and transferred to the laboratory within 15 minutes of collection. The blood was now placed in 10ml heparinized collection tubes (maintained in ice) and centrifuged (2,000G for 10 minutes at $4^{O}C$). Samples of 10ml of the plasma were collected and retained on ice.

Prior to use, the Spectra/Por 2 Membrane had been washed thoroughly with distilled water. Lengths of membrane of approximately 35cm were cut and tied tightly at one end. 30µl of ^{125}I-SP* was mixed with each of the 10ml plasma samples. The mixed 10ml plasma samples were then pipetted into each of the lengths of membrane and the remaining open end was tied.

In each of the plasma dialysis procedures there were three dialysis tubes. These were incubated in physiological saline (250ml kept at $4^{O}C$ and constantly bubbled with N_2 to ensure mixing of the solutions). At various time points, 500µl samples of the saline were removed and the radioactive content determined in the LKB counter.

Once the level of radioactivity in the saline was no longer found to be increasing (*i.e.*, once equilibrium had been attained; approximately one day later), a 500µl sample of the plasma and a 500µl sample of the saline were removed from each plasma dialysis for determination of radioactive content. Then, unlabelled SP was added (to a concentration of 1µM) to the plasma and the dialysis membrane returned to the saline. At various time points 500µl samples of plasma were removed for determination of radioactive content.

Results have been expressed as the change in radioactive content (cpm) for the attainment of equilibrium and the displacement studies. Binding of ^{125}I-SP has been calculated from cpm determinations to pg/ml plasma (mean $\pm$ SEM). (For calculations converting cpm bound ^{125}I-SP to pg/ml bound ^{125}I-SP see *Appendix V*).

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* 31µl of ^{125}I-SP for the first dialysis experiment.

5.2.6 Normal Serum Albumin Dialysis.

5.2.6.i Normal Serum Albumin (NSA).

As an adjunct to the human plasma dialysis experiments, dialysis was performed on human albumin to determine whether this plasma protein may bind SP. Normal serum albumin (NSA) was obtained as a 20g per 100ml solution (*i.e.*, a concentration of 20% w/v). A 5% solution of albumin is osmotically equivalent to plasma (from Goodman and Gilman's *The Pharmacological Basis of Therapeutics*, 1985). Thus, the following protocol utilized a 5% NSA solution (in physiological saline) as a substitute for the human plasma in the dialysis procedure.

For this dialysis section, seven 5% NSA membranes were dialysed on three separate occasions. The first NSA dialysis used three membranes, whilst the remaining two dialysis experiments used two 5% NSA membranes each.

5.2.6.ii Materials and Chemicals for NSA Dialysis.

These were the same as for the human plasma dialysis (5.2.3.ii *Materials and Chemicals for Plasma Dialysis*), but with the addition of Normal Serum Albumin (Commonwealth Serum Laboratories, Adelaide, Australia; Batch number 0673-08901, expiry 10/96) and with the exclusion of the 10ml heparinized blood collection tubes.

5.2.7 NSA Dialysis Protocol.

Essentially this was the same as the protocol for the dialysis of human plasma (*5.2.5 Plasma Dialysis Protocol*), but with the substitution of the NSA (5%) for the plasma.

To dilute the 20% NSA solution, 2.5ml of the 20% solution was mixed with 7.5ml physiological saline to yield 10ml of a 5% NSA solution. Two or three of these 5% NSA solutions were prepared on each occasion. 30μl of ^{125}I-SP was added to each of these 5% NSA solutions. The resultant was mixed thoroughly before pipetting into the prepared dialysis membrane, the end of which was then tied.

These membranes were incubated in physiological saline (250ml kept at $4^{o}C$ and constantly bubbled with N_2 to ensure mixing of the solutions). At various time points, 500μl samples of the saline were removed and the radioactive content determined in the LKB counter.

Once equilibrium had been attained (approximately two to three days later), a 500μl sample of the 5% NSA and a 500μl sample of the saline were removed from each dialysis membrane for determination of radioactive content. Then, unlabelled SP was added (to a concentration of 1μM) to the 5% NSA and the dialysis membrane returned to the saline. At various time points 500μl samples of the 5% NSA were removed for determination of radioactive content.

Results have been expressed as the change in radioactive content (cpm) for the attainment of equilibrium and the displacement studies. Binding of ^{125}I-SP has been calculated from cpm determinations to pg/ml 5% NSA (mean $\pm$ SEM). (For calculations converting cpm bound ^{125}I-SP to pg/ml bound ^{125}I-SP see *Appendix V*).

5.3 RESULTS

5.3.1 SDS-PAGE.

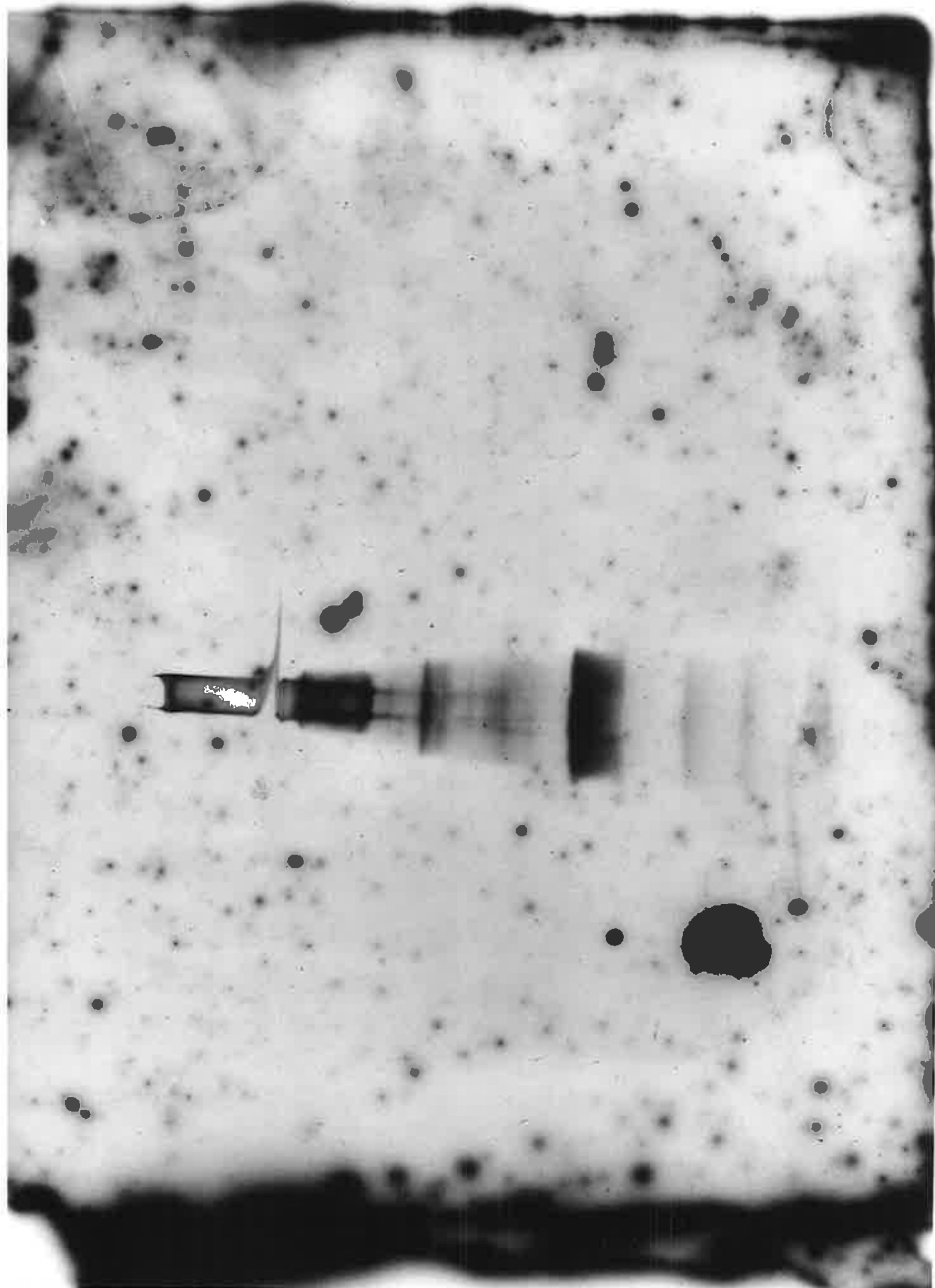
Photographs of the original X-rays of the SDS-PAGE gels may be seen in Figure 5.1a and 5.1b (next page). Figure 5.1a shows the gel obtained from the four human and two rat (one WKY and one SHR-SP) plasma samples. The MW marker may be seen on the far left of the gel. The next twelve lanes to the right of the MW marker held the human plasma samples in the first eight lanes (the 5μl sample followed by the 10μl sample for each individual) and the two rat plasma samples in the last four lanes on the far right (again with the 5μl sample followed by the 10μl sample). No band was seen for any of the sample lanes, whilst the MW marker was visible but not very distinct.

On the second gel, four human plasma samples were analysed (three female, one male; Fig.5.1b). Due to the indistinct development of the previous gel,

Fig. 5.1.a (above) and 5.1.b (below). A photograph of the autoradiographs for a possible binding protein for substance P in plasma.

Fig. 5.1.a. The MW marker is visible on the far left of the autoradiograph. The next eight lanes held the human plasma samples (5μl and 10μl), while the last four lanes on the far right held the rat plasma samples (5μl and 10μl).

Fig. 5.1.b. The MW marker is clearly visible slightly off-centre on the autoradiograph. The human plasma samples (5μl and 10μl) were run in the lanes on the far left of the autoradiograph. No samples were placed in the lanes on the right hand side of the MW marker.



a higher concentration of the probe was used (approximately 10^6 cpm) as well as allowing the X-ray film to develop for a longer period (around two weeks).

The MW marker may be seen slightly off centre towards the right of the gel (Fig.5.1b). The eight lanes used for the human plasma samples were on the left hand side of the MW marker. Again they alternated with a 5µl and 10µl plasma sample from each individual. Nevertheless, no bands were observed for any of these plasma sample lanes (Fig. 5.1b).

In essence, the SDS-PAGE technique did not reveal the potential presence of a binding protein for substance P in the human or rat plasma.

5.3.2 Dialysis.

5.3.2.i Plasma Dialysis.

The time taken to reach equilibrium for the dialysis of human plasma may be seen in Figure 5.2. This graph shows the increase in cpm per 500µl of saline taken at various time points. In general, equilibrium was attained at around 22 hours. The time point at which the saline and plasma samples were taken for determination of cpm and subsequently for calculation of binding (pg/ml) was 25.1 ± 0.8 hours.

At equilibrium, the radioactivity in plasma was far in excess of that observed in the saline (*Appendix V*, Table AV.1). Calculations revealed binding of 61.9 ± 4.4 pg/ml of ^{125}I-SP (N=4; *Appendix V*). The calculated values from each plasma dialysis membrane have been presented in Table 5.1 (next page).

The results of the displacement studies which involved the addition of unlabelled SP (1µM) to the plasma after equilibrium had been attained may be seen in Figure 5.3. This graph shows the decrease in radioactivity in the plasma following the addition of the cold SP. In most cases there was a decline of at least 50% of the original counts over the time period tested (Fig. 5.3). Consequently, there was an increase in cpm in the saline (of around 1.8 times the cpm at the original equilibrium determination) as summarized in Table 5.2 (next page).

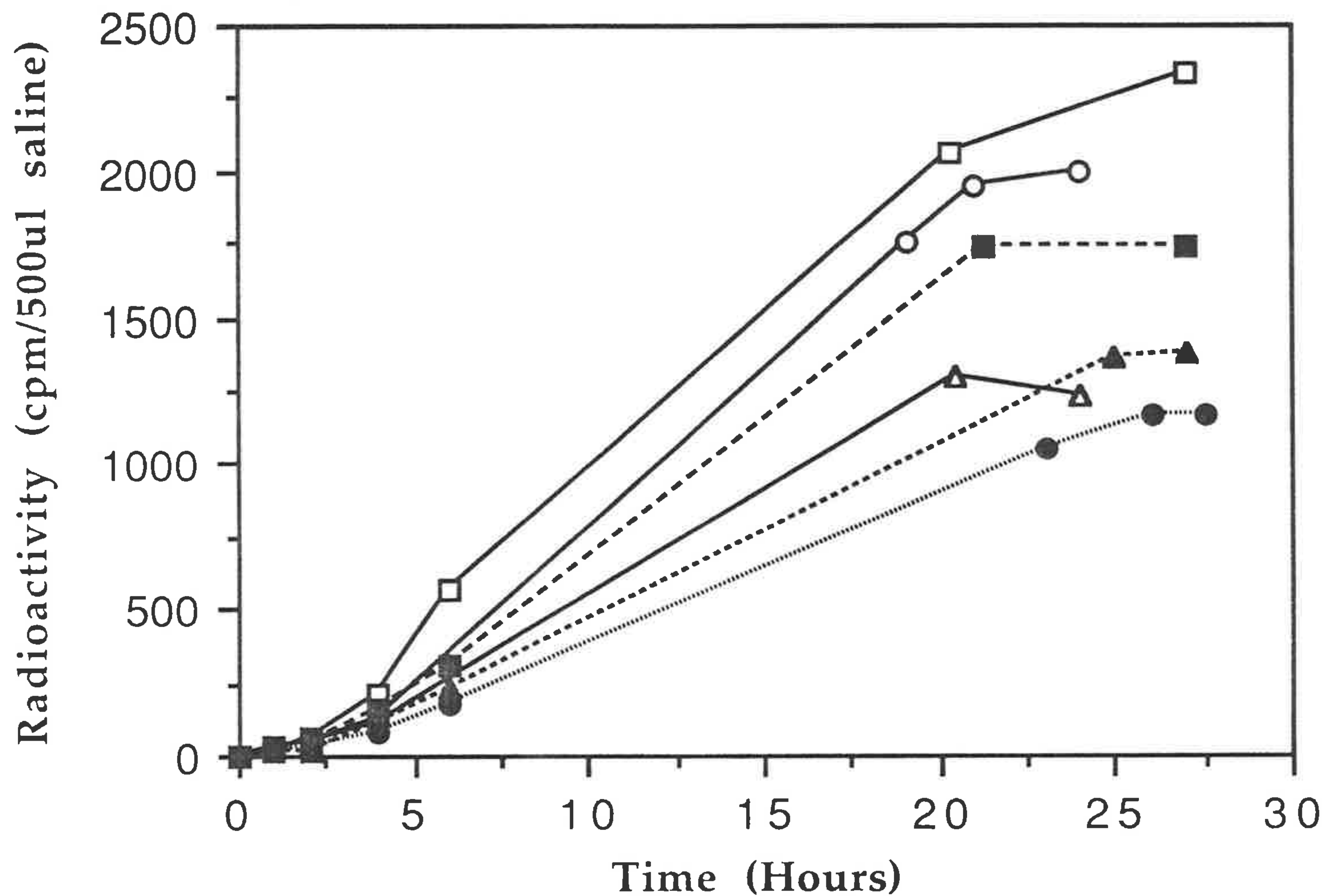


Fig. 5.2. Time course of human plasma dialysis.

^{125}I-SP was mixed with human plasma (N=4) and 10ml of the mix was placed in dialysis tubes which were then sealed. The tubes were immersed in physiological saline (250ml at 4°C mixed with N_2) and 500µl samples of the saline were taken for determination of radioactive content at various time points. The figure shows the radioactive content of the saline (cpm/500µl saline) at the various times points for each plasma sample. Legend; Sample number 1a —○— , *1b* ·····●····· , *1c* —△— , *2* - - -▲- - - , *3* —□— , *4* - - -■- - - .

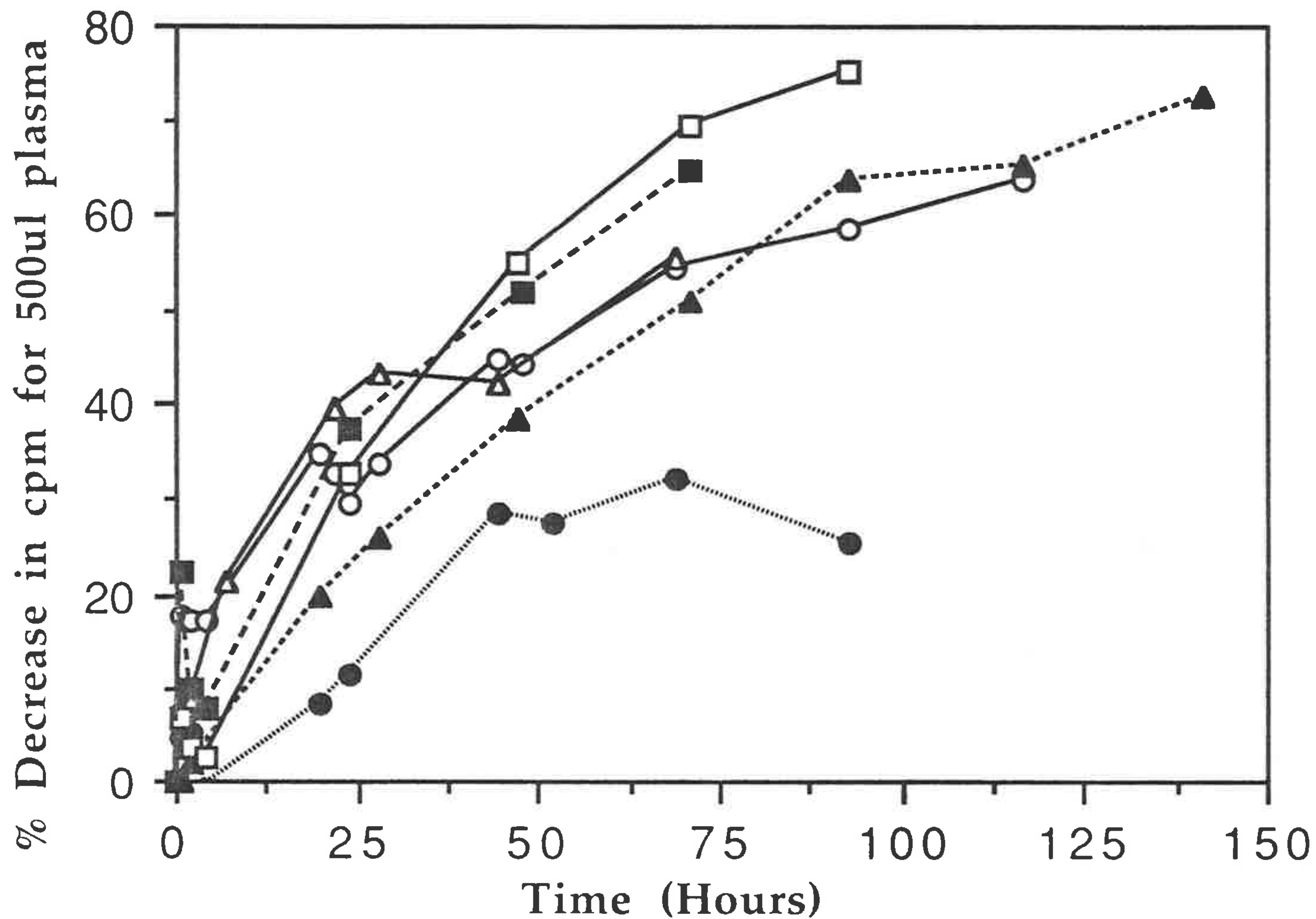


Fig. 5.3. Displacement of bound ^{125}I-SP from human plasma.

After equilibrium was attained (i.e., there was no further increase in the radioactive content of the saline), 1µM unlabelled substance P was added to the plasma samples in the dialysis tubes and the tubes were returned to the physiological saline. At various time points 500µl samples of plasma were removed and the level of radioactivity determined. The figure shows the decrease in the radioactive content of the human plasma samples (% decrease in cpm for 500µl plasma) at various time points after the addition of 1µM SP. Legend; Sample number 1a —○—, *1b* ·······●·······, *1c* —△—, *2* ----▲----, *3* —□—, *4* ---■--- .

Subject	Calculated Binding (pg/ml)
1a	55.7
1b	64.5
1c	56.5
2	62.0
3	54.3
4	72.2

Table 5.1. Summary of results for the calculations of the binding of ^{125}I-SP in human plasma. Note 1a, b and c were plasma samples obtained from the same individual. The mean ± SEM value expressed above used the mean value (pg/ml) obtained for this subject plus the values for each of the other individuals thereby giving N=4.

Subject	cpm at eq.	cpm after 1 M SP
1a	2,003.1	3,343.6
1b	1,168.5	1,927.7
1c	1,234.8	2,216.8
2	1,376.1	2,542.3
3	2,337.9	4,243.0
4	1,737.5	3,880.1

Table 5.2. Summary of cpm in saline (500µl) at equilibrium (eq.) and at least 60 hours after the addition of 1µM cold SP.

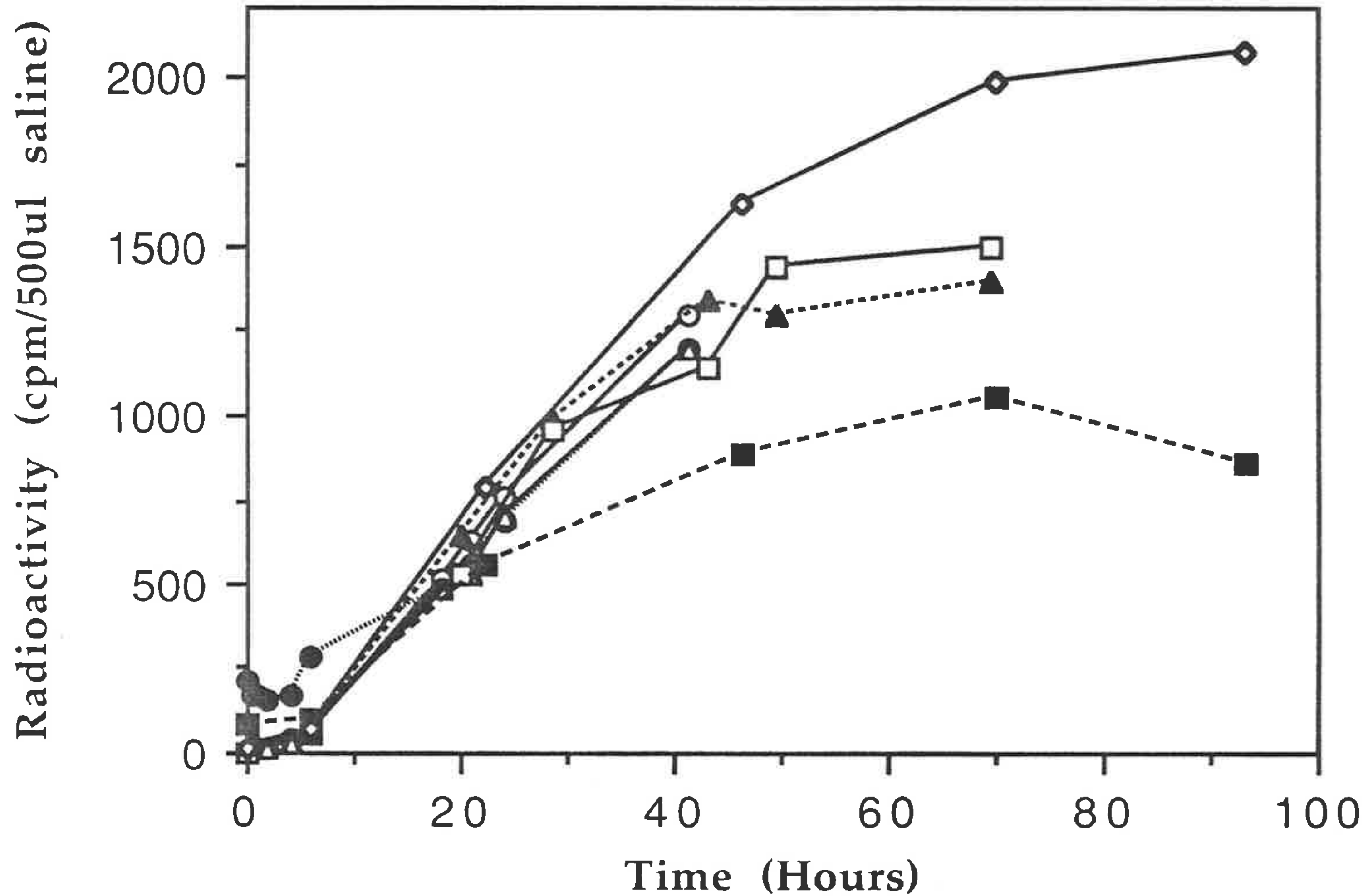


Fig. 5.4. Time course of normal serum albumin (NSA) dialysis.

