AN INVESTIGATION ON OPIOID PEPTIDE PRECURSORS

IN STRIATUM

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ABSTRACT

Proenkephalin contains four copies of methionine^5-enkephalin (ME) and one copy each of leucine^5-enkephalin (LE), methionine^5-enkephalin-arginine^6-phenylalanine^7 (ME-Arg^6-Phe^7) and methionine^5-enkephalin-arginine^6-glycine^7-leucine^8 (ME-Arg^6-Gly^7-Leu^8). Recent studies have supported the suggestion that proenkephalin is a precursor for enkephalin in both adrenal medulla and brain. However, while previous investigations have characterised enkephalin-containing adrenal intermediates derived from proenkephalin, as yet no such intermediates have been isolated from the brain. This has led to the belief that the processing of proenkephalin in the brain is extremely rapid and enkephalin-containing intermediates do not accumulate.

Sephacryl-300 gel filtration of guinea pig striata, extracted and chromatographed in 8M urea, demonstrated several peaks of both bioassayable and immunoreactive enkephalin-like peptides over the apparent molecular weight (M_r) range 2000->70000 following sequential digestion with trypsin and carboxypeptidase B. The major opioid-containing species emerged with an apparent M_r of 29000 and represented approximately 10% of all ME immunoreactivity after digestion. Comparable profiles were obtained using rat and bovine striatal tissue, although in the latter tissue considerably lower levels of putative opioid precursors were present. In contrast, guinea pig adrenal gland extracts showed substantial differences.

The major 29 kDalton species was subjected to pI analysis by isoelectricfocusing and partially purified by chromatofocusing. Chromatofocusing yielded a single peak of ME immunoreactive material,

after digestion, emerging with a pI 5.0. This species was further characterised using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and nitrocellulose blotting techniques as well as highly specific radioimmunoassays to ME, LE and ME-Arg^{6}-Phe^{7} in conjunction with reverse phase HPLC. It was found to contain ME, LE and ME-Arg^{6}-Phe^{7} in an approximate 5.2:1:0.7 ratio and to have an apparant molecular weight of 31000 Dalton. It was also indicated that ME-Arg^{6}-Phe^{7} constituted the carboxyl-terminal seven residues of this molecule.

Abbreviations

central nervous system	CNS
rough endoplasmic reticulum	RER
amino	NH_2
carboxyl	COOH
Methionine^5-enkephalin	ME
Leucine^5-enkephalin	LE
β-endorphin	β-END
β-lipotropin	β-LPH
α-melanotropin	α-MSH
adrenocorticotropic hormone	ACTH
pro-opiomelanocortin	POMC
dynorphin-(1-8)	DYN-(1-8)
dynorphin-(1-9)	DYN-(1-9)
dynorphin-(1-17)	DYN-(1-17)
dynorphin-A	DYN-A
dynorphin-B	DYN-B
Staphylococcus aureus (strain V8) protease	S. aureus protease
N,N,N',N'-tetramethylethylenediamine	TEMED
sodium dodecyl sulphate	SDS
radioimmunoassay	RIA
immunoreactivity	IR
mouse vas deferens	MVD
high performance liquid chromatography	HPLC
isoelectricfocusing	IEF
sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE

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CHAPTER ONE

INTRODUCTION

Chapter one

Introduction

The first endogenous opioid peptides to be isolated and sequenced were the pentapeptides Methionine^5-enkephalin (ME) and Leucine^5-enkephalin (LE) (Hughes et al., 1975b). Since then it has been established that there are at least three precursors , pro-opiomelanocortin, proenkephalin and prodynorphin, which can give rise to a variety of endogenous opioid peptides (Nakanishi et al., 1979; Noda et al., 1982 ; Kakidani et al., 1982). The opioid peptides all contain, at their amino (NH_2)-terminus, the enkephalin amino acid sequence Tyr-Gly-Gly-Phe-$\begin{matrix}\text{Met}\\\text{Leu}\end{matrix}$ which is necessary for opioid receptor recognition .

Many intermediates in the processing of proenkephalin, which contains six copies of ME and one copy of LE, have been isolated from bovine adrenal medulla (Udenfriend and Kilpatrick, 1983). In this investigation guinea pig striatum was chosen to investigate the biosynthesis of enkephalins in neural tissue. This is a tissue rich in enkephalin and enkephalin-containing peptides (Beaumont et al., 1979).

A general discussion on the biosynthesis of biologically active peptides will form the first section of this introduction. This will be followed by a detailed account of current knowledge concerning the biosynthesis of opioid peptides, with particular emphasis on those derived from proenkephalin. Finally, several other aspects of opioid biochemistry will be considered, in particular the existence of

multiple opioid receptor subtypes and their relationship to the endogenous opioid ligands.

1.2 Biosynthesis of biologically active peptides

Biologically active peptides have been identified in many different invertebrate and vertebrate tissues including the mammalian central nervous system (CNS) (Krieger, 1983). These peptides can function as paracrine factors, hormones, neurohormones or neuropeptide transmitters. It was therefore of interest to study their biosynthesis, in order to understand more precisely their physiological role.