^{125}I-SP was mixed with 5% NSA (N=7) and 10ml of the mix was placed in dialysis tubes which were then sealed. The tubes were immersed in physiological saline (250ml at 4°C mixed with N_2) and 500µl samples of the saline were taken for determination of radioactive content at various time points. The figure shows the radioactive content of the saline (cpm/500µl saline) at the various times points for each NSA sample. Legend; Sample number 1 —○— , 2 ·····●····· , 3 —△— , 4 - - -▲- - - , 5 —□— , 6 - - -■- - - , 7 —◇— .

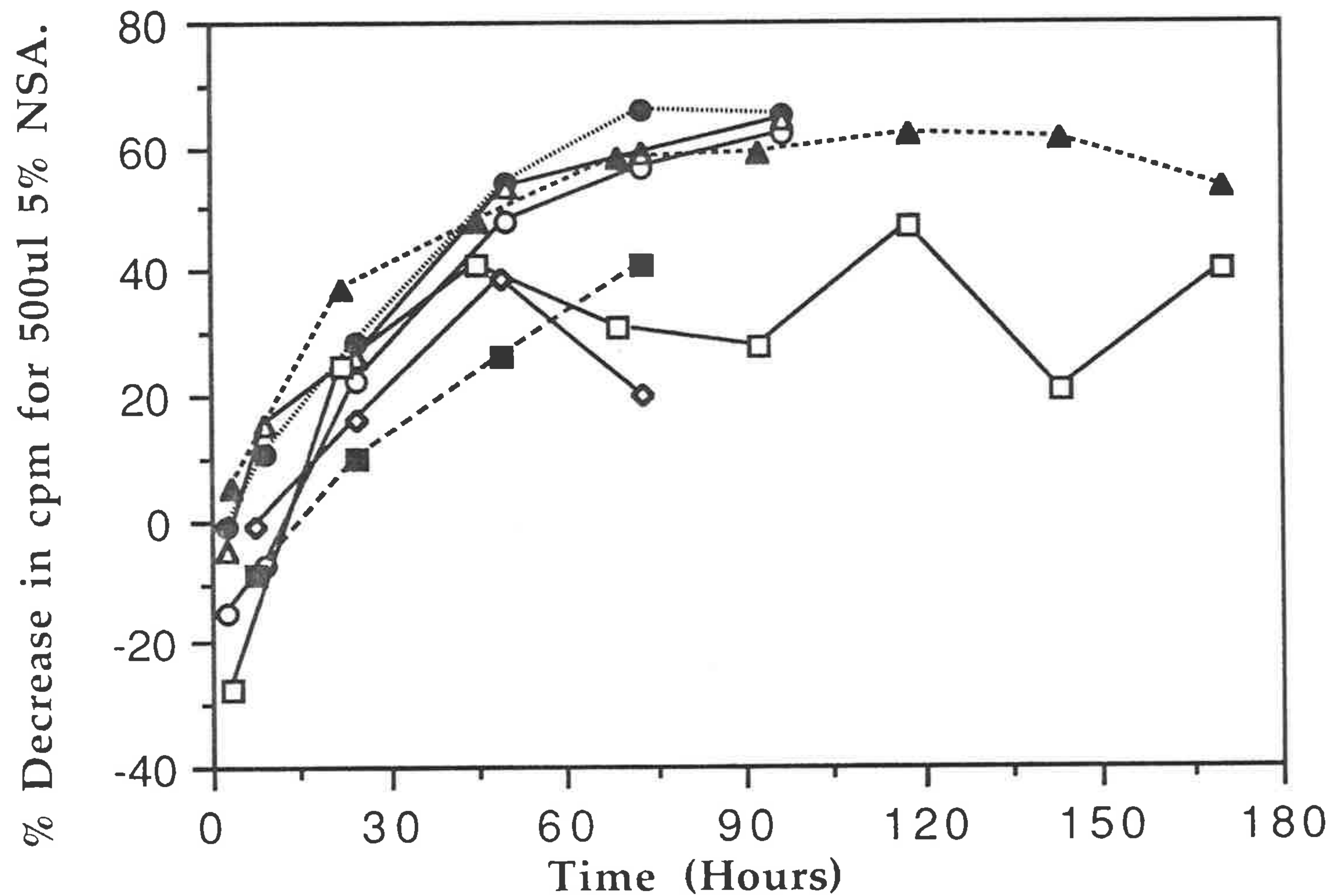


Fig. 5.5. Displacement of bound ^{125}I-SP from normal serum albumin (NSA).

After equilibrium was attained (i.e., there was no further increase in the radioactive content of the saline), 1µM unlabelled substance P was added to the 5% NSA samples in the dialysis tubes and the tubes were returned to the physiological saline. At various time points 500µl samples of 5% NSA were removed and the level of radioactivity determined. The figure shows the decrease in the radioactive content of the 5% NSA samples (% decrease in cpm for 500µl plasma) at various time points after the addition of 1µM SP. Legend; Sample number 1 —○—, 2 ···●···, 3 —△—, 4 ---▲---, 5 —□—, 6 ---■---, 7 —◇—.

5.3.2.ii NSA Dialysis.

The time taken to reach equilibrium for the dialysis of 5% NSA may be seen in Figure 5.4. This graph shows the increase in cpm per 500µl of saline taken at various time points. Equilibrium was attained at around 60 to 70 hours, which was around three times longer than that for whole plasma (Fig. 5.2). The saline and 5% NSA samples were taken for determination of cpm and subsequently for calculation of binding (pg/ml) at 64.6 ± 9.6 hours.

At equilibrium, the radioactivity in 5% NSA was far in excess of that observed in the saline (*Appendix V*, Table AV.2). Calculations revealed binding of 14.6 ± 1.8 pg/ml of ^{125}I-SP (N=7; *Appendix V*). The calculated values from each plasma dialysis membrane have been presented in Table 5.3 (below).

Sample	Calculated Binding (pg/ml)
1	16.1
2	22.8
3	16.5
4	9.3
5	14.1
6	11.8
7	11.4

Table 5.3. Summary of results for the calculations of the binding of ^{125}I-SP in 5% NSA (for calculations see *Appendix V*).

The results of the displacement studies for 5% NSA may be seen in Figure 5.5. This graph shows the decrease in radioactivity in the 5% NSA following the addition of the unlabelled SP (1µM). In most cases, there was a decline of at least 50% of the original counts in the 5% NSA (Fig. 5.5). Consequently, there was an increase in cpm in the saline (of around 1.6 times the cpm at equilibrium) as summarized in Table 5.4 (next page) for all samples, except sample 7.

Sample	cpm at eq.	cpm after 1μM SP
1	1,294.5	2,842.1
2	1,206.3	2,456.1
3	1,201.2	2,427.9
4	1,399.7	2,010.2
5	1,506.0	1,546.7
6	863.0	1,559.4
7	2,077.3	1,757.9

Table 5.4 Summary of cpm in saline (500μl) at equilibrium (eq.) and at least 60 hours after the addition of 1μM unlabelled SP.

5.4 DISCUSSION

Prior to the commencement of the SDS-PAGE and dialysis studies summarized above, little literature evidence was available suggesting the existence of a binding protein for SP. However in 1990 two separate groups working in this area provided data which suggest that such a binding protein may exist. Corbally *et al.* (1990) used crosslinking techniques in associaton with gel filtration to demonstrate a high molecular weight complex (HMWC) in human plasma that bound SP. Degradation determinations revealed rapid degradation of exogenous SP in plasma, whereas the endogenous form of the peptide was found to be more stable (Corbally *et al.*, 1990). It was concluded that the HMWC protected the endogenous form of the peptide from degradation. Furthermore, SDS-PAGE experiments indicated that human serum albumin could bind SP (Corbally *et al.*, 1990).

Attempts to purify SP from horse salivary glands using affinity chromatography identified a protein which bound SP (Oblas *et al.*, 1990). This polypeptide was thought to be GRP 78, a member of the family of heat shock proteins. However, the significance of this relationship between GRP 78 and SP *in*

vivo are unknown, let alone whether this protein-peptide association is significant in humans.

Studies by a third group of researchers (Nakata *et al.*, 1992) have also isolated a binding protein for SP from rat brain (essentially this binding protein is a receptor for SP). They calculated the molecular weight of the protein to be 76-74 kDa. Nakata *et al.* (1992) also produced polyclonal antibodies to this binding protein suggesting that such an antibody would be a useful tool for future SP receptor studies.

The SDS-PAGE evaluations performed in the current study did not suggest the existence of a binding protein for SP in human or rat plasma (Fig. 5.1a and 5.1b). Nevertheless, a negative result from these electrophoresis studies did not necessarily exclude the existence of such a binding protein. However, Corbally *et al.* (1990), Oblas *et al.* (1990) and Nakata *et al.* (1992) utilized SDS-PAGE techniques in their studies investigating a binding protein for SP. Therefore, it is possible that modification of the current protocol may alter the results.

Potential modifications to the SDS-PAGE protocol include the composition of gels and buffers as well as a review of sample preparation/handling. Perhaps even the probe (^{125}I-SP) was not appropriate under the outlined experimental conditions. Alternatively, it is possible that the binding sites on the protein were already saturated and the SDS-PAGE conditions did not favour dissociation. Furthermore, the probe may not have possessed a high affinity for the protein, when compared with the endogenous peptide.

Dialysis techniques provide an alternative method for identifying a binding protein for SP. The dialysis protocol outlined in this chapter revealed binding of the radiolabelled form of the peptide to be 61.9 $\pm$ 4.4 pg/ml in human plasma.

From data obtained in Chapter 4 (*The Metabolism of Substance P*), it was determined that the endogenous level of SP in human plasma was in the range of 15 to 25 pg/ml (Fig. 4.6a). This range was well below that determined as the binding capacity of human plasma for ^{125}I-SP (61.9 $\pm$ 4.4 pg/ml). Therefore, it may be expected from these results that a large proportion of endogenous

circulating SP may be bound to a plasma protein. This protein may then protect SP from various degradative enzymes. This could explain why the endogenous form of the peptide appears to be stable under conditions which render exogenous forms of the peptide very susceptible to enzymatic degradation (Figs. 4.1a, 4.1b and 4.4).

Moreover, degradation studies involving the addition of 800pg/ml of exogenous SP to human plasma revealed significant metabolism of the peptide when incubated at room temperature (Fig. 4.4). Thus, if the binding capacity of the protective protein was in the vicinity of 60pg SP/ml plasma, it is obvious that a concentration of 800pg/ml would far exceed the binding capacity of this protein. This would leave the majority of the peptide unbound and susceptible to enzymatic degradation.

Furthermore, examination of Figure 4.4 reveals that the degradation of the peptide at room temperature appears to plateau around the 100pg/ml SP concentration. In fact, the last determination of SP at 240 minutes found 86.2 ± 26.2 pg SP/ml. Thus, as the concentration of SP approached the 60pg/ml level, the activity or degradative potential of the plasma enzymes appeared to diminish. This suggests that the binding protein has a binding capacity (and consequently a protective effect) below (but not much less than) the 85pg/ml level. The degradation profile (Fig. 4.4) suggests that not much more degradation of SP would occur to decrease the concentration very much below the 85pg/ml level. Hence, the degradation studies in Chapter 4 roughly agree with the binding capacity of the plasma protein for the radiolabelled SP as determined by the dialysis experiments.

Addition of 1μM unlabelled SP to the plasma after the attainment of equilibrium decreased the radioactivity in the plasma (Fig. 5.3), indicating displacement of bound ^{125}I-SP from the binding protein. Note that the calculated binding capacity of the protein was approximately 60pg/ml, whilst the added unlabelled SP was at a concentration of 1μM (*i.e.*, 1ng/ml), over 15 times the binding capacity of the protein. Thus, it would appear from the dialysis data that

human plasma may contain a SP binding protein, which is saturable and from which SP may dissociate.

At the commencement of the 5% NSA dialysis studies, it was expected that equilibrium would be attained at roughly the same time as that seen with whole plasma. However, this was not the case (Figs. 5.2 and 5.4). Equilibrium for the 5% NSA samples was observed at around 60 to 70 hours whilst equilibrium for human plasma was observed at around 22 hours. A possible explanation for this phenomenon is that the principal binding protein for SP in human plasma may not be albumin and that this unidentified protein has a different affinity or binding characteristics for SP. Alternatively, plasma may contain a component which enhances the affinity of the binding protein for SP.

Because of the longer than expected equilibration time (Fig. 5.4), the first three determinations of binding in 5% NSA were performed slightly before equilibrium was attained. Calculations for binding were performed on samples taken at 41.5, 41.6 and 41.7 hours for samples 1, 2 and 3 respectively.

It is possible that as equilibrium had not been attained, more cpm were present in the 5% NSA solution than would have been found at equilibrium, since not all of the unbound ^{125}I-SP had had enough time to diffuse across the membrane. This would have influenced the calculations to determine the extent of binding and would have resulted in an overestimation of the actual binding.

Examining Table 5.3 it may be seen that the first three calculations determining the extent of binding in the 5% NSA solution revealed proportionately higher values than the remaining four determinations (*i.e.*, roughly 18 pg/ml versus 11 pg/ml). The latter four determinations were performed on samples taken after 70.3, 70.3, 93.5 and 93.6 hours of dialysis. Therefore,it may be more prudent to discard the first three determinations and accept the determinations of 9.3, 14.1, 11.8 and 11.4 pg/ml (*i.e.*, 11.7 $\pm$ 1.1 pg/ml; N=4) as the extent of binding in the 5% NSA solution.

This calculation for the binding of ^{125}I-SP in the 5% NSA solution was significantly below that determined for whole plasma (61.9 $\pm$ 4.4 pg/ml). This

suggests that although human plasma albumin possesses the ability to bind SP, it probably is not the principal binding protein for this peptide in blood.

The graph for the displacement component of the 5% NSA study, in which 1μM unlabelled SP was added to the 5% NSA solutions after equilibrium, is shown in Figure 5.5. When the unlabelled SP was added to the membrane, there appeared to be a flux of the free radiolabel into the 5% NSA solution, thereby resulting in an initial increase in cpm in the 5% NSA solution. No explanation for this occurrence is readily apparent. However, this effect was soon reversed, with a time-dependent decrease in cpm observed for the 5% NSA samples after about 10 hours.

This decrease in cpm in the 5% NSA solution after the addition of 1μM SP was again much slower than that observed with whole plasma (Figs. 5.5 and 5.3 respectively). These results reinforce the concept that another plasma protein may be the principal protein involved in binding SP (apart from albumin) and that this unidentified protein possesses an affinity which is different to that of albumin.

Hence, in summary, data obtained from the degradation studies of the various forms of substance P (Chapter 4, *Metabolism of Substance P*) in conjunction with the dialysis experiments above suggest that human plasma contains a binding protein for SP. This protein has a binding capacity of 61.9 $\pm$ 4.4 pg/ml for SP under the experimental conditions used. The binding of SP appears to be saturable and reversible. The human plasma protein albumin also possesses the capacity to bind SP in a saturable and reversible manner, although the capacity determined for this protein was lower than that found for whole plasma (11.7 $\pm$ 1.1 pg/ml). This suggests that although albumin may bind SP, it is most likely that it is not the principal binding protein for the peptide in whole plasma.

CHAPTER 6

IN VITRO AND *IN VIVO* STUDIES WITH SUBSTANCE P AND ITS FRAGMENTS.

6.1 BACKGROUND AND AIMS

Extensive research continues to examine the biological activity and significance of SP in health and disease states (Chapter 1, *1.3.3* and *1.4*). Nevertheless, researchers have concentrated less on the possible influence of the biological metabolites of SP in physiological systems. Both the C-terminal and the N-terminal fragments of SP have been recognized as possessing biological activity and some of these fragments have been found to be more potent than the parent peptide itself (Bury and Mashford, 1976; Lei *et al.*, 1991; Boix *et al.*, 1992; Halliday *et al.*, 1993 and Chapter 1, *1.5.4*).

Furthermore, in Chapter 4 (*The Metabolism of Substance P*) evidence was presented to show that there is present in human plasma a fragment (or fragments) of SP which may be separated from the parent peptide by the IEC technique in the 0.1M IEC fraction. These fragments have been identified as SP 4-11 and/or SP 5-11 and are found at a concentration of approximately ten times that of endogenous SP (Chapter 4, 4.4 *Discussion*). Since plasma concentrations of some of these fragments approach and, in some cases, exceed those of the parent peptide, it is possible that the physiological effects of these fragments may be important and further research is warranted.

The experiments described in this chapter were performed to compare the biological activity of SP with selected fragments in both an *in vitro* and an *in vivo* system; *i.e.* on the contractile responses in the longitudinal muscle/myenteric plexus of guinea pig ileum (GPI) and the blood pressure responses in the freely moving, non-anaesthetized Wistar Kyoto rat* respectively. (It must be noted that the determination of heart rate during the hypotensive phase was a secondary

* Note; preliminary BP studies using SHRs, SHR-SPs, Hooded Wistars and WKY rats revealed that only the WKY was sensitive to the hypotensive effects of SP and its fragments. The other strains were insensitive to the blood pressure altering effects of these peptides.

consideration and was not always possible). It is perhaps appropriate that the present choice of biological system is the same as that used by von Euler and Gaddum (1931) when they first discovered SP.

6.2 MATERIALS AND METHODS

6.2.1 Materials and Chemicals for the In Vitro GPI Studies.

Krebs-Henseleit buffer (see *Appendix VI*). Substance P 1-11, SP 4-11 (octa-substance P), SP 5-11 (hepta-substance P), SP 6-11 (hexa-substance P), SP 7-11 (penta-substance P), Tyrosine^{8}-substance P, SP 1-6, SP 1-7, SP 1-9 (Peninsula Laboratories, CA, USA) and SP 1-4 (Auspep, Melbourne, Australia). Physiological saline (0.9% sodium chloride w/v, Ajax Chemicals, Sydney, Australia). Graphtec Linearcorder F WR3701 (Graphtec Corp., Japan), JRAK Type RK1 Pressure Amplifier (JRAK Biosignals, Sydney, Australia).

6.2.2 Animals for the In Vitro GPI Studies.

All preparations were obtained from five adult guinea pigs from the animal colony at the University of Adelaide.

6.2.3 Tissue Collection and Preparation for the In Vitro GPI Studies.

The guinea pigs were stunned and killed by exsanguination. The method for collecting the longitudinal muscle/myenteric plexus preparation was similar to that described by Taylor *et al.* (1988). The ileum was located and the first 10cm discarded, the next 10-15cm of ileum was removed and cleaned by perfusion with Krebs-Henseleit buffer (Krebs, *Appendix VI*). Sections of 1cm were then cut and threaded onto a glass pipette and the longitudinal muscle/myenteric plexus component of the ileum removed by careful teasing with cotton balls soaked in Krebs. As soon as the longitudinal muscle/myenteric plexus preparation was obtained, it was set up in the organ bath and the equilibration procedure initiated.

6.2.4 Protocol for the In Vitro GPI Studies.

Once the longitudinal muscle/myenteric plexus preparations were placed in the organ baths, they were perfused with Krebs (at 37°C) and attached to a force transducer (Graphtec Linearcorder F WR3701 with pre-pressure amplifier). All preparations were placed under an initial tension of 1g and allowed a 1 hour equilibration period (with flushing of fresh Krebs every 20 minutes) before testing commenced.

The peptides were diluted in physiological saline such that a 15µl addition to the 15ml organ bath would yield a final concentration of between 0.1pM and 100nM. [Note; final organ bath concentrations of the peptides used were 0.1pM, 1.0pM, 10.0pM, 100pM, 1.0nM, 10.0nM, 100.0nM and 300.0nM]. This study was conducted in two separate subsections; *i.e.* a) first the effect of the C-terminal fragments SP 4-11, 5-11, 6-11 and 7-11 and then b) the effect of the N-terminal fragments SP 1-4, 1-6, 1-7 and 1-9 (as well as Tyr^{8}-SP) were examined.

After the peptide had been added to the organ bath and the maximum contractile response recorded (usually within two to three seconds), the organ bath was flushed with fresh Krebs and the tissue allowed 5-10 minutes to fully relax before the next dose was added. When changing to a different peptide, the apparatus was flushed with fresh Krebs and rested for 15-20 minutes before the next round of dosing.

Prior to the setting up of the longitudinal muscle/myenteric plexus preparation in the organ bath, calibration of the transducer with counter-weights revealed that a 1mm deflection corresponded to 64.5mg of tension. Therefore, a simple calculation converted the deflection (recorded in mm) to tension (mg). Measurement of the contractile response deflection was obtained by assessing the distance from the baseline of the trace to the peak of the maximum response recorded.

The contractile response of the longitudinal muscle/myenteric plexus preparation was recorded as the mean of the tension developed (mg) $\pm$ the SEM and represents the data from between four and fourteen longitudinal

muscle/myenteric plexus preparations obtained from each of the five adult guinea pigs.

Statistical significance was assessed using two-way ANOVA and Tukey-Kramer Multiple Comparisons Test (*i.e.*, the post test; if required). A value of $p < 0.05$ was considered significant.

6.2.5 Materials and Chemicals for the In Vivo Blood Pressure Studies.

Methohexitone sodium (Brietal Sodium, Eli Lilly, Sydney, Australia), pentobarbitone sodium (Nembutal, Bomac Laboratories, NSW, Australia), benzalkonium chloride (Apex Laboratories, Sydney, Australia) and heparin (Commonwealth Serum Laboratories, Sydney, Australia). Substance P 1-11, SP 4-11 (octa-substance P), SP 5-11 (hepta-substance P), SP 6-11 (hexa-substance P), SP 7-11 (penta-substance P) (Peninsula Laboratories, CA, USA) and substance P 1-4 (Auspep, Australia). Physiological saline (0.9% sodium chloride w/v, Ajax Chemicals, Sydney, Australia). Graphtec Linearcorder F WR3701 (Graphtec Corp., Japan), JRAK Type RK1 Pressure Amplifier (JRAK Biosignals, Sydney, Australia).

6.2.6 Animals for the In Vivo Blood Pressure Studies.

Five adult Wistar Kyoto rats (WKY; mean weight 479.2 ± 7.9g) were obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO) Adelaide. Rats were maintained on standard rat chow and water *ad libitum* and a standard light-dark cycle, until required.

6.2.7 Animal Preparation for the In Vivo Blood Pressure Studies.

Indwelling venous and arterial catheters were placed in the WKY rats in a manner similar to that described earlier by Howe *et.al.* (1986). Rats were anaesthetized with a 2:1 Brietal/Nembutal mixture (i.p.; 0.25ml/100g body weight) and the aorta identified. A catheter (which had been soaked overnight in 0.05% benzalkonium chloride) was inserted into the aorta and sealed with tissue adhesive (Instant Glue; cyanomethacrylate-ester) before anchoring the catheter to

adjacent muscle. In a similar manner, the jugular vein was located and catheterized. Both catheters were externalized to emerge at the nape of the neck and flushed with heparin (200U/ml in saline, 1-2ml) before they were sealed with a removable plug. Rats were allowed at least a one day recovery prior to infusion of the peptides and the catheters were flushed daily with heparin (200U/ml in saline) to inhibit clotting.

6.2.8 Protocol for the In Vivo Blood Pressure Studies.

The peptides were dissolved and diluted in physiological saline such that an injection volume of 0.1ml/500g body weight would yield a final dose of 0.1pmole/500g body weight up to a dose of 10nmole/500g body weight.

The transducer (Graphtec Linearcorder F WR3701 and pressure amplifier) was calibrated prior to the attachment of the aortic catheter of the WKY. It was determined that a 1mm deflection corresponded to a change of 8mmHg of pressure. The chart speed was 5mm/sec to record the heart rate during the infusion of the peptides (for recovery periods chart speed was 5mm/min).

Following calibration, the aortic catheter was connected for direct determination of blood pressure (BP; mean arterial pressure in this instance) and heart rate (HR). Once basal BP and HR levels had been determined, administration of the peptides commenced. Peptides were introduced directly into the circulation via the jugular catheter. (Note; to ensure that the full dose of peptide entered the circulation the jugular catheter was immediately flushed with no more than 500µl of physiological saline). Dosing intervals were dependent upon the time taken for BP to return to basal levels. (This was usually around two minutes with a further minute required, so as to ensure full recovery).

Conversion of pressure responses (recorded as mm deflection) to mmHg pressure simply involved multiplying the deflection (in mm) by 8 to yield the equivalent pressure change (in mmHg). Quantification of the hypotensive reponse was obtained by measuring the distance from the baseline of the normal trace to

the maximum drop in pressure recorded. Heart rate was determined directly from the chart (as beats per second) and subsequently converted to bpm.

The blood pressure response was recorded as the mean decrease in BP (mmHg) $\pm$ the SEM from the recorded basal BP. Heart rate was recorded as the mean beats per minute (bpm) $\pm$ the SEM.

Statistical significance was assessed using two-way ANOVA (for BP) and one-way ANOVA (for HR) with Tukey-Kramer Multiple Comparisons Test (*i.e.*, the post test, if required). A value of $p < 0.05$ was considered significant.

6.3 RESULTS

6.3.1 Results for the In Vitro GPI Studies

The addition of SP to the organ bath resulted in contractile activity in the longitudinal muscle/myenteric plexus preparations from a concentration of approximately 1nM upwards (Fig. 6.1). Statistical analysis (two-way ANOVA and post test) comparing the tension generated at the 1nM concentration with the higher concentrations revealed a significant difference for the 100 and 300 nM concentrations ($p < 0.05$).

The C-terminal fragments SP 4-11, 5-11 and 6-11 elicited a contractile response which was similar to that of SP (Fig. 6.1). The fragment SP 7-11 did not possess significant contractile activity (Fig. 6.1). Results of the post test comparing the response obtained with SP with those of its fragments at the same dose may be seen in Table 6.1 (next page).

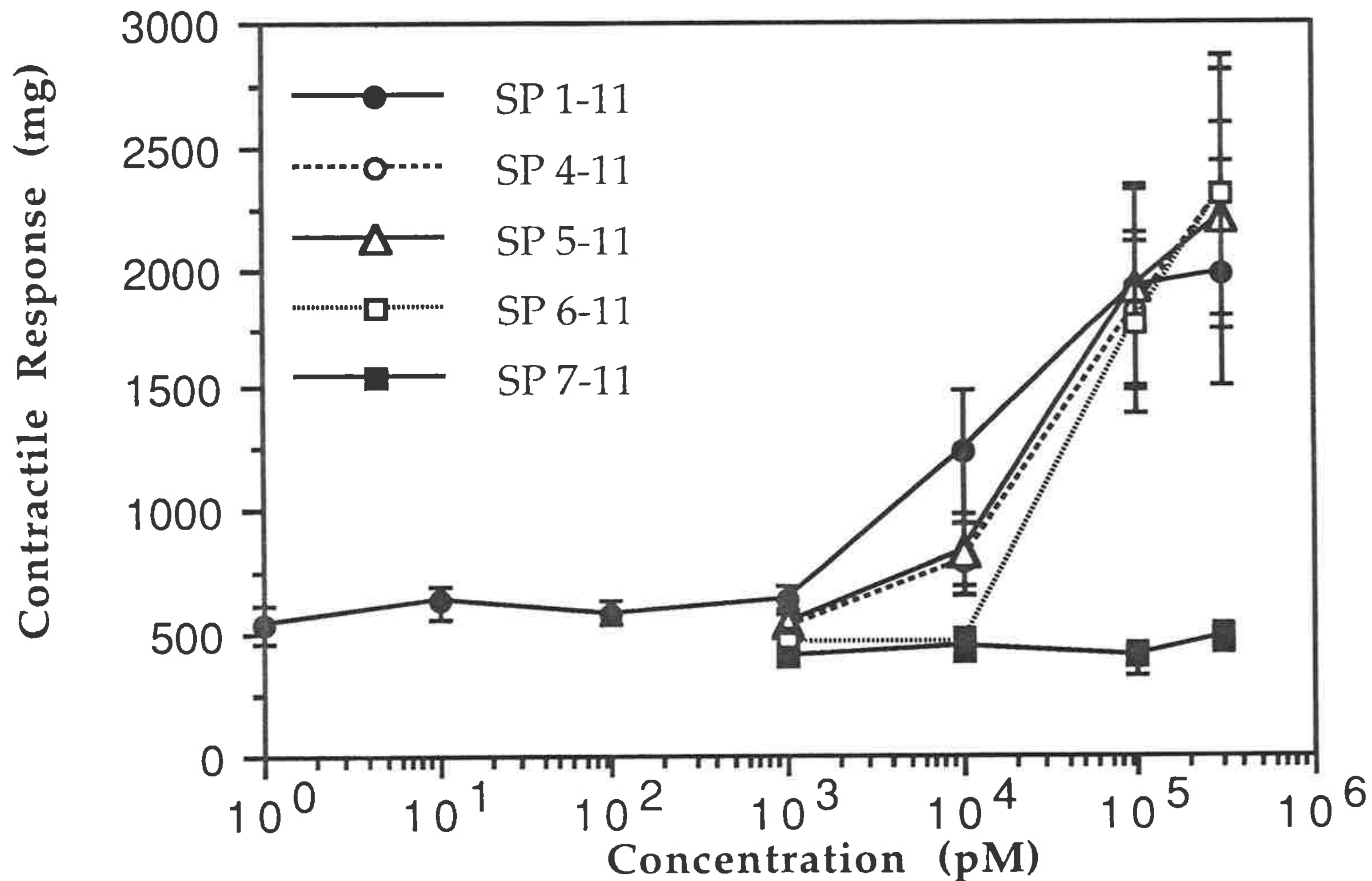


Fig. 6.1. Contractile effect of C-terminal fragments of substance P (SP) on the longitudinal muscle/myenteric plexus of the Guinea Pig.

Sections of longitudinalmuscle/myenteric plexus of untreated Guinea Pig ileum (N=6) were placed in organ baths attached to force transducers to determine the contractile effect of various C-terminal SP fragments. The figure shows the contractile effects (mean tension developed [mg] ± SEM) produced by substance P (SP 1-11), SP 4-11, SP 5-11, SP 6-11 and SP 7-11 between the concentration range of 1pM to 300µM. (See legend in figure).

SP 1-11 versus	1nM	10nM	100nM	300nM
SP 4-11	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
SP 5-11	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
SP 6-11	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
SP 7-11	$p > 0.05$	$p > 0.05$	$p < 0.01$	$p < 0.01$

Table 6.1. Summary of results for the post test comparing the contractile response obtained with SP 1-11 with one of its fragments at the same concentration in the longitudinal muscle/myenteric plexus of the guinea pig.

SP 1-11 versus	1nM	10nM	100nM	300nM
Tyr^8-SP	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
SP 1-4	$p > 0.05$	*	* *	@
SP 1-6	$p > 0.05$	*	* *	* *
SP 1-7	$p > 0.05$	* *	* *	* *
SP 1-9	$p > 0.05$	* *	* *	* *

Table 6.2. Summary of results for the post test comparing the contractile response obtained with SP 1-11 with one of its fragments at the same concentration in the longitudinal muscle/myenteric plexus of the guinea pig. * denotes $p < 0.01$, * * denotes $p < 0.001$ and @ denotes a sample size too small to perform the post test.

The effects of the N-terminal fragments on the guinea pig longitudinal muscle/myenteric plexus preparation may be seen in Figure 6.2. Substance P elicited contractile activity from the approximate concentration of 1nM. Tyr^8-SP (although not an endogenous peptide) produced a very similar response to that seen with SP (Table 6.2; previous page). However, the N-terminal fragments (*i.e.*, SP 1-4, 1-6, 1-7 or 1-9) appeared not to possess any contractile activity in this system. These fragments generated a significantly lower contractile response than that observed with SP for concentrations above 1nM (Table 6.2; previous page).

6.3.2 Results for the In Vivo Blood Pressure Studies.

Mean basal blood pressure (BP) and heart rate (HR) for the five WKYs were 135 ± 6.5 mmHg and 360 ± 13.4 bpm respectively. Infusions of SP and the fragments SP 4-11, 5-11 and 6-11 all resulted in a similar, significant, dose-related hypotensive response, particularly with doses above 10pmole/500g body weight (Fig. 6.3). Statistically, the hypotensive responses elicited by infusion of these fragments were not significantly different from those seen with SP at the same dose (two-way ANOVA and post test; $p > 0.05$: Table 6.3, next page).

In contrast, both SP 7-11 and SP 1-4 had little effect on BP at the lower dose range. However, at the highest dose employed (*i.e.*, 10^4pmoles/500g body weight), the effect of SP 7-11 and SP 1-4 did not differ significantly from the hypotensive response obtained with SP at the same dose (post test; $p > 0.05$; Table 6.3, next page).

None of the peptides significantly increased HR above basal levels for any of the doses tested (one-way ANOVA and post test, $p > 0.05$; Fig. 6.4).

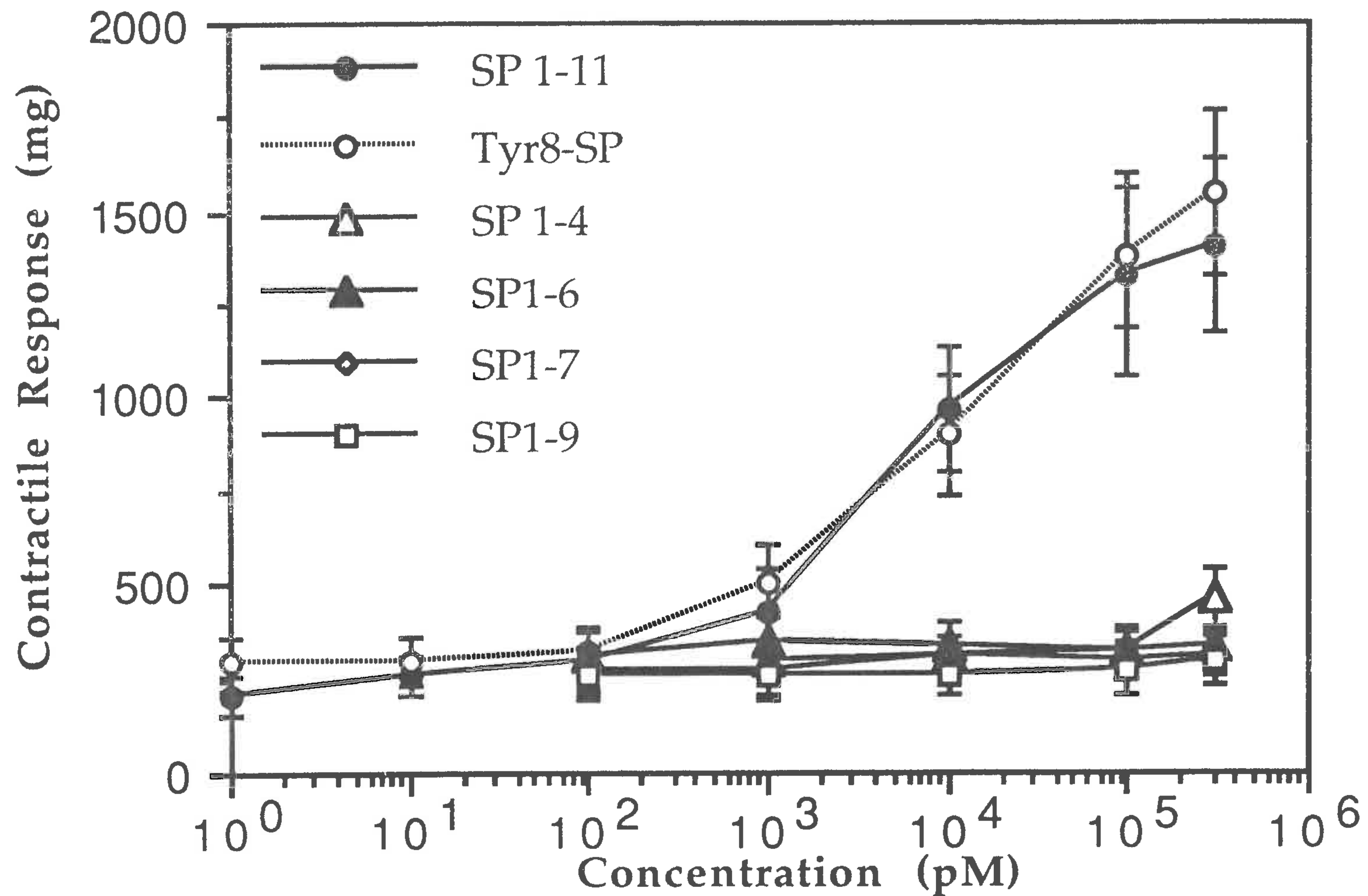


Fig. 6.2. Contractile effect of N-terminal fragments of substance P (SP) on the longitudinal muscle/myenteric plexus of the Guinea Pig.

Sections of longitudinalmuscle/myenteric plexus of untreated Guinea Pig ileum (N=6) were placed in organ baths attached to force transducers to determine the contractile effect of various C-terminal SP fragments. The figure shows the contractile effects (mean tension developed [mg] ± SEM) produced by substance P (SP 1-11), Tyr^{8}-SP, SP 1-4, SP 1-6, SP 1-7 and SP 1-9 between the concentration range of 1pM to 300µM. (See legend in figure).

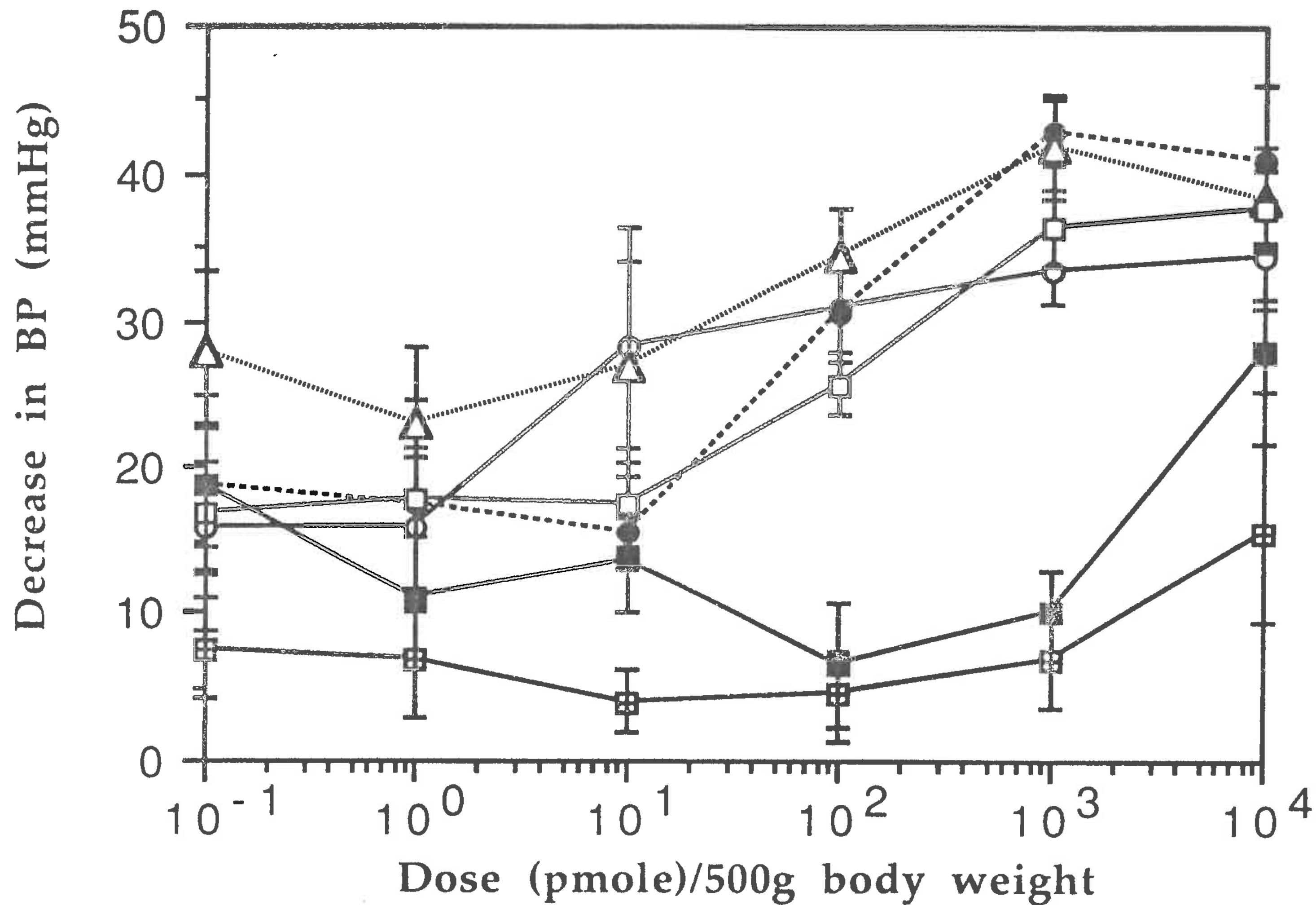


Fig. 6.3. Hypotensive effect of substance P and some of its fragments in the Wistar Kyoto Rat (WKY).

The in vivo blood pressure (BP) responses to the intravenous infusion of various fragments of substance P were determined in the freely moving, non-anaesthetized WKY (N=5). Basal BP levels were 135.0 ± 6.5 mmHg. The figure shows the mean decrease in BP (mmHg ± SEM) elicited by the various fragments over the concentration range of 0.1pmole to 10nmole per 500g body weight. Legend; SP 1-11 —○— *, SP 4-11* ----●---- *, SP 5-11* ·····△····· *, SP 6-11* —□— *, SP 7-11* ═■— *and SP 1-4* —⊞— *.*

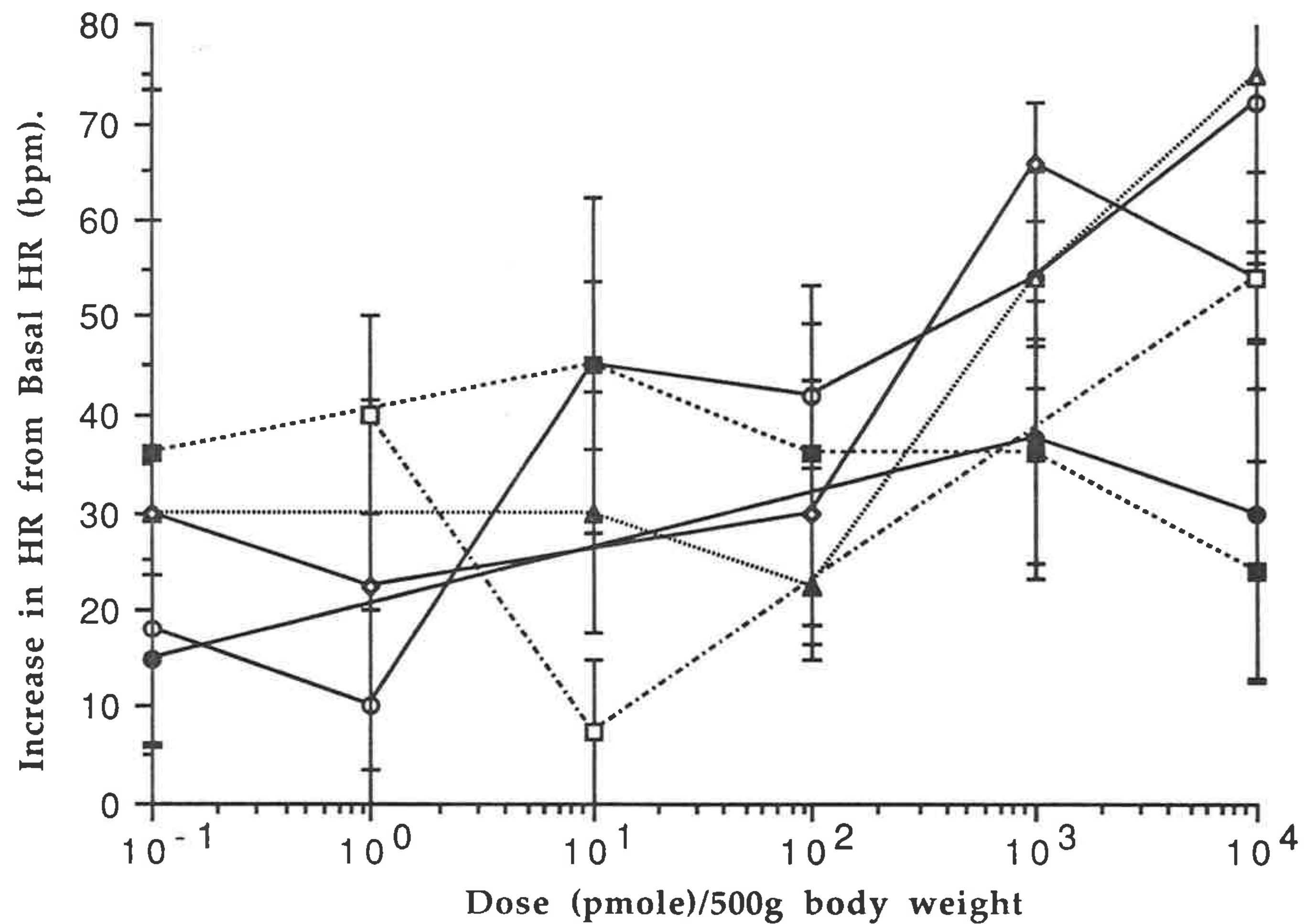


Fig. 6.4. Tachycardia effect of substance P and some of its fragments in the Wistar Kyoto Rat (WKY).

The in vivo heart rate (HR) responses to the intravenous infusion of various fragments of substance P were determined in the freely moving, non-anaesthetized WKY (N=5). Basal HR levels were 360.0 ± 13.4 bpm. The figure shows the mean increase in HR (from basal HR; mean bpm ± SEM) elicited by the various fragments over the concentration range of 0.1pmole to 10nmole per 500g body weight. Legend; SP 1-11 —○— , SP 4-11 ·····▲····· , SP 5-11 ----■---- , SP 6-11 —◇— , SP 7-11 ----□---- and SP 1-4 —●— .

Dose	SP 4-11	SP 5-11	SP 6-11	SP 7-11	SP 1-4
0.1	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
1	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
10	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	*
100	$p > 0.05$	$p > 0.05$	$p > 0.05$	*	* *
10^3	$p > 0.05$	$p > 0.05$	$p > 0.05$	*	* *
10^4	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$

Table 6.3. Summary of results for the post test comparing the hypotensive response obtained with SP with one of its fragments at the same dose in the freely moving WKY. The dose has been expressed as pmole/500g body weight.

* denotes $p < 0.01$ and * * denotes $p < 0.001$.

6.4 DISCUSSION

Previous studies have shown that the C-terminal fragments (and analogues of SP) possess varying degrees of activity in numerous preparations (Bury and Mashford, 1976; Teichberg and Blumberg, 1980; Couture and Regoli, 1982; Escher *et al.*, 1982; Pernow, 1983; Hall *et al.*, 1987; Lei *et al.*, 1991 and Stavropoulos *et al.*, 1991).

As more specific analogues and antagonists of SP have become available, knowledge of the actions of SP in the guinea pig ileum has grown (Tousignant *et al.*, 1991; Legat *et al.*, 1992; Mochizuki *et al.*, 1993). However, disagreement exists regarding the type of receptors present in the guinea pig ileum. Most studies have examined the receptors in the circular muscle layer of the ileum (Maggi *et al.*, 1990; Maggi *et al.*, 1993; Santicioli *et al.*, 1993), with some examining receptor subtypes in the myenteric plexus (Guard *et al.*, 1991; Schemann *et al.*, 1991). Nevertheless, for SP to be active in this particular system the presence of the NK_1 receptor subtype is required.

The C-terminal fragments SP 4-11, 5-11 and 6-11 elicited comparable responses in the longitudinal muscle/myenteric plexus preparation to SP itself (Fig. 6.1). However, in decreasing the chain length by one amino acid from SP 6-11 to SP 7-11, the activity of the resulting fragment was severely reduced (Fig. 6.1). Hence, it appears that the sixth amino acid is important for biological activity in this *in vitro* system. It might be expected the larger N-terminal fragments incorporating the fourth, fifth and sixth amino acids should be biologically active as well. However, all of the N-terminal fragments tested (SP 1-4, SP 1-6, SP 1-7 and SP 1-9) were ineffective in eliciting contractile activity in the longitudinal muscle/myenteric plexus preparation (Fig. 6.2). Therefore, the presence of the amino acid sequence Pro^4-Gln^5-Gln^6 in part or whole, appears to be essential for biological activity in this preparation in combination with the remainder of the C-terminal.

When the peptide was shortened, but the Pro^4-Gln^5-Gln^6 sequence was still present (as in SP 1-6, 1-7 and 1-9), the contractile activity was minimal (Fig.

6.2). Thus, the C-terminal fragments are important in this preparation. Without this fragment, the conformation could be altered such that the Pro^4-Gln^5-Gln^6 sequence may not be able to bind to the specific receptors. A similar observation has been made by Teichberg and Blumberg (1980) who concluded that the sequences SP 5-11 and SP 6-11 were the optimal size and sequence for a SP analogue.

Nevertheless, in the light of the results of these biological activity studies, the elevated endogenous plasma levels of the C-terminal fragments SP 4-11 and SP 5-11 (outlined in Chapter 4) would not be considered to be physiologically significant. [Note: the endogenous plasma level of approximately 450pg/ml for the fragments SP 4-11/5-11 roughly correlates to 0.47nM for SP 4-11 and 0.54nM for SP 5-11. These values are well below the threshold of 1nM required to elicit a significant contractile response in the longitudinal muscle/myenteric plexus preparation (Fig. 6.1)].

Substance P is also known to be an effective hypotensive agent in dogs, rats, rabbits and man when administered by intravenous infusion (Bury and Mashford, 1977b; Pernow 1983; Maggi *et al.*, 1985; Fuller *et al.*, 1987; Evans *et al.*, 1988), although intracerebroventricular injection in rats elicits a pressor response (Trimarchi *et al.*, 1986). Nevertheless, it was not surprising to find that intravenous infusion of the C-terminal fragments SP 4-11, SP 5-11 or SP 6-11 decreased blood pressure to the same extent as SP (Fig. 6.3, Table 6.3). These are the same C-terminal fragments which elicited a contractile response similar to SP in the longitudinal muscle/myenteric plexus of the guinea pig. Thus, some of the C-terminal fragments of SP may be as effective as the parent peptide in producing a response in a biological system.

SP 7-11 was not as effective as SP, SP 4-11, SP 5-11 or SP 6-11 in eliciting the hypotensive response (Fig. 6.3), suggesting that the fourth, fifth and sixth amino acids in the sequence (*i.e.*, Pro^4-Gln^5-Gln^6) are important in the *in vivo* blood pressure responses. In contrast, N-terminal fragment SP 1-4 only produced a significant depressor response at the highest dose tested (*i.e,* 10^4pmole/500g body

weight), suggesting that the sequence Arg^1-Pro^2-Lys^3-Pro^4 is relatively unimportant for conferring biological activity in this system. This further substantiates the importance of the middle Pro^4-Gln^5-Gln^6 sequence in the biological activity of the peptide for both the contractile response of the longitudinal muscle/myenteric plexus of the guinea pig and the hypotensive reponse of the WKY.

Heart rate was not significantly increased from basal readings by the administration of any of the peptides (one-way ANOVA and post test, $p > 0.05$). This suggests that SP and its fragments do not possess a direct chronotropic effect in this system.

Species differences in responses to SP have been alluded to from the preliminary studies (6.1 *Background and Aims*; data not presented). SHR-SPs are more sensitive than WKYs, whilst SHRs appear to be less sensitive than WKYs to intracerebroventricular SP (Unger *et al.*, 1980; Pompei, Tayebati, Massi *et al.*, 1992; Pompei, Tayebati, Polidori, *et al.* 1992). Nevertheless, little research has been undertaken to investigate this phenomenon further.

In conclusion, the major aim of this study was to determine the effects of SP and its fragments on both the contractile responses of the longitudinal muscle/myenteric plexus of the guinea pig ileum and the blood pressure responses of the freely moving WKY. The C-terminal fragments SP 4-11, 5-11 and 6-11 presented a similar dose-response profile when compared to SP in both systems. Minimal contractile activity was noted in the longitudinal muscle/myenteric plexus preparation from the guinea pig ileum for the fragments SP 7-11, 1-6, 1-7 and 1-9 and the fragments SP 7-11 and SP 1-4 elicited a significant hypotensive response in the WKY only at the highest dose tested (10^4 pmole/500g body weight). These results suggest that the presence of the sequence Pro^4-Gln^5-Gln^6 is essential to SP for conferring biological activity in these systems.

CHAPTER 7

DISTRIBUTION OF SUBSTANCE P IN THE WISTAR KYOTO RAT (WKY) AND THE SPONTANEOUSLY HYPERTENSIVE RAT (SHR).

7.1 BACKGROUND AND AIMS

Substance P has a widespread distribution throughout the CNS and the periphery (Chapter 1, *1.3* and Pernow, 1983). It is not known whether the distribution and/or the concentration of substance P may be altered in models of hypertension such as the spontaneously hypertensive rat (SHR). One of the factors believed to contribute to the development of hypertension is sympathetic hyperinnervation, which itself is mediated via the trophic factor, NGF (Chapter 1, *1.7.3.vi*). NGF is also a trophic factor for SP-containing nerves (Chapter 1; *1.3.3.ii*). If NGF is elevated in the hypertensive animal and mediates the development of sympathetic hyperinnervation, then it may also increase the number of SP-fibres and therefore the SP content of tissues innervated by these nerves.

Hence, the aim of the following study was to determine and compare the distribution of SP in the genetic animal model of hypertension, the SHR, with its normotensive control the Wistar Kyoto rat (WKY). Tissues selected for this purpose were the dorsal root ganglia (a major site of SP biosynthesis [Pernow, 1983] and expected to contain comparatively high concentrations of SP), the superior cervical ganglia, a selection of blood vessels (mesenteric artery, caudal artery and aorta) as well as the heart, kidney, adrenal glands, liver and salivary gland (the submaxillary gland).

Substance P is a potent natriuretic, diuretic and sialagogue (Chapter 1, *1.4*). Mouse salivary glands contain high levels of NGF (Levi-Montalcini and Angeletti, 1968). The kidney and adrenal glands have a role in the maintenance of blood pressure and, in some instances, the development of hypertension. Thus, the SP content of the kidneys, adrenal glands and submaxillary glands may be altered in

the hypertensive animal when compared to the WKY. The heart was selected due to its importance in the circulatory system and therefore blood pressure control, whilst the liver was selected as a representative of an organ not primarily involved in the circulatory system and not highly innervated by SP-fibres (*i.e.*, a form of control tissue).

7.2 MATERIALS AND METHODS

7.2.1 Materials and Chemicals.

Blood pressure recording apparatus consisted of a manual scanner (Model 65-12), pulse amplifier/recorder (Model 129) and cuff pump (Model 20-NW) all from IITC. Inc. C.A., U.S.A and heated racks (around 32°C) with individual restraining canisters (CSIRO workshop, Adelaide).

Homogenizations were performed with a motor-driven glass homogenizer (1ml; blood vessels, ganglia and adrenals) or hand-held glass homogenizer (10ml; all other tissues); (both from Wheaton, U.S.A).

Pentobarbitone sodium (Nembutal, Bomac Laboratories, NSW, Australia), physiological saline (0.9% sodium chloride w/v, Ajax Chemicals, Sydney, Australia); methanol and hydrochloric acid (HCl; concentrated) (Ajax Chemicals, Australia); Eppendorf tubes (1.5ml) and 10ml polypropylene tubes (Labsupply, Adelaide, Australia).

Reagents and apparatus required as per the IEC technique (Chapter 3; *3.2.1.i Materials and Chemicals for the Basic IEC*) and the RIA method (Chapter 2; *2.2.1.i Materials and Chemicals for the Basic RIA*).

7.2.2 *Animals.*

Nine WKYs and nine SHRs (all male) were obtained from the CSIRO Glenthorne facility and transferred to the Kintore Avenue site at between 10 and 12 weeks of age. Rats were maintained on standard rat chow and water *ad libitum* and a standard light-dark cycle, until sacrificed.

7.2.3 *Blood Pressure Recordings.*

Blood pressure readings were obtained using the indirect tail-cuff pulse detector (IITC) procedure which was performed between 7am and 12 noon on the required day.

WKYs were 15 weeks of age and SHRs were 17 weeks of age at the determination of BP. Two trial runs were performed on the rats, to accustom them to the apparatus and procedure. Each rat had 20-25 separate recordings taken during the 5 hour procedure with the best (clear) readings taken (as determined by visual inspection of the chart recordings).

Results have been expressed as the mean and SEM of the recorded blood pressures (mmHg) for the WKYs as a group and the SHRs as a separate group. The unpaired t-test was used as the statistical test of choice ($p < 0.05$ for significance).

7.2.4 *Tissue Collection and Processing.*

Rats were anaesthetized with a 15mg/ml Nembutal solution (diluted 1:4 with physiological saline) and injected *i.p.* at a dose of 0.35ml/100g body weight. The various required tissues were located, (*i.e.*; superior cervical ganglia [SCG; 2 per rat], dorsal root ganglia [DRG; 6 per rat], mesenteric artery, aorta, heart, kidney [only 1 was removed], adrenal glands [2 per rat], spleen, caudal artery, liver and submaxillary glands [2 per rat]), removed and cleaned of adhering fat and other connective tissue. Tissues were immediately rinsed in ice-cold 0.9% saline, blotted and weighed. Samples were either homogenized on the day and the

homogenate frozen for later IEC processing and SP determination, or frozen immediately and homogenized on the day of the IEC procedure (see below).

The IEC procedure was conducted in the usual manner (SP Sephadex C-25 resin was used for these IEC procedues). The fractions which were run were 0.018M pyridine/0.1 M formic acid, 0.1M pyridine/0.1M formic acid, 0.35M pyridine/0.35M formic acid and 1.5M pyridine/1.5M formic acid. All of the fractions (except for 0.018M pyridine/0.1 M formic acid) were collected and dried and the SP content determined by the SP RIA (Chapter 2; 2.2.2 *The Basic RIA Method*).

Results have been expressed as the substance P content per weight of tissue (either pg/mg or pg/g tissue). The statistical test used was the unpaired t-test ($p < 0.05$ for significance).

7.2.4.i Blood Vessels and Ganglia.

Due to the relatively small size and large surface area of the blood vessels and ganglia, it was believed that the endogenous enzymic degradation of substance P would be inhibited by the immediate addition of an ice-cold MeOH/HCl solution and storage at -80°C. Thus, once these tissues had been removed (*i.e.;* SCG, DRG, mesenteric artery, aorta and caudal artery) cleaned and weighed, they were immediately placed in an ice-cold Eppendorf tube containing 1ml of 1:1 MeOH/0.1M HCl and promptly frozen at -80°C.

On the day of the IEC, samples were removed from the freezer and stored on ice during the homogenization procedure. Tissues were transferred to the 1ml glass motor-driven homogenizer along with the MeOH/0.1M HCl for homogenization purposes.

The homogenate was transferred back to the original Eppendorf tube. The homogenizer was rinsed with a further 0.5ml ice-cold 1:1 MeOH/0.1M HCl and the rinse added to the Eppendorf tube. Homogenates were then centrifuged (at 13G for 20 mins at 4°C) and the supernatant decanted. 1.5mls of the 0.018M IEC buffer were added to the supernatant and the resultant mixture was loaded on to

the IEC column and run in the usual manner. Fractions were collected and dried and the SP content determined by RIA.

7.2.4.ii Adrenal Glands.

Both adrenal glands from each rat were immediately placed in an ice-cold Eppendorf tube containing 1ml of 1:1 MeOH/0.1M HCl and promptly frozen at -80°C.

The homogenization procedure was identical to that outlined above for the blood vessels and ganglia, except homogenates were transferred into a 5ml polypropylene tube. The homogenizer was rinsed twice with 0.5ml 1:1 MeOH/0.1M HCl with both rinsings added to the 5ml tube. The adrenals required extra rinsing due to the larger quantity of cellular debris obtained during homogenization.

Homogenates were centrifuged (at 13G for 20 mins and 4°C) and the supernatant decanted. 2mls of 0.018M IEC buffer was added to the supernatant which was mixed and then run on the IEC columns as outlined above. The fractions were collected and dried before determination of SP content by RIA.

7.2.4.iii All Other Organs.

The remaining tissues removed for SP determinations were substantially larger than the blood vessels, ganglia and adrenals and a significant degree of enzymic degradation of SP may be possible. These tissues (*viz.* the heart, kidney, spleen, liver* and submaxillary glands) were homogenized at the time of removal.

Immediately after weighing, the tissue was placed in an ice-cold hand-held 10ml homogenizer containing 10ml 1:1 MeOH/0.1M HCl. After homogenization, the homogenate was transferred to a 10ml polypropylene tube and centrifuged (at 13G for 20 mins and 4°C). The supernatant was decanted and stored at -80°C until the IEC was performed.

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*The total weight of the livers was approximately 11-12g per animal (for both WKYs and SHRs) and were subsequently too large for total homogenization in a 10ml volume. Sections of liver (of approximately 1g) were used in homogenizations and subsequent SP determination.

On the day of the IEC, samples were removed from the -80^oC freezer, defrosted and a 2ml aliquot taken. 2ml of 0.018M IEC buffer was added and the resultant solution mixed before centrifugation (at 13G for 20 mins and 4^oC). The supernatant was loaded onto the IEC column which was run in the manner outlined above. The fractions were collected and dried with subsequent RIA determination of the SP content.

7.3 RESULTS

7.3.1 Animal Weights, Ages and Blood Pressures.

The rats were 27.2 $\pm$ 0.2 weeks of age for WKYs and 28.7 $\pm$ 0.3 weeks of age for the SHRs when the tissues were harvested. The total body weight of the animals was 430.8 $\pm$ 4.5 g and 375.8 $\pm$ 8.6 g for the WKYs and SHRs respectively (Fig. 7.1). This was a significant difference in total body weight ($p < 0.0001$) with the WKYs tending to be heavier than the SHRs.

Determination of blood pressures (at 15 weeks and 17 weeks of age respectively) revealed pressures of 137.7 $\pm$ 4.7 mmHg for the WKYs and 187.2 $\pm$ 3.2 mmHg for the SHRs (Fig. 7.2). Again, this difference in blood pressures* was found to be statistically significant ($p < 0.0001$).

7.3.2 Harvested Tissue Weights.

Table 7.1 summarizes the data obtained for the tissue weights in both WKYs and SHRs (next page). There was no significant difference in tissue weights between WKYs and SHRs in the superior cervical ganglia, heart, adrenal glands (when N=8 for WKYs; one animal had a single adrenal gland), spleen or the liver sample. A significant difference in tissue weights ($p < 0.05$) was observed for the dorsal root ganglia, mesenteric artery, caudal artery, thoracic aorta, submaxillary glands, kidney and *total* liver between WKYs and SHRs.

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*BP recordings were obtained around 11 to 12 weeks prior to tissue harvesting. These readings were taken to determine that the SHRs did possess elevated BP readings when compared with those of WKYs. Knowledge of the exact difference in BPs at the time of sacrifice of the animals was not essential to the study.

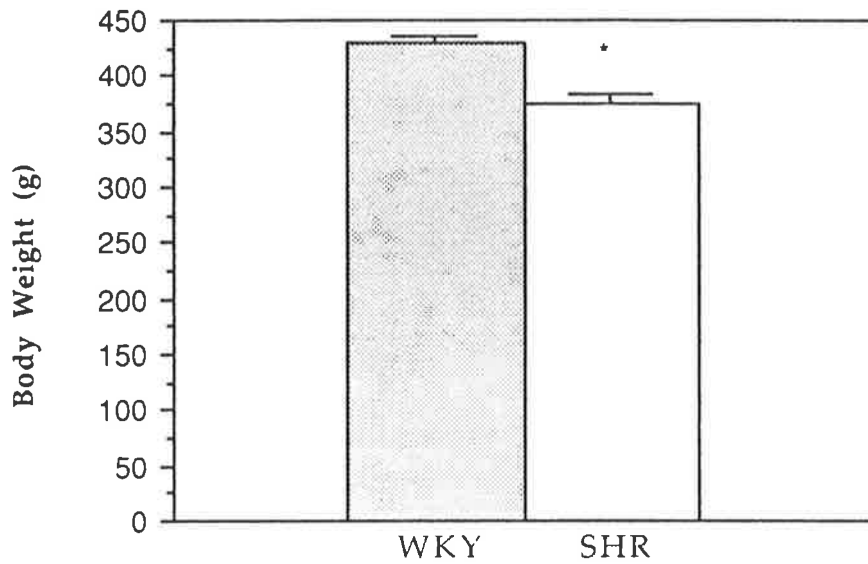

Fig. 7.1. Body weights for the WKYs and SHRs.

This figure shows the body weight (mean g ± SEM) of the untreated WKY (mean age 27.2 ± 0.2 weeks; N=9) and SHR (mean age 28.7 ± 0.3 weeks; N=9).
** $p < 0.0001$ WKY versus SHR (unpaired t-test).*

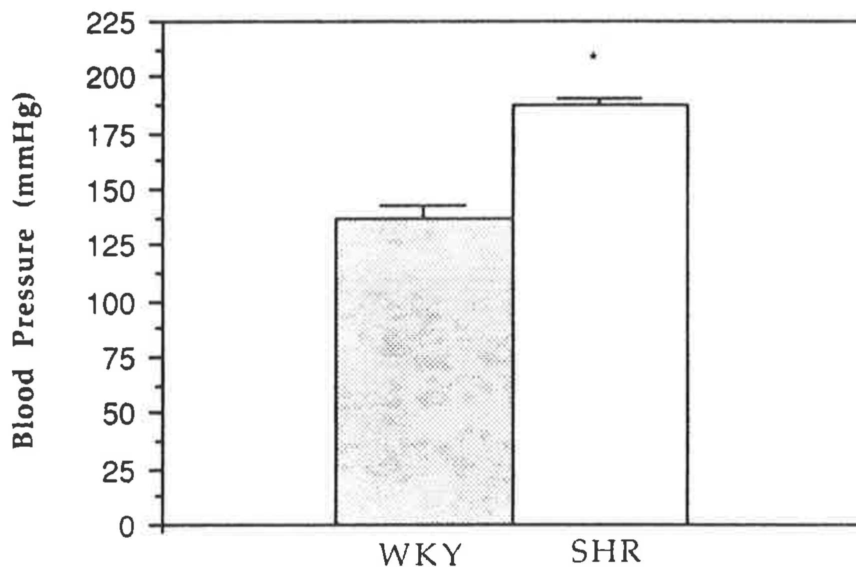

Fig. 7.2. Blood pressure determinations for the WKYs and SHRs.

Blood pressures (mean mmHg ± SEM; N=9 each) of the untreated WKY and SHR were measured by the indirect tail cuff method.
** $p < 0.001$ WKY versus SHR (unpaired t-test).*

MEAN TISSUE WEIGHT

Tissue	WKY	SHR
SCG	3.4 ± 0.4 mg	3.3 ± 0.2 mg
DRG	4.7 ± 0.2 mg	3.8 ± 0.2 mg
Mesenteric Artery	29.0 ± 3.4 mg	39.0 ± 1.8 mg
Caudal Artery	17.7 ± 1.1 mg	21.9 ± 1.6 mg
Thoracic Aorta	38.3 ± 1.3 mg	46.7 ± 1.5 mg
Heart	1.26 ± 0.01 g	1.28 ± 0.03 g
Adrenal Glands	35.5 ± 1.4 mg[A] 33.5 ± 2.3 mg[B]	42.1 ± 3.0 mg
Submaxillary Glands	771.3 ± 6.8 mg	719.4 ± 16.9 mg
Kidney	1.37 ± 0.02 g	1.17 ± 0.03 g
Spleen	592.1 ± 4.4 mg	565.5 ± 23.7 mg
Total Liver	11.59 ± 0.17 g	12.41 ± 0.31 g
Liver Assayed	0.90 ± 0.08 g	0.94 ± 0.06 g

Table 7.1 A summary of the wet weights for tissue samples taken from WKYs and SHRs. No significant differences were observed between WKYs and SHRs in the weights of the SCG, heart, adrenal gland (N = 8 for WKY) or liver assayed (unpaired t-test, $p > 0.05$).

7.3.3 SP-Like Immunoreactivity per Tissue.

Results have been presented in graphical form for each of the tissue types assayed and each of the IEC fractions collected (Figs. 7.3-7.13). The SP determinations for the WKYs and SHRs have been shown as pg/mg for all tissues assayed. The substance P-containing IEC elution was the 1.5M fraction. The sample size was nine, unless otherwise indicated (some samples were lost during processing).

[A] 8 WKYs had both adrenal glands. This value pertains to those 8 animals only.

[B] One WKY had only 1 adrenal gland. This value pertains to the data for all WKYs including the animal with only one adrenal.

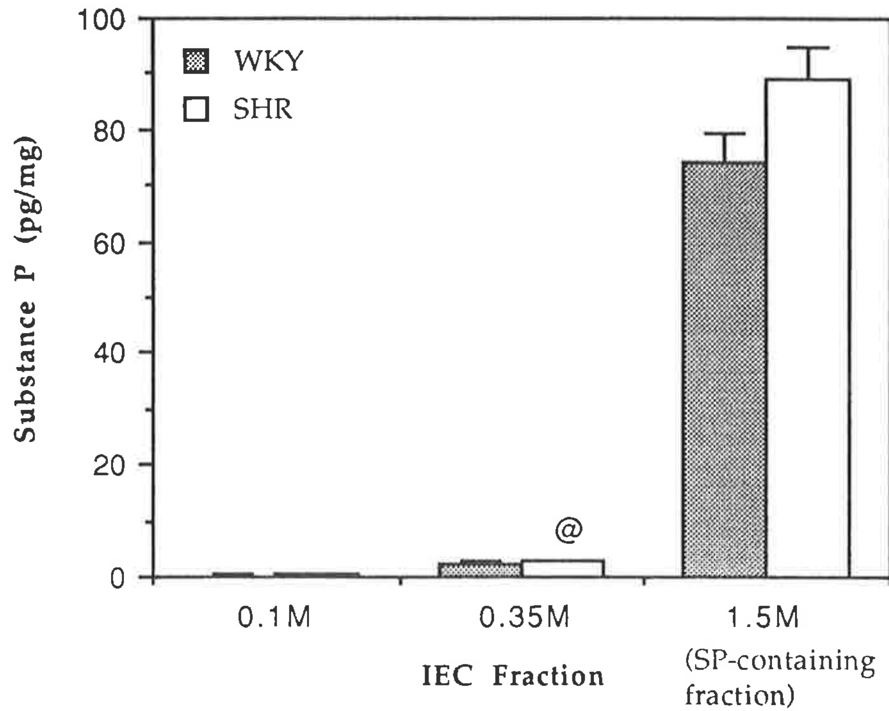

Fig. 7.3. Determination of substance P (SP) content in the dorsal root ganglia (DRG).

DRG were removed from WKY and SHR and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the DRG for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction). N=9, except at @ where N=8.

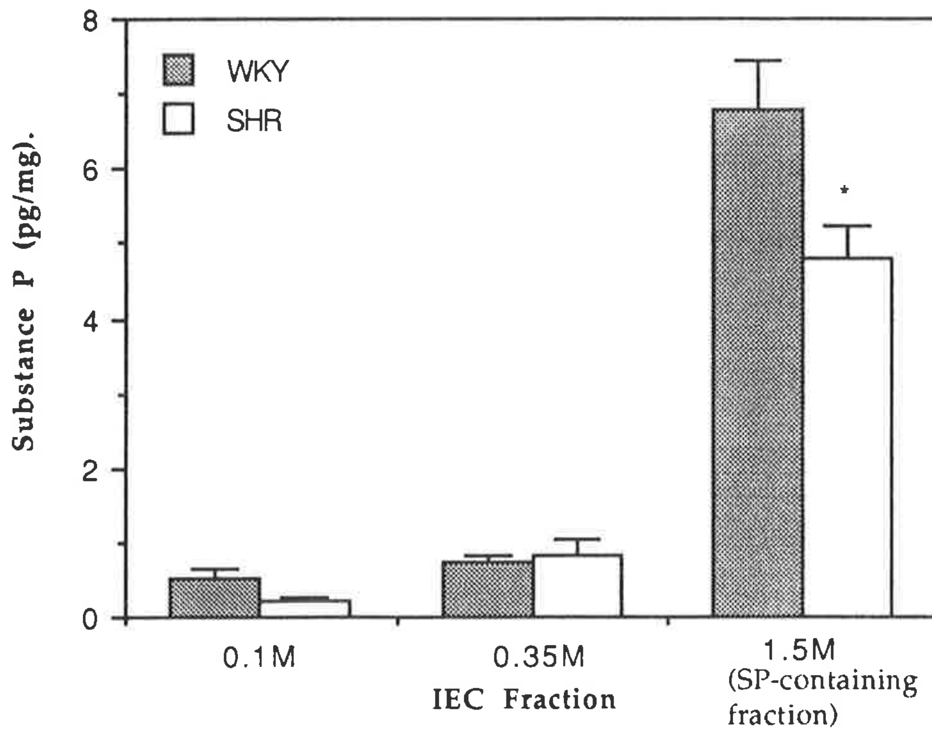

Fig. 7.4. Determination of substance P (SP) content in the superior cervical ganglia (SCG).

SCG were removed from WKY and SHR (N=9 each) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the SCG for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction).

***** $p < 0.05$ **WKY versus SHR (unpaired t-test).**

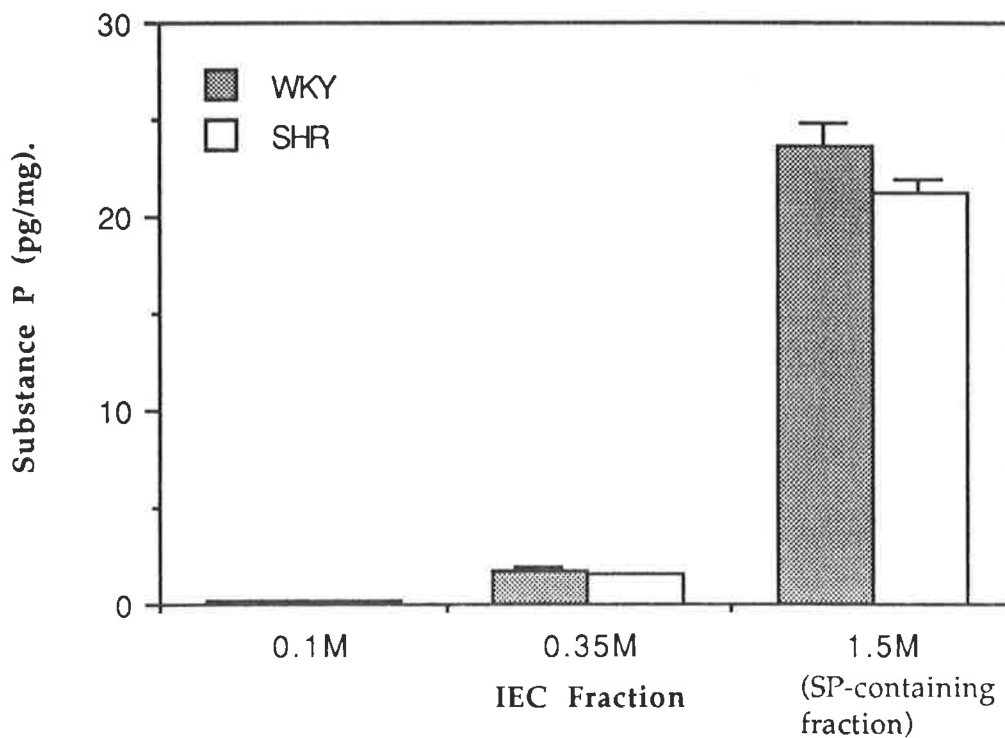

Fig. 7.5. Determination of substance P (SP) content in the mesenteric artery.

The mesenteric artery was removed from WKY and SHR (N=9 each) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the mesenteric artery for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction).

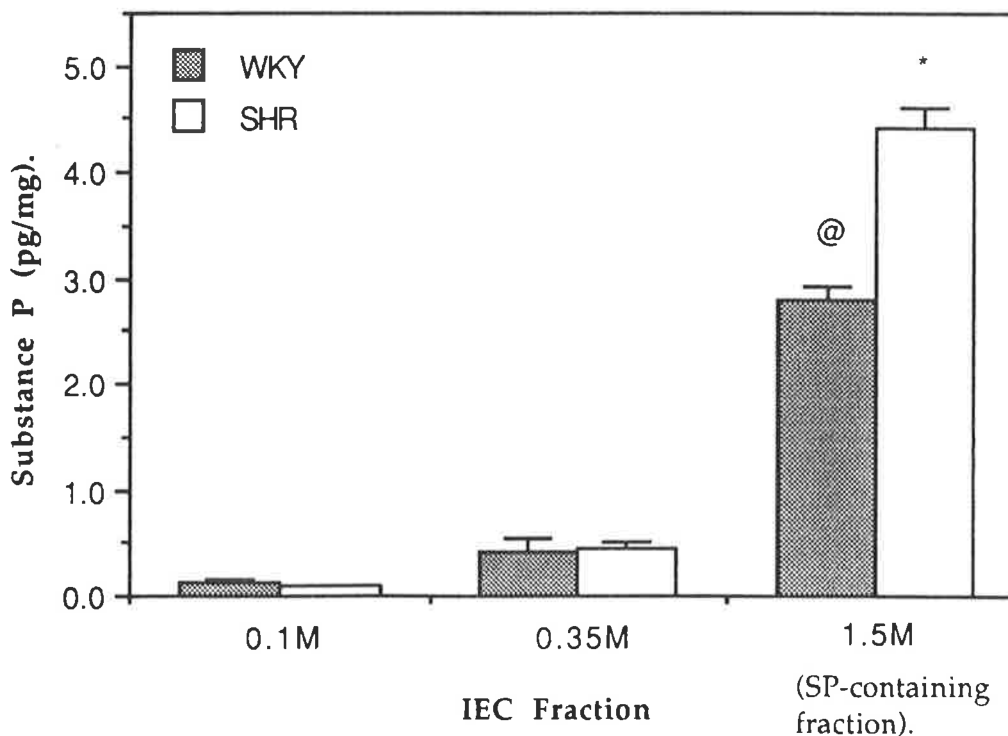

Fig. 7.6. Determination of substance P (SP) content in the caudal artery.

The caudal artery was removed from WKY and SHR and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the caudal artery for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction). N=9, except at @ where N=8.

** $p < 0.05$ WKY versus SHR (unpaired t-test).*

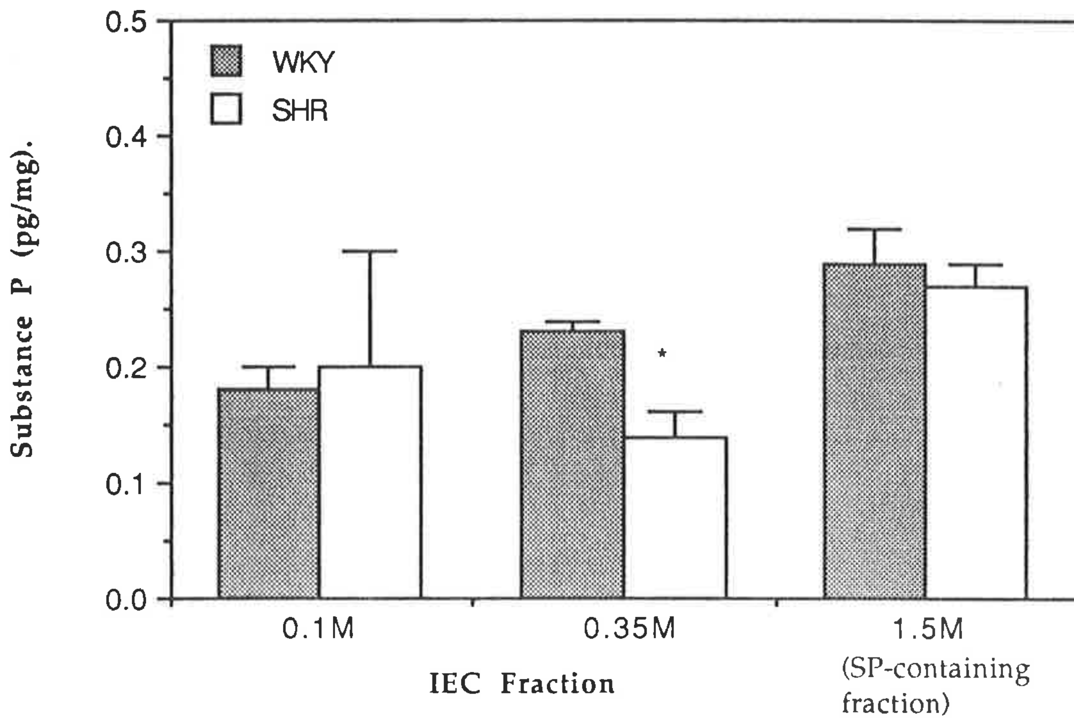

Fig. 7.7. Determination of substance P (SP) content in the thoracic aorta.

The thoracic aorta was removed from WKY and SHR (N=9 each) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the thoracic aorta for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction).
** $p < 0.05$ WKY versus SHR (unpaired t-test).*

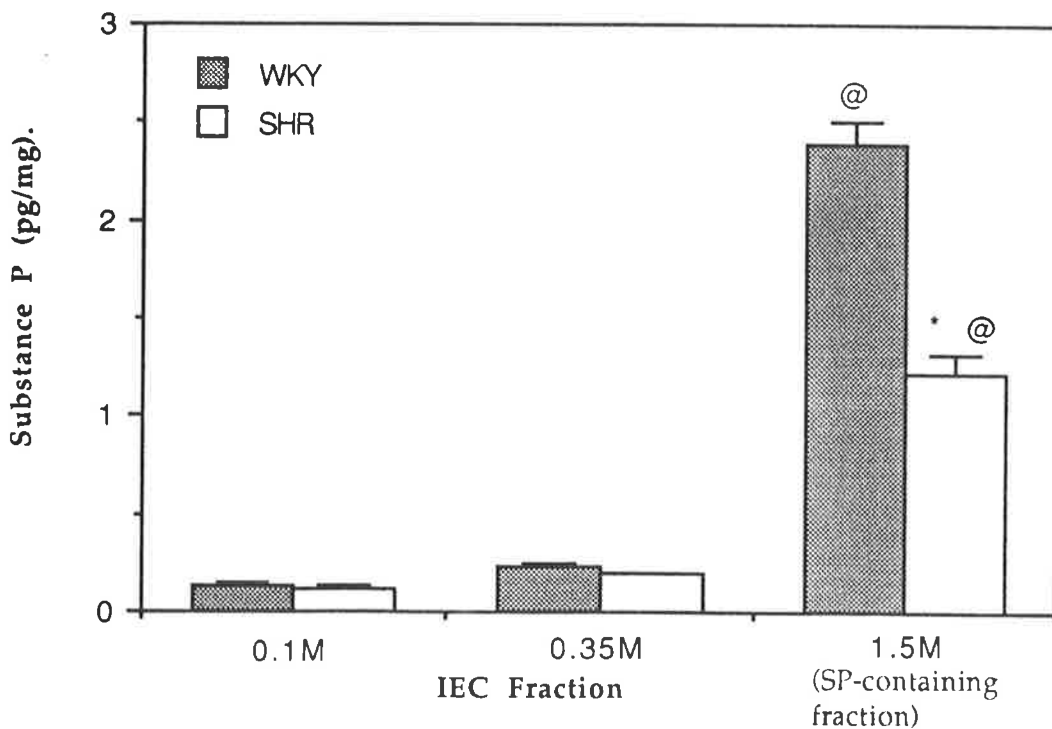

Fig. 7.8. Determination of substance P (SP) content in the adrenal glands.

The adrenal glands were removed from WKY and SHR and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the adrenal glands for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction). N=9, except at @ where N=8.
** $p < 0.05$ WKY versus SHR (unpaired t-test).*

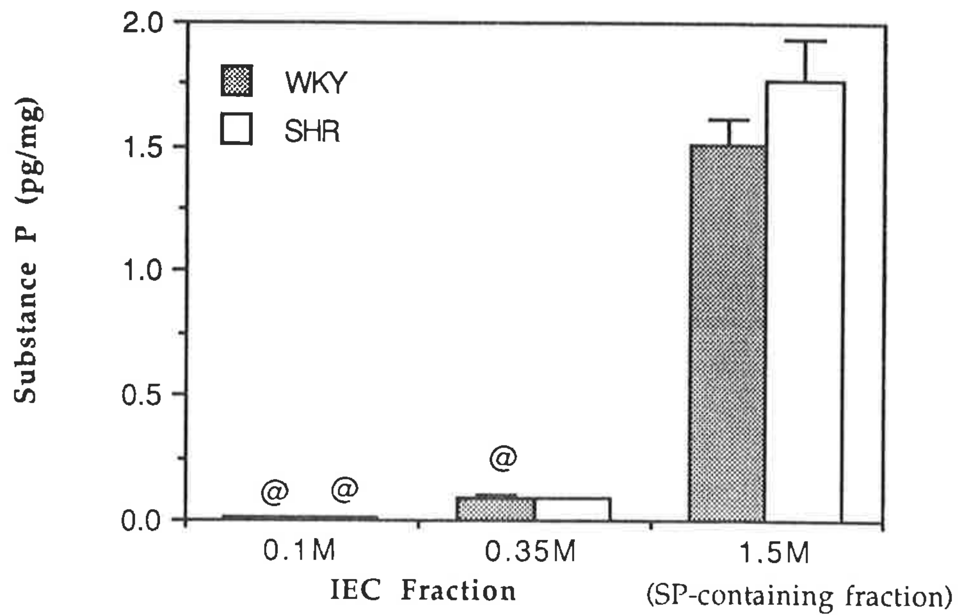

Fig. 7.9. Determination of substance P (SP) content in the submaxillary glands.

The submaxillary glands were removed from WKY and SHR and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the submaxillary glands for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction). N=9, except at @ where N=8.

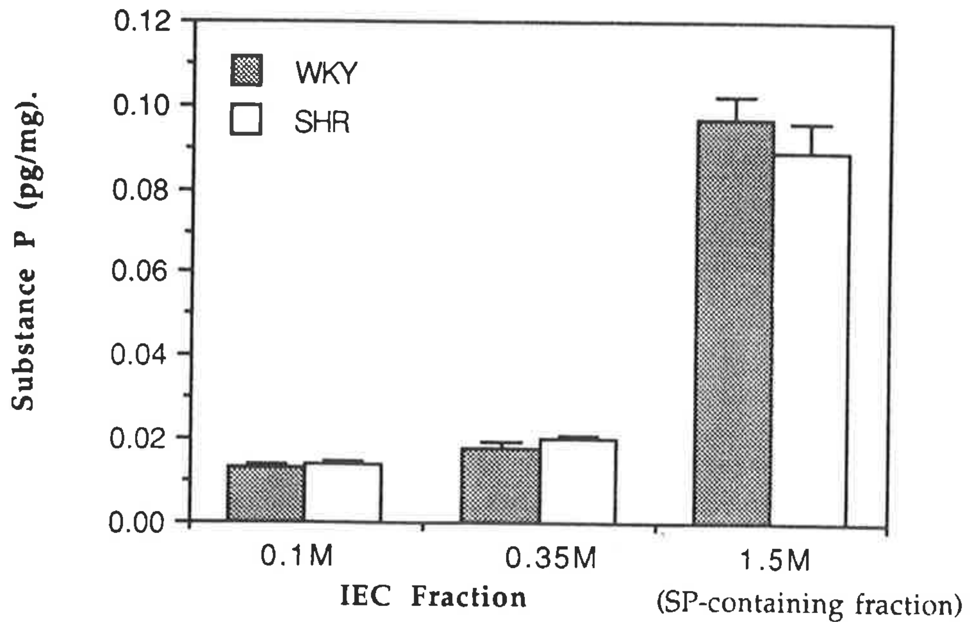

Fig. 7.10. Determination of substance P (SP) content in the heart.

The heart was removed from WKY and SHR (N=9 each) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the heart for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction).

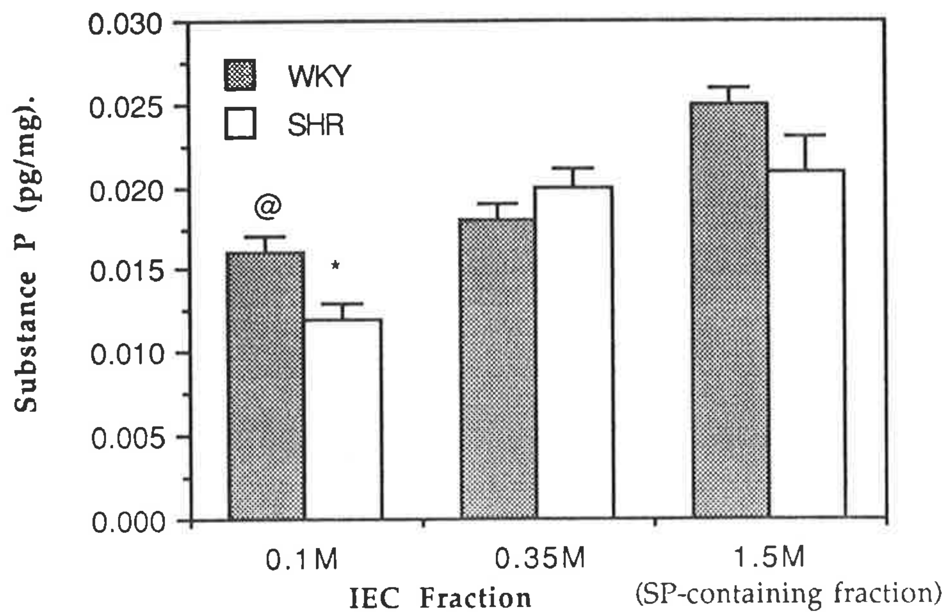

Fig. 7.11. Determination of substance P (SP) content in the kidney.

The kidney was removed from WKY and SHR and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the kidney for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction). N=9, except at @ where N=8.
** $p < 0.05$ WKY versus SHR (unpaired t-test).*

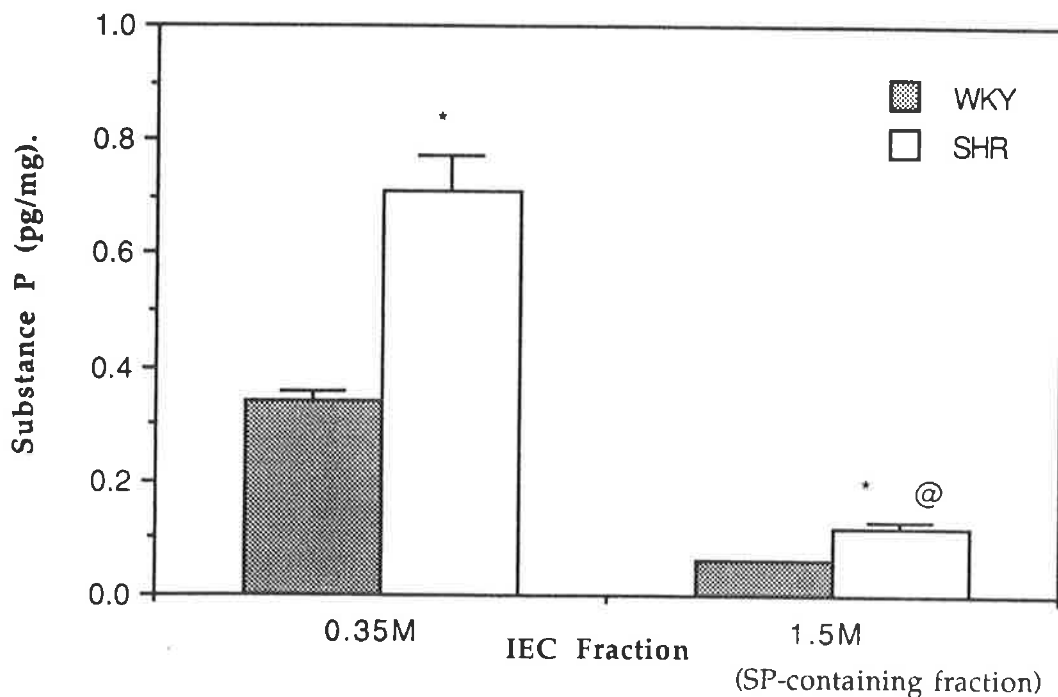

Fig. 7.12. Determination of substance P (SP) content in the spleen.

The spleen was removed from WKY and SHR and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the spleen for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction). N=9, except at @ where N=8.
** $p < 0.05$ WKY versus SHR (unpaired t-test).*

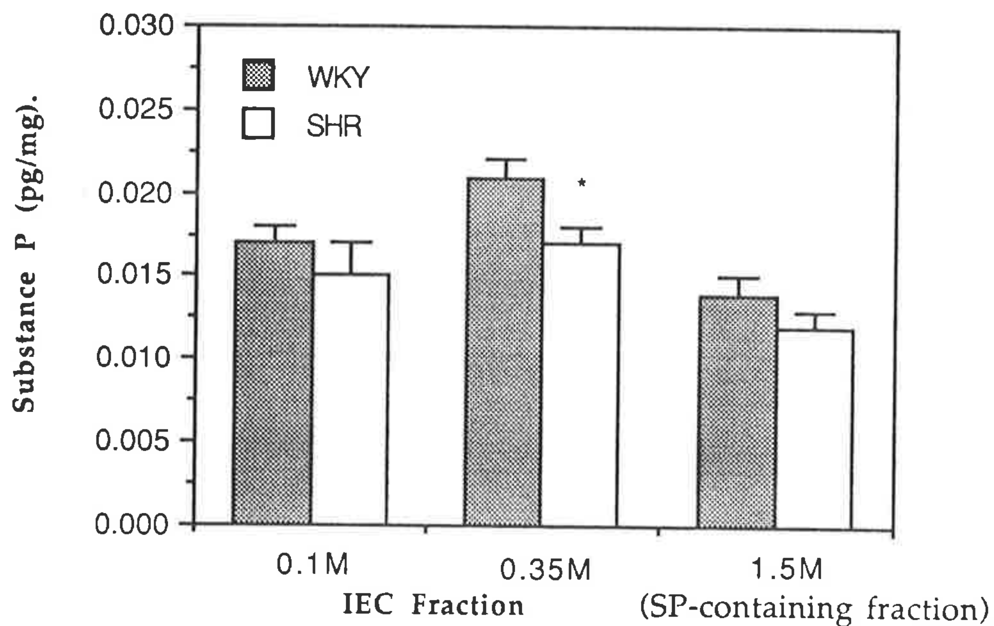

Fig. 7.13. Determination of substance P (SP) content in the liver.

The liver was removed from WKY and SHR (N=9 each) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the liver for WKY and SHR for each IEC fraction as determined by RIA. (Note; the 1.5M IEC fraction was the SP-containing fraction).

$^{*}p < 0.05$ WKY versus SHR (unpaired t-test).

7.3.4 Comparison of Tissue Contents of Substance P Between WKYs and SHRs.

All WKY results for each tissue determination of SP and each IEC fraction have been compared with those of the SHRs using the unpaired t-test ($p < 0.05$). The only difference in SP content between WKYs and SHRs in the 0.1M IEC fraction was in the kidney (Fig 7.11). There was a significant difference in SP between WKYs and SHRs in the spleen, thoracic aorta and liver for the 0.35M IEC fraction (Figs. 7.7, 7.12 and 7.13). Significant differences in SP levels in the 1.5M IEC fraction (*i.e.*, the SP-containing fraction) between WKYs and SHRs were seen in the superior cervical ganglia (WKY > SHR), caudal artery (SHR > WKY), adrenal glands (WKY > SHR) and the spleen (SHR > WKY) (Figs. 7.4, 7.6, 7.8 and 7.12). The SP values (pg/mg) for the 1.5M IEC fractions for both SHRs and WKYs have been better illustrated in Figures 7.14 (results for the ganglia), 7.15 (results for the arteries), 7.16 (submaxillary and adrenal glands) and 7.17 (all other tissues).

7.4 DISCUSSION

Indirect tail-cuff measurements revealed a significantly elevated blood pressure in SHRs (187.2 $\pm$ 3.2 mmHg) when compared with WKYs (137.7 $\pm$ 4.7 mmHg) (Fig. 7.2). These results correspond well with literature reports (Warshaw *et al.*, 1980; Lee *et al.*, 1987; Donohue *et al.*, 1988; Owens *et al.*, 1988).

As the BP measurements in the present study were made at 15 and 17 weeks of age and reports suggest that the established phase in SHRs may not be attained until 19 weeks of age (Lee *et al.*, 1987), it was possible that the actual SHR BPs were even higher at the time that the tissues were removed (at age 28.7 $\pm$ 0.3 weeks), but this was not confirmed at the time. WKYs would not be likely to increase their BP in this time period.

The difference in total body weight observed between the strains is commonly seen. WKYs tend to be heavier than SHRs (Warshaw *et al.*, 1980; Donohue *et al.*, 1988) and this was also observed with our animals (Fig. 7.1).

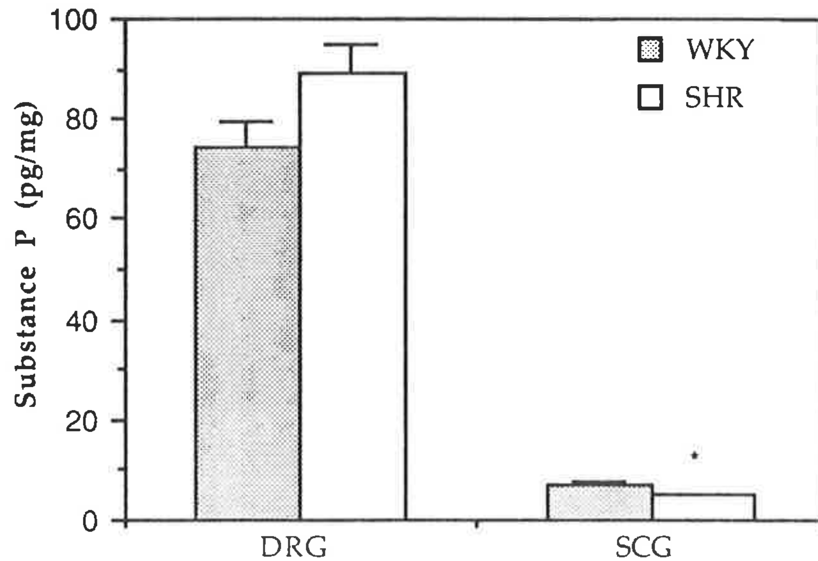

Fig. 7.14. Comparison of the determination of substance P (SP) content in the dorsal root ganglia (DRG) and superior cervical ganglia (SCG).

The DRG and SCG were removed from WKY and SHR (N=9 for all) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the DRG and SCG for WKY and SHR as determined by RIA.

** $p < 0.05$ WKY versus SHR (unpaired t-test).*

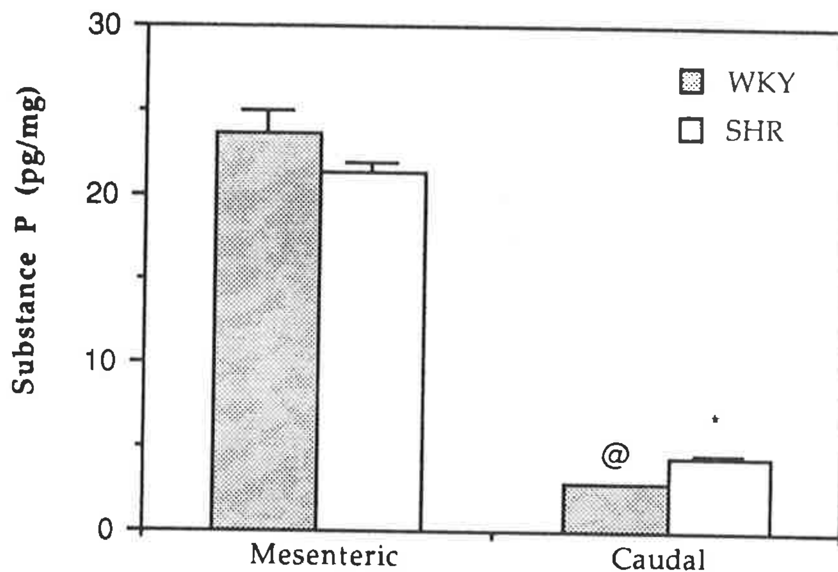

Fig. 7.15. Comparison of the determination of substance P (SP) content in the mesenteric artery and caudal artery.

The mesenteric artery and caudal artery were removed from WKY and SHR (N=9 except at @ where N=8) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the mesenteric artery and caudal artery for WKY and SHR as determined by RIA.

** $p < 0.05$ WKY versus SHR (unpaired t-test).*

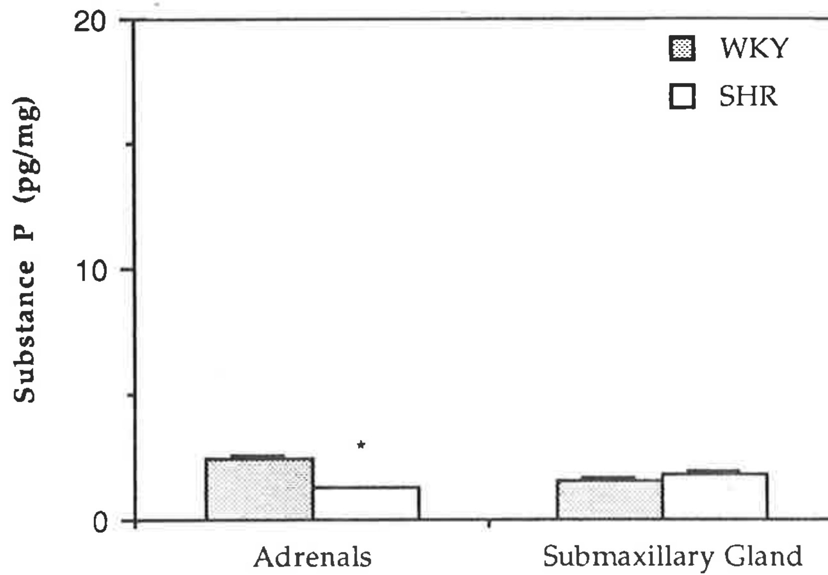

Fig. 7.16. Comparison of the determination of substance P (SP) content in the adrenal glands and submaxillary glands.

The adrenal glands and submaxillary glands were removed from WKY and SHR (N=9 for all) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the adrenal glands and submaxillary glands for WKY and SHR as determined by RIA.
** $p < 0.05$ WKY versus SHR (unpaired t-test).*

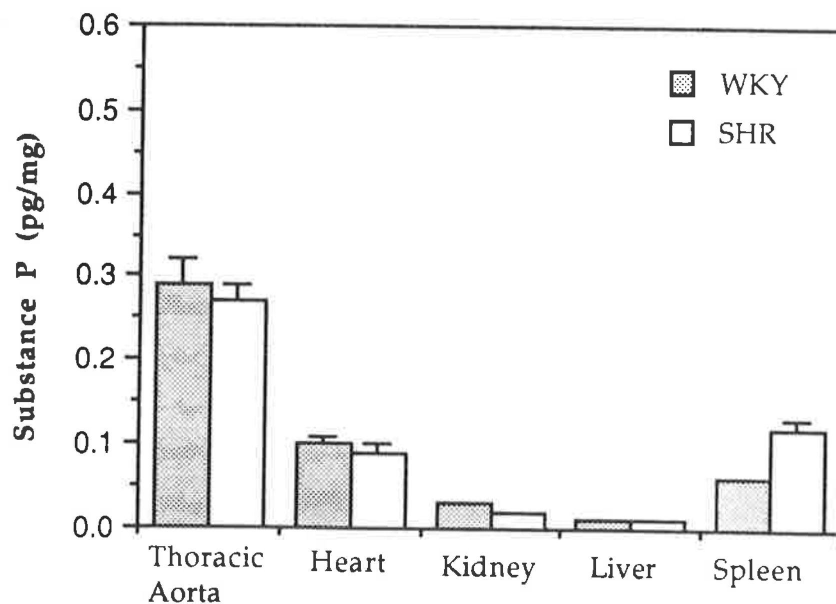

Fig. 7.17. Comparison of the determination of substance P (SP) content in a variety of tissues.

The thoracic aorta, heart, kidney, liver and spleen were removed from WKY and SHR (N=9 except at @ where N=8) and homogenized ready for processing by ion exchange chromatography and RIA. The figure shows the SP content (mean pg/mg ± SEM) of the thoracic aorta, heart, kidney, liver and spleen for WKY and SHR as determined by RIA.

There were strain differences in individual tissue weights (Table 7.1), but whether this was secondary to differences in total body weight is unknown. Significant differences in tissue weight between WKYs and SHRs were seen in the dorsal root ganglia, mesenteric artery, caudal artery, thoracic aorta, submaxillary glands, kidney and liver. The mesenteric artery, caudal artery and thoracic aorta were heavier in the SHRs (along with the liver). This finding may be indicative of the vascular smooth muscle cell hyperplasia and hypertrophy which is associated with hypertension in the SHR. Superior cervical ganglia, heart, adrenal glands (when all pairs of adrenal glands were included) and the spleen were not significantly different in their weights when the WKY and SHR tissues were compared.

The SP determinations for the 0.1M IEC fraction revealed low levels for all tissues assayed, for both WKYs and SHRs (Figs 7.3-7.13). All SP levels obtained were less than 1pg/mg tissue. These results suggest extremely low endogenous levels of the C-terminal fragments SP 4-11, 5-11 and 6-11 which elute into this fraction (Chapter 3, *3.3.1.ii*) and which may also interfere with this assay. This IEC fraction may contain other endogenous peptides and proteins, but, if present, these compounds are not detected and quantified as substance P by the RIA.

There were similar SP levels in the 0.35M IEC fraction. Almost all of the tissues recorded SP levels of less than 1 pg/mg for both the WKY and SHR tissues (Figs 7.3-7.13). These SP determinations did not reflect endogenous SP levels since SP does not elute into this IEC fraction. This IEC fraction did contain the C-terminal fragments SP 4-11, 5-11 and 6-11 (Chapter 3, *3.3.1.ii*) which do have affinity for the RIA antibody. Thus, it was possibly a combination of these C-terminal fragments which have actually been quantified as "substance P" in the RIA for this fraction. Whether these endogenous levels of the C-terminal fragments have physiological significance in these tissues is unknown, although it is unlikely that these fragments are in a high enough concentration to be of any consequence.

Of greater interest was the data obtained from the 1.5M IEC fraction. Several of the tissues analysed for SP revealed low concentrations of this peptide. Such tissues (*i.e.*, those containing less than 1pg SPLI/mg tissue) were the thoracic aorta, heart, kidney, liver and spleen (Figs. 7.7, 7.10, 7.11, 7.12 and 7.13). None of these tissues contain high levels of SP-fibre innervation and some are known to be sites of degradation for the peptide (Lembeck *et al., 1978*) or of enzymes such as NEP and ACE (Erdos and Skidgel, 1989; Phillips *et al.*, 1993). The remaining tissues (*i.e.*, DRG, SCG, caudal and mesenteric arteries, adrenal glands and submaxillary glands) had SP determinations above 1pg/mg (Figs. 7.3, 7.4, 7.5, 7.6, 7.8 and 7.9). Of these determinations, species differences in SP were noted for the superior cervical ganglia, caudal artery, adrenal glands and spleen ($p < 0.05$ in each case).

It was expected that the dorsal root ganglia SP determinations would be substantial, since the DRG are known to be a site of biosynthesis for substance P (Pernow, 1983). The relatively high SP levels in the DRG (Fig. 7.14) agree with published reports (Warden and Young, 1988; Shimonaka and Kream, 1991; Smith *et al.*, (1993).

The superior cervical ganglia are also acknowledged to contain SP, although not to the same extent as the DRG (Pernow, 1983; Kessler *et al.*, 1993; Rao *et al.*, 1993). Thus, the SP levels obtained for the SCG (Fig. 7.14) were within the expected range observed in the literature.

Previous published determinations of SP in the mesenteric artery were substantially lower than that observed with the present determinations for the WKYs and SHRs (Duckles, 1985 and Fig. 7.15). Whether this was due to species differences or differences in techniques is unknown.

Little published evidence exists for quantitative analysis of SP in the other tissues examined. The caudal artery, adrenal glands and submaxillary glands did contain appreciable levels of SP (Figs. 7.15 and 7.16). It is known that SP-fibres are found in close association with the vasculature (Pernow, 1983), salivary glands

(Virta *et al.*, 1992) and adrenal glands (Vaupel *et al.*, 1988), hence these results were not unexpected.

It was an interesting exercise to compare the substance P content for each tissue with an estimate of the C-terminal fragments of substance P (SP 4-11, 5-11 and 6-11) plus substance P. This was possible by taking the aggregate of the mean RIA determinations for the 0.1M, 0.35M and 1.5M IEC fractions together (*i.e.*, the RIA determinations of SP 4-11, 5-11, 6-11 and 1-11 together). Such a calculation has been performed and presented in Table 7.2 (next page).

Essentially, the overall results were no different for the substance P determinations alone when compared with the determinations for the fragments and substance P combined (Table 7.2). The values were comparable for the dorsal root ganglia, mesenteric artery, superior cervical ganglia, caudal artery, adrenal gland and submaxillary gland for substance P and the fragments combined in comparison with substance P alone (Table 7.2). This was mainly due to the comparatively low levels of the fragments found in these tissues compared to substance P itself (see Figs. 7.3, 7.4, 7.5, 7.6, 7.8 and 7.9).

The thoracic aorta, heart, kidney and liver showed slight increases in the total fragment and substance P content compared to substance P content alone (Table 7.2). However, the total substance P levels were already very low in these tissues (Figs. 7.7, 7.10, 7.11 and 7.13) and the combination with the fragments still results in concentrations below 1pg/mg (Table 7.2).

Thus, although the fragments SP 4-11, 5-11 and 6-11 are biologically active in their own right, it appears that they are not present in the tissues assayed in any appreciable concentration and therefore would not augment any of the actions attributed to substance P in these tissues.

	Substance P alone (pg/mg)		SP + fragments (pg/mg)	
Tissue	**WKY**	**SHR**	**WKY**	**SHR**
Dorsal Root Ganglia	74.11 ± 5.20	89.10 ± 5.84	76.71 ± 5.58	92.2 ± 6.12
Mesenteric artery	23.69 ± 1.22	21.29 ± 0.54	25.63 ± 1.46	22.9 ± 0.68
Superior Cervical Ganglia	6.78 ± 0.65	4.81 ± 0.43	8.01 ± 0.89	5.86 ± 0.67
Caudal Artery	2.79 ± 0.13	4.42 ± 0.18	3.35 ± 0.27	4.94 ± 0.24
Adrenal Gland	2.39 ± 0.11	1.22 ± 0.10	2.76 ± 0.14	1.53 ± 0.12
Submaxillary Gland	1.51 ± 0.10	1.77 ± 0.16	1.61 ± 0.11	1.86 ± 0.17
Thoracic Aorta	0.29 ± 0.03	0.27 ± 0.02	0.70 ± 0.06	0.61 ± 0.14
Heart	0.10 ± 0.01	0.09 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
Kidney	0.03 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	0.05 ± 0.00
Liver	0.01 ± 0.00	0.01 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
Spleen	0.06 ± 0.00	0.12 ± 0.01	NA*	NA*

Table 7.2. A comparison of the substance P tissue content (substance P alone [pg/mg]) with the tissue content of the sum of the C-terminal fragments SP 4-11, 5-11 and 6-11 plus substance P (SP + fragments [pg/mg]) in WKY and SHR rats (N = 9 animals; mean ± SEM). NA*: the 0.1M fraction for the spleen was lost during processing, thus this calculation was not possible.

Tissue distribution of the catecholamine noradrenaline (NA)* reveals the following rank order; adrenal glands >> caudal artery > mesenteric artery > heart, spleen > aorta, kidney (Donohue *et al.*, 1988). Elevated NA levels (*i.e.* SHR values

*Enhanced NA levels observed in the SHR are a consequence of the sympathetic hyperinnervation observed in this species.

which were significantly greater than WKY determinations) were observed in the mesenteric artery, kidney, caudal artery and aorta. The rank order of NA levels in another study revealed; coeliac ganglia, SCG >> mesenteric artery > heart (Mano *et al.*, 1992). Elevated NA levels (for SHRs) were observed for all tissues except the SCG.

Rank order of SP tissue levels for the current study reveals the following; DRG >> mesenteric artery >> SCG > caudal artery > adrenal glands, submaxillary glands > thoracic aorta, heart, kidney, spleen and liver. Elevated SP levels (for SHRs) were seen in the caudal artery and the spleen.

Both NA and SP levels appear to be higher in ganglia and blood vessels as a general rule, with average levels found in the adrenal glands. Low levels of NA and SP were observed in the heart, spleen, aorta and kidney. These results suggest that the pattern of distribution of SP innervation generally follows that of the catecholamine NA in the tissues studied.

Significantly elevated levels of NA (in the SHR when compared with the WKY) have been found in the caudal artery, mesenteric artery, aorta and kidney (Donohue *et al.*, 1988); coeliac ganglia, mesenteric artery and heart (but not SCG: Mano *et al.*, 1992). Elevated SP levels were only seen in the caudal artery and spleen, with decreased SP levels seen in SCG and adrenal glands of SHRs. Thus, it appears that the pattern of *elevated* levels of NA in the SHR (when compared with the WKY) is not maintained when comparing SP levels.

It has been noted that there is an increase in SP in the SCG in the SHR-SP (the stroke-prone variant of the SHR) when compared with the WKY (Ariano and Kenny, 1987). This result is contrary to the decrease of SP in the SCG in the SHR observed in the current study (Fig. 7.14). This may be due to the strain difference (SHR versus SHR-SP) or differences in techniques.

A small number of other studies have examined potential differences in SP levels of SHRs with those of WKYs. Immunofluorescence studies revealed no significant difference in SP levels of nerves associated with the peripheral blood vessels of the WKY when compared with the SHR-SP (Lee *e .al.*, 1988). Similar

results (*i.e.* no significant differences) have been found for comparisons of SP-fibre innervation in the mesenteric arteries of WKYs and SHRs (Kawamura *et al.*, 1989). These results do agree closely with those of the current study with the exception of the caudal artery and spleen (elevated in the SHR) and the SCG and adrenal glands (decreased in the SHR).

In summary, the primary aim of the present study was to compare SP levels in the animal model for hypertension, the SHR, with those of its genetic normotensive cousin, the WKY. The hypothesis stated that SP levels should be increased in the majority of the SHR tissues as a reflection of the sympathetic hyperinnervation and increased NGF levels observed in this animal model for hypertension. This should have been particularly true for the ganglia and blood vessels. The present data suggest that there is no significant difference in SP levels in SHRs when compared with WKYs. Of the eleven representative tissues assayed, only two displayed elevated SP levels in the SHR. In direct contradiction to the hypothesis, another two tissues had significantly lower SP levels in the SHR. Despite no cohesive difference in SPLI levels being detected between the SHRs and WKYs, the pattern of distribution of SP throughout the various tissues of both strains was similar to that which has been reported for the catecholamine NA.

CHAPTER 8

GENERAL DISCUSSION.

It has been established that NGF is a prerequisite for the normal growth and development of primary sensory neurons (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). Direct application of NGF, or of antibodies to NGF to SP-containing neurons has been shown to significantly increase or decrease the levels of the peptide in the neurons respectively (Kessler and Black, 1980; Otten *et al.*, 1980; Goedert *et al.*, 1981). Furthermore, the presence or absence of NGF in cultured adult sensory neurons has been found to significantly alter the levels of both SP (Lindsay *et al.*, 1989) and PPT mRNA (Lindsay and Harmar, 1989). Thus, alterations in the levels of NGF have been directly linked to changes in SP levels in primary sensory neurons.

It has been noted that in the animal model for hypertension, the SHR, that enhanced sympathetic innervation, vascular smooth muscle cell hyperplasia and hypertrophy as well as increased levels of NGF have been associated with the development of hypertension (Chapter 1, *1.7.3.ii*, *1.7.3.iii*, *1.7.3.iv* and *1.7.3.v*). It is also known that most tissues and organs are innervated by SP neurons (Leeman and Gamse, 1981; Pernow, 1983). Therefore, it is possible in the SHR which is characterized by sympathetic hyperinnervation and increased levels of NGF, that there is a concomitant increase of SP in certain organs or tissues. Therefore, the basic theory upon which this body of work has been based is that the levels of SP in the SHR may potentially reflect the enhanced levels of NGF in this animal model.

Prior to testing this theory, it was essential to develop a reliable and accurate assay for quantifying SP. A basic RIA procedure to measure SP in plasma had been developed by Mark T. Mano (CSIRO, Division of Human Nutrition, Adelaide), but it was still necessary to determine the optimum concentration of antibody, select the most appropriate tracer and examine the cross-reactivity of the antibody for fragments of SP and related peptides.

Analysis of RIA standard curves generated with either ^{125}I-SP or ^{3}H-SP as the tracer (Figs. 2.3 and 2.4) and at various antibody dilutions (Fig. 2.2), revealed that ^{125}I-SP (for the tracer) and the 1:50,000 Ab dilution were the optimal conditions for the RIA.

Antibody affinity studies assessed the potential for cross-reactivity with fragments of SP or related peptides, since proprietary literature only cited the cross-reactivity with SP and other tachykinins (Auspep Product Catalogue, 1992/1993). Only the C-terminal fragments SP 3-11, 4-11 and 5-11 (and the non-endogenous Tyr^{8}-SP) possessed affinity for the Auspep Ab (Fig. 2.5). The C-terminal fragments SP 6-11 and 7-11 and the N-terminal fragments SP 1-4, 1-6, 1-7 and 1-9 had little or no affinity for the Ab (Fig. 2.6a). Neuropeptide K, neuropeptide Y, α-neurokinin, α-neurokinin 4-10, bradykinin, methionine enkephalin, leucine enkephalin and angiotensin II did not possess any affinity for the Ab (Fig. 2.6b).

Thus, the Ab was not specific for SP alone. Analysis of the minimum requisite peptide sequence for binding to the Ab revealed that the sequence of amino acids five through to eleven [*i.e.*, the fragment SP 5-11] was the minimum requirement.

Nevertheless, it was still not certain that the RIA would accurately measure tissue SP levels. Little agreement was evident in the literature regarding endogenous SP plasma levels (Chapter 1, *1.7.5* and Table 1.5). This was possibly due to the presence of components which cross-reacted with the antibody used in these RIAs resulting in erroneous determinations of true SP concentrations. Consequently, it was essential to establish a technique which would isolate SP prior to quantification by RIA.

Ion exchange chromatography (IEC) was the technique of choice. A previously cited technique (Bergstrom *et al.*, 1983) was used for the basic methodology with slight modifications. Nevertheless, one modification was to prove extremely significant in later experiments,; *i.e.*, the selection of SP Trisacryl M resin over Bergstrom *et al*'s. choice of SP Sephadex C-25 resin.

Initial HPLC experiments comparing the elution profiles of substance P from SP Trisacryl M or SP Sephadex C-25 revealed better recovery in the SP fraction (0.8M IEC fraction, Bergstrom *et al.*, 1983) from the SP Trisacryl M columns (99.5% as opposed to 87.8% for SP Sephadex C-25). However, Bergstrom *et al.* did not cite direct recovery levels for SP, although recovery of SP added to tissue samples yielded recoveries of 70 to 80%.

Recovery of SP from the IEC columns in the company of fragments of the peptide was better with SP Trisacryl M resin than SP Sephadex C-25 resin (Figs. 3.2 and 3.3). In addition, there were lower levels of these fragments in the 0.8M fraction of the SP Trisacryl M resin when compared with the SP Sephadex C-25 resin (Figs. 3.2 and 3.3). These elution profile results, in conjunction with the claims of superior resolution and flow rates led to the selection of SP Trisacryl M as the resin for the IEC procedure.

As the studies progressed, it was noticed that unusual data were being generated from the IEC technique. Thus, it was imperative to determine the underlying cause of the aberrant elution of substance P from the IEC columns.

Variables excluded as the cause for the shift of the elution profile were; resin washing conditions, possible contamination of pyridine stock, formic acid stock, IEC buffers, water source or ^{125}I-SP, composition of the column, effect of pH, volume of buffer, resin bed volume, antioxidants or chelating agents. However, it was noted that with each new batch of SP Trisacryl M resin the elution profile of SP shifted considerably (Fig. 3.4).

The theory of IEC is complicated and involves detailed mathematical equations (Yamamoto *et al.*, 1988). Essentially, the chromatographic separation of proteins or peptides is primarily determined by the distribution coefficient (K) of components in the sample mixture. K is dependent upon various types of non-covalent binding such as hydrogen bonding, hydrophobic interactions, electrostatic interaction, van der Waals forces and a size-exclusion effect. However, with IEC in particular, K is principally dependent upon the ionic

strength of the elution buffers, pH and protein concentration (Yamamoto *et al.*, 1988a).

During the course of the study involving the SP Trisacryl M resin in the IEC procedure, the ionic concentration of the elution buffers was unchanged, the pH was unchanged (however, experimental modifications to the pH in Chapter 3 did not seriously affect the elution profiles) and the protein concentration did not change substantially. Thus, there was no outstanding explanation for the elution profile shift of SP on the resin.

Other factors to be examined when assessing the separation of components by IEC procedures are complex equations encompassing concentration components, electrostatic components and pressure components (Yamamoto *et al.*, 1988a). However, many other factors not directly expressed in the equation also affect the separation behaviour, such as the number and pK of charged groups associated with the sample and the resin (Yamamoto *et al.*, 1988a). A more detailed section regarding factors affecting IEC separation is presented in *Theoretical Aspects* (Yamamoto *et al.*, 1988b) and *Factors Affecting Separation Behaviour* (Yamamoto *et al.*, 1988c).

Despite the consideration of many variables which were interdependent and could potentially affect the elution profile of SP, only two components of the IEC procedure were altered in any significant way. The first component was the source of the sample and the second was the batch of resin. The elution profile determinations using ^{125}I-SP did reveal a definite batch-dependent shift of the IEC elution profile. Thus, there was an inexplicable change in the *inherent* characteristics of the resin itself.

Rather than deviate from the primary aim of this study and assess (in detail) the variable components of the IEC procedure in relation to SP Trisacryl M, it was decided to return to the resin originally used in the method of Bergstrom *et al.* (1983), *i.e.*, SP Sephadex C-25 and continue with the original course of the study. Thus, SP Sephadex C-25 was used for the IEC procedures in Chapter 7 (*i.e.*, the WKY and SHR data). It must be noted that in this method the 0.8M IEC elution

buffer was not used. The elution buffers progressed from the 0.35M IEC buffer directly to the 1.5M IEC buffer, to ensure total collection of SP from the column (SP eluted into both the 0.8M and 1.5M IEC fractions when using the SP Sephadex C-25 resin).

An understanding of the stability of the peptide was also essential prior to determining SP tissue concentrations. Synthetic SP is degraded in both animal and human blood or plasma in a time- and temperature-dependent manner (Boileau *et al.*, 1970; Bury and Mashford, 1977a; Lembeck *et al.*, 1978; Berger *et al.*, 1979; Couture and Regoli, 1981; Conlon and Sheehan, 1983; Conlon and Goke, 1984 and Theodorsson-Norheim *et al.*, 1987). Furthermore, endogenous SP may be degraded in plasma in a manner which is different to that observed with the exogenous peptide (Pernow, 1983).

Chapter 4 studies did reveal a time- and temperature-dependent degradation of exogenous forms of the peptide (Figs. 4.1a, 4.1b, 4.2a, 4.2b, 4.3 and 4.4). However, the degradation of endogenous SP was not temperature- or time-dependent (for up to 4 hours, Fig. 4.6a). As suggested by Pernow (1983), the difference in the stability of exogenous and endogenous SP may be due to the presence of a protective binding protein (to be discussed shortly).

Furthermore, RIA analysis of IEC plasma fractions suggested the presence of fragments of SP in the 0.1M IEC fraction at a concentration around ten times that of endogenous SP. Examination of HPLC data in Chapter 3 in association with the antibody affinity data (Chapter 2) revealed that the 0.1M IEC fraction of human plasma contained the fragments SP 4-11 and/or SP 5-11. This elevated plasma concentration of SP 4-11 and/or SP 5-11 suggests that they may be important (if not more important) than SP in some physiological systems.

Despite the apparent stability of the endogenous form of the peptide in human plasma, precautions were taken to minimize potential tissue degradation of substance P during collection and processing of the various tissue samples (Chapter 7).

Substance P is a substrate for a number of peptides which are found in the various tissues assayed. These include NEP (found mainly in kidney, in low concentrations in vascular endothelial cells and is believed to be bound to membranes in most tissues and organs), ACE (high levels are found in the kidney and it is present in the endothelial cells of all vascular beds) and DPPIV (in the kidney, liver and adrenal glands and believed to be found on the vascular epithelium of most organs) (Chapter 1; *1.6 Metabolism of Substance P*).

NEP may cleave substance P at any of three cleavage sites yielding the fragments SP 1-6, 1-7, 1-9, 7-11, 8-11 and 10-11 and ACE may cleave substance P at two sites giving SP 1-8, 1-9, 9-11 and 10-11 (Fig. 1.5). However, the antibody used in the RIA does have affinity for some of the C-terminal fragments although the minimum amino acid recognition sequence required for the antibody was SP 5-11 (Figs. 2.5 and 2.6a). Thus, any possible tissue degradation of substance P by either NEP or ACE would not yield fragments which would be detected by the RIA procedure.

In contrast, DPPIV may cleave substance P to produce SP 1-2, 1-4, 3-11 and 5-11 (Fig. 1.5). The C-terminal fragments SP 3-11 and 5-11 may be detected by the RIA. Thus, if NEP or ACE have been active in any of the tissues assayed, the resultant fragments could not be detected using the combined IEC/RIA procedure. However, if DPPIV was active, it would be possible to detect the fragment SP 5-11 in the non-substance P-containing (*i.e.*, not the 1.5M) IEC fraction with the RIA.

Nevertheless, collected and processed tissue samples were kept on ice (in a methanol/hydrochloric mixture) and stored at -80°C and the samples were kept under these conditions until processed by IEC. Large tissues were homogenized (on ice) immediately upon removal before storing at -80°C. These steps were taken to minimize and inhibit potential enzyme activity on endogenous substance P.

As substance P determinations were in concurrence with literature values for dorsal root ganglia and superior cervical ganglia and above previously

published values for the mesenteric artery (Chapter 7, 7.4 Discussion), it appears that sufficient enzymatic inhibition was achieved with these tissue determinations. Thus, it is reasonable to assume that the substance P tissue determinations performed in the current study do reflect an accurate measurement reasonably free from enzymatic influences.

Initial investigations in the determination of a binding protein for SP utilizing SDS-PAGE techniques provided no evidence for a binding protein under conditions used (Figs. 5.1a and b). However, literature reports published since this study have indicated the existence of a binding protein for SP using the SDS-PAGE technique (Corbally *et al.*, 1990; Oblas *et al.*, 1990, Nakata *et al.*, 1992). Chapter 5 lists some experimental conditions and procedures which may have been modified to improve the SDS-PAGE results.

As the SDS-PAGE studies proved unsuccessful at the time, dialysis techniques were used to find evidence for a binding protein for SP. Whereas the previously listed researchers have shown binding of a protein with SP (sometimes under extreme non-physiological conditions), the presence of a binding protein in human plasma has been demonstrated under more consistent physiological conditions (however, at a reduced temperature [$4^{O}C$] to suppress enzymatic activity). Furthermore, the binding capacity of this plasma protein has been calculated to be 61.9 ± 4.4 pg SP/ml plasma (there is no other evidence as yet, of binding capacity in the literature). Displacement studies indicated that the binding of this protein to SP was both saturable and reversible (there are no other displacement studies in the literature).

The calculated binding capacity of this plasma protein taken together with the endogenous plasma determinations of SP (Chapter 4, *i.e.*, 15 to 25 pg SP/ml plasma) suggest the capacity of the protein is in excess of the concentration of SP and offers full protection for the bound peptide against enzymatic degradation. Thus, endogenous plasma SP is totally protein bound and therefore appears fully protected from degradation.

Additional dialysis studies assessed the binding potential of human serum albumin (NSA) for SP. NSA (at the physiological concentration) has a binding capacity of 11.7 ± 1.1 pg/ml for SP which was both saturable and reversible (although the time-frame of displacement was much greater with SP bound to NSA than with SP bound to the unidentified plasma protein). The potential of serum albumin to bind SP has been shown by another group, although determination of the binding capacity was not attempted (Corbally *et al.* 1990).

Examining the dialysis results obtained, there is evidence for a binding protein for SP in human plasma. NSA may bind SP, but does not account for the total binding capacity of whole plasma (*i.e.*, less than 20% of the whole plasma binding capacity). Thus, the identity of the principal binding protein in plasma is unknown. Future studies of possible plasma binding protein candidates may include the globulins, fibrinogen, free fatty acids, amino acids and plasma hormones.

Biological activity studies were conducted to determine the physiological significance of the greater plasma concentrations of SP 4-11 and SP 5-11 compared to the parent peptide, SP (see above). Previous studies have shown that the C-terminal fragments (and analogues of SP) possess varying degrees of activity in numerous preparations (Bury and Mashford, 1976; Teichberg and Blumberg, 1980; Couture and Regoli, 1982; Escher *et al.*, 1982; Pernow, 1983; Hall *et al.*, 1987; Lei *et al.*, 1991 and Stavropoulos *et al.*, 1991).

The present study focussed on the activity of the C-terminal (SP 4-11, 5-11, 6-11 and 7-11) and N-terminal (SP 1-4, 1-6, 1-7 and 1-9) peptides when compared with SP in the longitudinal muscle/myenteric plexus of the guinea pig and blood pressure (BP) responses in the WKY.

In both systems, the C-terminal fragments SP 4-11, 5-11 and 6-11 elicited a similar dose-response profile when compared to SP (Figs. 6.1 and 6.3). However, in shortening the fragment SP 6-11 by one amino acid (*i.e.*, to SP 7-11), the activity of the resulting fragment was severely reduced in both systems (Figs. 6.1 and 6.3). All of the N-terminal fragments tested in the longitudinal muscle/myenteric

plexus of the guinea pig (*i.e*, SP 1-4, SP 1-6, SP 1-7 and SP 1-9) were ineffective in eliciting contractile activity (Fig. 6.2), whilst the fragments SP 7-11 and SP 1-4 produced a significant blood pressure lowering effect only at the highest dose tested in the WKY (Fig. 6.3). These results suggest that in terms of both the contractile response of the longitudinal muscle/myenteric plexus of the guinea pig and the hypotensive response of the WKY, that the peptide sequence Pro^{4}-Gln^{5}-Gln^{6} (in association with the remainder of the fragment) is essential for conferring biological activity in these systems.

Nevertheless, the elevated endogenous plasma levels of the C-terminal fragments SP 4-11 and SP 5-11 (discussed above) would not be considered to be physiologically significant in these biological systems. The calculated endogenous plasma concentrations of SP 4-11 and 5-11 were below the threshold of at least 1nM required to elicit a significant contractile response in the longitudinal muscle/myenteric preparation and the 10pmole/500g body weight dose required to produce a hypotensive response in the WKY (Chapter 6). No evidence could be found in the literature to establish whether the circulating concentration of these bioactive fragments was sufficient to promote physiological responses in these systems.

Preliminary studies had suggested a species difference in BP responses to SP. Several papers have also cited strain differences also (Unger *et al.*, 1980; Pompei, Tayebati, Massi, *et al.*, 1992; Pompei, Tayebati, Polidori, *et al.*, 1992). Nevertheless, little research has been undertaken to investigate this phenomenon further. Future research could be directed towards determining whether this difference in sensitivity to SP occurs between normotensive and hypertensive humans or in SP-implicated disease states.

The final and primary aspect of the study was to determine and compare the levels of SP in various tissues of the SHR and the WKY. For the hypothesis to hold true, the majority of the SHR SP determinations should be significantly greater than the SP determinations for the WKY.

Significantly greater SP determinations were observed for the caudal artery and spleen in the SHR compared with the WKY (Figs. 7.4 and 7.6). However, significantly lower SP values were observed for the superior cervical ganglia and adrenal glands in the SHR compared with the WKY (Figs. 7.3 and 7.5). No difference in SP levels was observed in the dorsal root ganglia, mesenteric artery, thoracic aorta, submaxillary glands, heart, kidney or liver between SHRs and WKYs. Thus, there was no distinct pattern of increased SP in the SHR when compared with the WKY. Therefore, the original hypothesis (as postulated in the introductory chapter of this thesis) may not hold true.

Nevertheless, the pattern of distribution of the catecholamine noradrenaline (NA) does tend to be roughly similar to that observed for SP (Table 8.1, next page). As a general rule, the blood vessels and ganglia contained comparatively higher levels of both NA and SP. In the adrenal glands, high NA levels were observed (this was to be expected since the adrenal medulla is a site of biosynthesis of NA), but only average levels of SP were observed. Low levels of NA and SP were seen in the heart, spleen, aorta and kidney. Thus, the pattern of distribution of SP does tend to follow that of NA.

Comparison of NA levels in the SHR with the WKY (Donohue *et al.*, 1988) and of SP levels in the SHR with the WKY does not follow a similar trend. Elevated levels of NA were found in the caudal artery, mesenteric artery, aorta and kidney (Donohue *et al.*, 1988) and for the coeliac ganglia, mesenteric artery and heart (Mano *et al.*, 1992) for SHRs when compared with the WKYs. Elevated levels of SP in the SHR (when compared with the WKY) were only observed for the SCG and adrenal glands.

Donohue *et al.*, (1988) NA	Mano *et al.*, (1992) NA	Current Study SP
Adrenal gland	Coeliac ganglia & SCG	Dorsal root ganglia
Caudal artery	Mesenteric artery	Mesenteric artery
Mesenteric artery	Heart	SCG
Heart & Spleen		Caudal artery
Aorta		Submaxillary & Adrenal gland
Kidney		Aorta, Heart, Kidney, Spleen & Liver

Table 8.1. Comparison of the relative tissue distribution of noradrenaline (NA) with substance P. Tissues with the highest NA or SP have been listed at the top of the table, whereas tissues with the lowest determined levels of NA or SP have been listed at the bottom of the table.

Two other studies which have cited no significant difference in SP innervation of blood vessels in SHRs compared with WKYs include Lee *et al.* (1988) and Kawamura *et al* (1989). Nevertheless, elevated levels of SP have been reported in the SHR and the Otago Wistar rat (another genetically hypertensive strain) when compared with the WKY in SCG and coeliac ganglia (Virus *et al.*, 1982; Gurusinghe and Bell, 1989a). Thus, although there is some evidence for an increase in SP levels in SHRs compared with WKYs, the majority of the evidence from this study suggests no significant difference in SP levels between the hypertensive and normotensive rat strains when using an assay for SP optimized for specificity.

It is therefore appropriate to re-examine the original hypothesis; *i.e.*, that enhanced levels of NGF in the hypertensive animal model may be reflected by enhanced substance P levels. It is known that vascular smooth muscle cell hyperplasia and hypernoradrenergic innervation each exert trophic influences on the other, a consequence of which is enhanced NGF levels associated with the

vascular smooth muscle cells (Fig. 1.6). Furthermore, the presence or absence of NGF has been found to significantly increase or reduce substance P levels (1.7.4.i *Potential Biochemical Markers for the Development of Hypertension*). It was this enhanced NGF associated with the vascular smooth muscle cells in the hypertensive animal which was proposed to exert an enhanced trophic effect on neighbouring substance P-containing neurons (Fig. 1.7). Hence, NGF exerts a trophic effect on both the nearby sympathetic neurons and the substance P-containing primary sensory neurons.

It may then be predicted that there is competition between the sympathetic neurons and substance P-containing neurons for the target derived NGF. Hill and associates (1988) have demonstrated *in vivo* evidence of competition between sensory and sympathetic neurons for target derived NGF. Given that substance P-containing neurons comprise only a small component of the general neuronal population in comparison with sympathetic nerves, it may be presumed that the sympathetic neurons may utilize the major proportion of available NGF, at the expense of the substance P-containing neurons. Thus, although there may be enhanced levels of NGF in the region, the majority of the available NGF is utilized by the larger population of sympathetic neurons.

Moreover, considering the feasibility of competition for NGF between sympathetic and sensory neurons, it may be expected that a decrease in sympathetic innervation would promote the development of sensory neurons containing substance P (as opposed to the sympathetic hyperinnervation proposed in the hypothesis). Evidence for this has been reported by Kessler and colleagues (1983) and Hill and associates (1988).

Nevertheless, it has been shown in the genetically hypertensive Otago Wistar rat that there is an increase of substance P-positive terminals in superior cervical ganglia (SCG) from these animals, when compared with normotensive rats (Gurusinghe and Bell, 1989a). However, this was not reflected in the current study where the substance P level of the SCG of the normotensive Wistar Kyoto rat was significantly greater than that of the hypertensive Spontaneously

Hypertensive Rat (Fig. 7.4). This was also shown in a study conducted by Virus *et al.* (1982).

Further studies by Gurusinghe and Bell (1989b) have suggested that the increased number of substance P-positive terminals in the SCG of the hypertensive Otago Wistar may be collaterals of thoracic sensory afferents (rather than preganglionic sympathetic neurons). However, these substance P-positive terminals were acknowledged to be less than 10% of the total ganglionic neuronal pool. Regardless, Gurusinghe and Bell believe that the excessive sympathetic drive observed in the genetically hypertensive Otago Wistar rat may be generated at the ganglionic level by the local release of substance P. Thus, Gurusinghe and Bell suggest that increased ganglionic substance P in hypertensive animals may act directly on sympathetic neurons to increase sympathetic drive and therefore increased blood pressure in these animals. More evidence for this theory has recently been shown by Bell and Bakhle (1993).

Nevertheless, it remains possible that there are strain differences in the substance P content of the SCG in genetically hypertensive animals. In the current study, the hypertensive SHRs exhibited significantly lower levels of substance P in the SCG than the normotensive animals (Fig. 7.4). This result has also been observed in SCG from SHRs by Virus and associates (1982). In contrast, elevated substance P levels have been observed in SCG from genetically hypertensive Otago Wistar rats (Gurusinghe and Bell, 1989a and b). It may therefore be of interest to direct future studies towards establishing the substance P content of the SCG in some of the later strains of genetically hypertensive rats.

Thus, in light of the current results in conjunction with a review of the relevant literature, it appears that the original hypothesis is now outdated and simplistic. Studies have suggested that there is competition for target derived NGF between sympathetic and substance P-containing neurons and that a decrease in sympathetic innervation (rather than the increase in innervation suggested in the hypothesis) would result in a concomitant increase in substance P neurons. Such a decrease in sympathetic innervation has been evidenced by a decrease in the

marker NPY-containing sympathetic neurons (Gurusinghe and Bell, 1989a) in the genetically hypertensive Otago Wistar rat. Furthermore, the increases in the substance P-positive terminals are believed to exert an excitatory effect on the sympathetic neurons, increasing sympathetic drive and ultimately resulting in elevated blood pressure in that strain. In contrast in the SHR where there is abundant evidence for an increase in sympathetic innervation this study has demonstrated no connected pattern of substance P alterations in sympathetically innervated tissues. The latter suggest NGF is exerting its influence upon sympathetic rather than sensory neurons in this model.

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APPENDICES

APPENDIX I

RADIOIMMUNOASSAY SOLUTIONS/REAGENTS.

Double strength assay buffer (DSAB); to make 1L:

CHEMICAL	WEIGHT	CHEMICAL	WEIGHT
$Na_2HPO_4.12H_2O$	28.66g	NaCl	11.68g
EDTA	5.84g	Bacitracin	56.0mg
Thiomersal	480.0mg	BSA	5.0g

Where;

EDTA = ethylenediaminetetraacetic acid [di-sodium] (Sigma, USA), **BSA** = bovine serum albumin (Boehringer Fraction V, No. 735108), di-sodium hydrogen orthophosphate and sodium chloride (Ajax Chemicals, Australia), thiomersal (BDH Chemicals, England) and bacitracin (53500 U/g; Sigma, U.S.A).

Single strength assay buffer (SSAB) was made by diluting DSAB 1:1 with glass distilled water. Diluent buffer (DB) was made by diluting DSAB 1:1 with 1% acetic acid (v/v; Ajax Chemicals, Australia).

DSAB, SSAB and DB were made on the commencement day of the RIA.

Preparation of RIA Standards.

The stock standard of 100µg/ml substance P (triacetate, Sigma, USA) in 1% acetic acid was stored at -80°C until required. Diluent buffer (DB) was used to dilute the stock standard to yield solutions of 10, 1, 0.1, and 0.01ng/ml substance P. These solutions were further diluted to make up the standards for the RIA in the manner outlined below

Preparation of RIA Standards;

Solution (ng/ml)	Volume(µl)	DB(µl)	Final Conc.(pg)
-	-	200	0
0.01	200	0	2
0.1	50	150	5
0.1	100	100	10
0.1	200	0	20
1.0	100	100	100
1.0	200	0	200
10	50	150	500
10	100	100	1000

Note that volume (µl) plus DB (µl) always totalled 200µl.

Preparation of the Substance P Antibody (Ab).

The substance P antibody (Ab; Auspep, Australia, code 8508I) was diluted 1:5,000 in SSAB and stored frozen at -20°C in 1ml aliquots until required.

On the day of the RIA, the Ab was diluted further with SSAB to yield a final dilution of 1:50,000 which was used for the assay.

Preparation of the Normal Rabbit Serum (NRS) and Sheep Anti-rabbit Immunoglobulin (SARS).

The normal rabbit serum (Silenus, Australia, code WNRS) was diluted 1:100 in SSAB for use in the RIA. The sheep anti-rabbit serum (Silenus, Australia, code RS) was diluted 1:25 with SSAB for use in the RIA.

Preparation of Polyethylene Glycol 6,000 (PEG).

The PEG was diluted to 5.5% w/v (*i.e.*, 55g PEG 6,000/L) in physiological saline (9g NaCl/L). This 5.5% PEG solution was kept refrigerated until required.

Preparation of the tracer (^{125}I-SP).

The tracer ([^{125}I-Tyr^{8}]-Substance P, NEN Dupont, Australia) was prepared on the day of use. The dilution of the ^{125}I-SP in SSAB was calculated according to the following equation;

Volume of undiluted tracer (µl) =

$$\frac{\text{final volume of diluted } ^{125}\text{I-SP required (ml)} \times 100}{\text{specific activity of } ^{125}\text{I-SP } (\mu\text{Ci/ml}) \times 1.78}$$

APPENDIX II

ION EXCHANGE CHROMATOGRAPHY SOLUTIONS AND REAGENTS.

Base buffers;

0.1M HCl (Ajax Chemicals, Australia)

[*i.e.*, take 1.74ml of concentrated HCl and make the total volume up to 200ml with GDW (glass distilled water)].

3M pyridine/1M formic acid (pyridine ; 88% formic acid, Ajax Chemicals, Australia)

[*i.e.*, take 24ml pyridine and 4.34ml of 88% formic acid and make the total volume up to 100ml with GDW].

3M pyridine

[*i.e.*, take 24ml pyridine and make the total volume up to 100ml with GDW].

3M formic acid (HCOOH)

[*i.e.*, take 13ml of 88% HCOOH and make the total volume up to 100ml with GDW].

0.018M pyridine/0.1M formic acid

[*i.e.*, take 725 l pyridine and 2.17ml 88% HCOOH and make the total volume up to 500ml with GDW].

Preparation of ion exchange chromatography buffers;

BUFFER	3M PYRIDINE	3M HCOOH	TOTAL VOLUME
0.1M P/0.1M HCOOH	3.3ml	3.3ml	100ml
0.35M P/0.35M HCOOH	11.7ml	11.7ml	100ml
0.8M P/0.8M HCOOH	26.7ml	26.7ml	100ml
1.5M P/1.5M HCOOH	50ml	50ml	100ml

Where P = pyridine and HCOOH = formic acid and the total volume is made up with GDW.

Note; all of these buffers may be made up in bulk and stored at room temperature.

APPENDIX III

Scintillant; to make 2.5L

CHEMICAL	WEIGHT	CHEMICAL	VOLUME
PPO	6.7g	toluene	1.67L
POPOP	333mg	triton	0.83L

Where;

PPO = 2,5-diphenyloxazole (Ajax Chemicals, Australia)

POPOP = (1,4-bis[2(4-methyl-5-phenyl-oxazolyl)]benzene)

(Dimethyl POPOP; Sigma USA)

toluene (Ajax Chemicals, Australia)

triton = iso-octylphenoxypolyethoxyethanol (x-100; BDH Chemicals, Australia)

APPENDIX IV

SDS-PAGE Gels and Buffers

To make **12.5% Gel** (quantities sufficient for the preparation of 10 gels);

REAGENT	VOLUME/WEIGHT
LGB	25ml
acrylamide*	31.25ml
GDW	43.75ml
APS	40mg
TEMED	35ml

To make **4% Stacking Gel** (quantities sufficient for the preparation of 10 gels);

REAGENT	VOLUME/WEIGHT
UGB	15ml
acrylamide*	6.6ml
GDW	38.4ml
APS	56.4mg
TEMED	25ml

Where;

LGB = lower gel buffer (1.5M Tris base, 0.4% SDS; pH = 8.8) [*i.e.*, 90.825g Tris base and 2g SDS in 500ml].

UGB = upper gel buffer (0.5M Tris base, 0.4% SDS; pH = 6.8) [*i.e.*, 30.275g Tris base and 2g SDS in 500ml]. (continued overleaf).

acrylamide* = 40% acrylamide, 1.07% MBA [*i.e.*, 40g acrylamide and 1.07g MBA in 100ml].

GDW = glass distilled water

The **Tris base**, **SDS** [lauryl sulfate; sodium salt], **APS** [ammonium persulfate], **acrylamide**, **TEMED** [N,N,N',N'-tetramethylethylenediamine] and **MBA** [N,N'-methylene-bis-acrylamide] were obtained from Sigma (Mo., USA).

To make the **Running Buffer** (4L);

REAGENT	WEIGHT (g)
Tris base	12.11
glycine	57.65
SDS	4.0
	pH = 8.3 - 8.4

To make the **Loading Buffer** (100ml);

REAGENT	WEIGHT/VOLUME
Tris base	3.02g
SDS	8.0g
glycerol	20ml
Bromophenol blue	4.0mg
	pH = 6.8

The **glycine**, **glycerol** and **Bromophenol blue** were obtained from BDH Chemicals (Sydney, Australia).

Rainbow Marker

The Rainbow Marker (Rainbow protein molecular weight markers, ^{14}C-labelled; Amersham, England) was diluted 1:1 with loading buffer and stored at -20°C in 5ml aliquots until required. When needed, a further 10-15ml of loading buffer was added to the 5ml aliquot before use.

Electrotransfer Buffers

Towbin Buffer = 20mM Tris base, 150mM glycine, 20% ethanol (pH = 8.3).

[*i.e.*, to make 5L take 500ml 10 x Towbin buffer and 1L ethanol; where 10 x Towbin buffer = 24.22g Tris base and 112.61g glycine in 1L].

Washing Buffers

The base buffer for the following washing buffers = 0.15M NaCl and 0.01M Tris base.

1% Triton (v/v; Triton x 100, Ajax Chemicals, Sydney, Australia) in base buffer.

1% BSA (w/v; Albumin, Bovine, Fraction V, Sigma, Mo., USA) in base buffer.

0.1% Tween (v/v; Tween 20 = polyoxyethylenesorbitan monolaurate, Sigma, Mo., USA) in base buffer.

APPENDIX V

Calculations for converting cpm bound ^{125}I-SP to pg/ml ^{125}I-SP.

Part One. Human Plasma Calculations.

Table AV.1 summarizes the cpm determinations for plasma and saline at equilibrium.

Subject	1. cpm/500μl saline	2. cpm/500μl plasma	cpm/500μl bound ^{125}I-SP	cpm/ml bound ^{125}I-SP
1a	2,003.1	70,663.7	68,660.6	137,321.2
1b	1,168.5	80,620.1	79,451.6	158,903.2
1c	1,234.8	70,836.7	69,601.9	139,203.8
2	1,376.1	77,608.3	76,232.2	152,464.4
3	2,337.9	69,110.4	66,772.5	133,545.0
4	1,737.5	90,557.0	88,819.5	177,639.0

Table AV.1. A summary of cpm determinations for saline and plasma at equilibrium. Cpm bound ^{125}I-SP per 500μl was obtained by determining the difference between plasma and saline values, *i.e.*, 2 - 1. The final column converts cpm/500μl to cpm/ml.

The next step was to convert cpm to pg. This was achieved by utilizing the specific activity of the radiolabel at the time of counting.

For **1a**, **1b** and **1c** the specific activity of ^{125}I-SP on the day of counting was 55.7 μCi/ml.

For **2**, **3** and **4** the specific activity of ^{125}I-SP on the day of counting was 57.1 μCi/ml.

The specific activity was also known to be 2200 Ci/mmol, which is the same as 2.2 μCi/pmol.

Thus 55.7 μCi = 25.3 pmol (55.7 divided by 2.2)

and 57.1 μCi = 26.0 pmol (57.1 divided by 2.2).

But, 1 μCi also = 2.22 x 10^6 DPM, however, with counting efficiency of the γ-counter being 75%, 2.22 x 10^6 DPM x 0.75 = 1.665 x 10^6 cpm. Thus, 1 μCi = 1.665 x 10^6 cpm.

Therefore, 55.7 μCi = 92.74 x 10^6 cpm and 57.1 μCi = 95.07 x 10^6 cpm.

Thus, 55.7 μCi = 92.74 x 10^6 cpm = 25.3 pmol, which converts to 3.666 x 10^6 cpm/pmol when the specific activity was 55.7 μCi.

Similarly, 57.1 μCi = 95.07 x 10^6 cpm = 26.0 pmol, which converts to 3.657 x 10^6 cpm/pmol when the specific activity was 57.1 μCi.

Hence, converting cpm/ml ^{125}I-SP bound to pmol/ml ^{125}I-SP bound yields;

1a 137,321.2cpm / 3.666 x 10^6 cpm/pmol = 0.03746 pmol/ml

1b 158,903.2cpm / 3.666 x 10^6 cpm/pmol = 0.04335 pmol/ml

1c 139,203.8cpm / 3.666 x 10^6 cpm/pmol = 0.03797 pmol/ml

2 152,464.4cpm / 3.657 x 10^6 cpm/pmol = 0.04169 pmol/ml

3 133,545.0cpm / 3.657 x 10^6 cpm/pmol = 0.03652 pmol/ml

4 177,639.0cpm / 3.657 x 10^6 cpm/pmol = 0.04858 pmol/ml

Now, the MW of ^{125}I-SP is 1487.

Thus, 1 mole of ^{125}I-SP weighs 1487g.

Similarly 1mmole = 1.487g

1mmole = 1.487mg

1nmole = 1.487mg

and 1pmole=1.487ng (or 1487pg).

So, calculations for bound ^{125}I-SP yielded;

1a = 0.03746 pmol/ml = 55.7 pg/ml

1b = 0.04335 pmol/ml = 64.5 pg/ml

1c = 0.03797 pmol/ml = 56.5 pg/ml

2 = 0.04169 pmol/ml = 62.0 pg/ml

3 = 0.03652 pmol/ml = 54.3 pg/ml

4 = 0.04858 pmol/ml = 72.2 pg/ml.

The mean value for subject number 1 was 58.9 ± 3.4 pg/ml.

Using this value, plus those obtained for the other subjects (2, 3, 4), the mean ± SEM for the binding of ^{125}I-SP in plasma was 61.9 ± 4.4 pg/ml (N=4).

Part Two. 5% NSA Calculations.

Table AV.2 summarizes the cpm determinations for plasma and saline at equilibrium (next page).

The next step was to convert cpm to pg. This was achieved by utilizing the specific activity of the radiolabel at the time of counting.

For **1**, **2** and **3** the specific activity of ^{125}I-SP on the day of counting was 49.4 μCi/ml.

For **4** and **5** the specific activity of ^{125}I-SP on the day of counting was 45.6 μCi/ml.

For **6** and **7** the specific activity of ^{125}I-SP on the day of counting was 69.4 μCi/ml.

The specific activity was also known to be 2200 Ci/mmol, which is the same as 2.2 μCi/pmol.

Thus 49.4 μCi = 22.5 pmol (49.4 divided by 2.2)

and 45.6 μCi = 20.7 pmol (45.6 divided by 2.2)

and 69.4 μCi = 31.5 pmol (69.4 divided by 2.2).

Subject	cpm/500µl saline	cpm/500µl 5% NSA	cpm/500µl bound ^{125}I-SP	cpm/ml bound ^{125}I-SP
1	1,294.5	21,082.0	19,787.5	39,575.0
2	1,206.3	29,275.5	28,069.2	56,138.4
3	1,201.2	21,520.3	20,319.1	40,638.2
4	1,399.7	12,844.3	11,444.6	22,889.2
5	1,506.0	18,951.6	17,445.6	34,891.2
6	863.0	15,394.9	14,531.9	29,063.8
7	2,077.3	16,172.9	14,095.6	28,191.2

Table AV.2. A summary of cpm determinations for saline and 5% NSA at equilibrium. Cpm bound ^{125}I-SP per 500µl was obtained by determining the difference between 5% NSA and saline values, *i.e.*, 2 - 1. The final column converts cpm/500 l to cpm/ml.

But, 1 µCi also = 2.22 x 10^6 DPM, however, with counting efficiency of the γ-counter being 75%, 2.22 x 10^6 DPM x 0.75 = 1.665 x 10^6 cpm. Thus, 1 µCi = 1.665 x 10^6 cpm.

Therefore, 49.4 µCi = 82.25 x 10^6 cpm, 45.6 µCi = 75.92 x 10^6 cpm and 69.4 µCi = 115.55 x 10^6 cpm.

Thus, 49.4 µCi = 82.25 x 10^6 cpm = 22.5 pmol, which converts to 3.656 x 10^6 cpm/pmol when the specific activity was 49.4 µCi.
Similarly, 45.6 µCi = 75.92 x 10^6 cpm = 20.7 pmol, which converts to 3.668 x 10^6 cpm/pmol when the specific activity was 45.6 µCi.
Also, 69.4 µCi = 115.55 x 10^6 cpm = 31.5 pmol, which converts to 3.668 x 10^6 cpm/pmol when the specific activity was 69.4 µCi.

Hence, converting cpm/ml ^{125}I-SP bound to pmol/ml ^{125}I-SP bound yields;

1 39,575.0cpm / 3.656 x 10^6 cpm/pmol = 0.01082 pmol/ml

2 56,138.4cpm / 3.656 x 10^6 cpm/pmol = 0.01536 pmol/ml

3 40,638.2cpm / 3.656 x 10^6 cpm/pmol = 0.01111 pmol/ml

4 22,889.2cpm / 3.668 x 10^6 cpm/pmol = 0.00624 pmol/ml

5 34,891.2cpm / 3.668 x 10^6 cpm/pmol = 0.00951 pmol/ml

6 29,063.8cpm / 3.668 x 10^6 cpm/pmol = 0.00792 pmol/ml

7 28,191.2cpm / 3.668 x 10^6 cpm/pmol = 0.00769 pmol/ml

Now, the MW of ^{125}I-SP is 1487.

Thus, 1 mole of ^{125}I-SP weighs 1487g and therefore 1pmole=1.487ng (or 1487pg).

So, calculations for bound ^{125}I-SP yielded;

1 = 0.01082 pmol/ml = 16.1 pg/ml

2 = 0.01536 pmol/ml = 22.8 pg/ml

3 = 0.01111 pmol/ml = 16.5 pg/ml

4 = 0.00624 pmol/ml = 9.3 pg/ml

5 = 0.00951 pmol/ml = 14.1 pg/ml

6 = 0.00792 pmol/ml = 11.8 pg/ml

7 = 0.00769 pmol/ml = 11.4 pg/ml

Hence, this yielded a value of 14.6 $\pm$ 1.8 pg/ml bound ^{125}I-SP in 5% NSA (N=7).

APPENDIX VI

Krebs-Henseleit Buffer (Krebs); to make 1L

CHEMICAL	WEIGHT	CHEMICAL	WEIGHT
NaCl	6.604g	KCl	358mg
KH_2PO_4	164mg	$MgSO_4.7H_2O$	296mg
$NaHCO_3$	2.1g	*Glucose**	2.0g
*Ascorbic Acid**	100mg	*$CaCl_2$**	2.5*ml*

* These reagents were only added on the day of use.

$CaCl_2$ = 2.5ml of a 1.0M solution.

The buffer was made up to a final volume of 1L using Glass Distilled Water (GDW).

All chemicals were from Ajax Chemicals, Sydney, Australia.

The Krebs was stored in the refrigerator until required.