Following experiments by Steiner et al., (1967) on the biosynthesis of insulin, it has been established that biologically active peptides are usually synthesised as part of large inactive precursor molecules and subsequently generated by sequential proteolytic cleavages. These peptide precursors are synthesised in the manner typical for secretory proteins. The primary gene translation product, synthesised intracellularly on an mRNA-ribosome complex bound to the rough endoplasmic reticulum (RER), is called the preproprotein. The 'pre' suffix denotes the signal peptide sequence, present at the NH_2-terminus of the nascent polypeptide chain, which is usually 15-30 amino acid residues in length and hydrophobic in nature. This signal peptide is necessary for transportation of the translation product into the lumen of the RER and is usually cleaved during translation . The proprotein then follows a maturation pathway, travelling from the RER to the Golgi vesicles, where sorting and packing into secretory vesicles occurs prior to exocytosis. Post-translational processing of the promoiety is thought to occur during migration from the RER to the outside of the cell, with individual maturation steps localised within

intracellular compartments containing the appropriate enzyme systems (Dautry-Varsat and Lodish, 1983).

Although proteolysis plays a major role in the formation of biologically active peptides, many other modifications can occur during maturation, such as glycosylation, O-sulphation, phosphorylation, amidation and N-acetylation (Mains et al., 1983). In some cases the modification(s) is essential for biological activity, for example sulphation of cholecystokinin is necessary before this peptide can promote emptying of the gall bladder and secretion of pancreatic enzymes (Rehfeld, 1981). Post-translational modifications can also inactivate biologically active peptides, β-endorphin (β-END) and LE have negligible opioid receptor activity after N-acetylation and O-sulphation respectively (Smyth et al., 1979 ; Unsworth et al., 1982).

It is also possible that processing occurs post-transcriptionally by splicing at the mRNA level as in the case of calcitonin and calcitonin gene related peptide (Amara et al., 1982) and α - and β-preprotachykinin (Nawa et al., 1984).

The rapid growth in DNA technology has allowed the cloning and sequencing of the cDNA which codes for many peptide precursor molecules. The strategy has been to isolate the mRNA from a tissue rich source and to use this as a template for the synthesis of double-stranded cDNA's using reverse transcriptase enzymes. The cDNA sequences are inserted into plasmids and this "library" of cDNA clones is screened for the ability of individual clones to hybridise to an oligodeoxynucleotide probe which codes for a known amino acid sequence within the precursor. The appropriate positive clones are selected and the cDNA within the plasmid sequenced. The primary amino acid structure

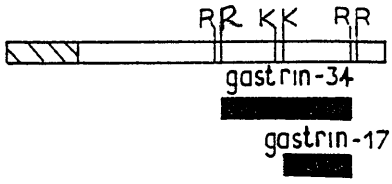
can then be deduced from the cDNA sequence. In many cases the amino acid sequence can be partially verified by comparison with known peptide and extended peptide structures obtained by classical protein isolation and sequencing techniques.

The structures of several preproproteins are shown in Fig. 1. Each precursor can contain more than one biologically active peptide, a striking example being pro-opiomelanocortin (POMC) which contains the sequences of many important products including adrenocorticotropic hormone (ACTH), α-melanotropin (α-MSH), β-lipotropin (β-LPH) and β-END (Nakanishi et al., 1979), while preproenkephalin contains multiple copies of the enkephalin peptide sequence (Noda et al., 1982).

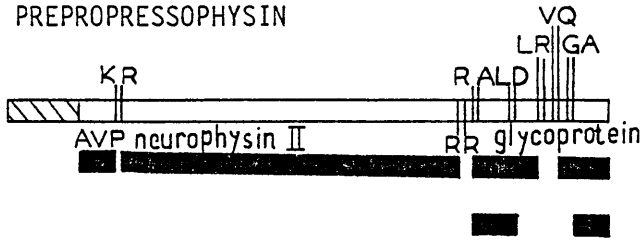
A most important observation has been that many of the cleavages necessary for the release of known biologically active peptides occur at pairs of basic residues which bracket the peptide sequences within the precursor (Lazure et al., 1982 ; Loh et al., 1984). The preferred sequence seems to be lysine-arginine although cleavage can occur at arginine-arginine and lysine-lysine sites as in preprogastrin (Yoo et al., 1982), argine-lysine as in bovine POMC (Nakanishi et al., 1979) or, less commonly, at single basic residues, for example between arginine and alanine residues in bovine prepropressophysin (Land et al., 1982). While this suggests a general processing mechanism at basic residues, the situation is complicated by the observation that not all basic residues are cleaved; although preprodynorphin contains three copies of the LE sequence this peptide does not appear to be the major product present in dynorphin and α-neoendorphin producing cells(Watson et al., 1982).

FIG. 1. BIOLOGICALLY ACTIVE PEPTIDES : STRUCTURES OF SELECTED PRECURSORS AND SOME OF THEIR PRODUCTS.

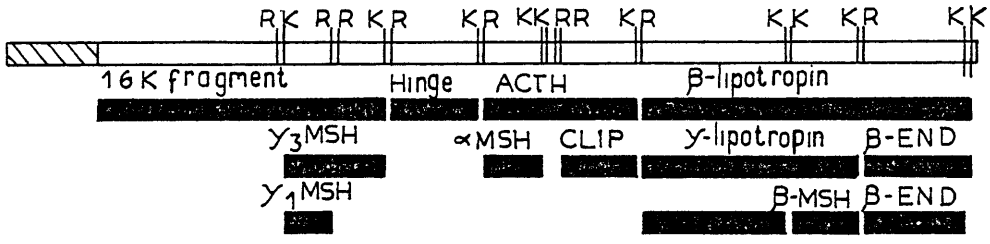
PREPROGASTRIN — Yoo et al.1982.



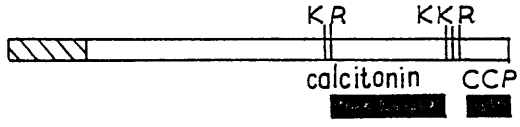
PREPROPRESSOPHYSIN — Land et al. 1982.



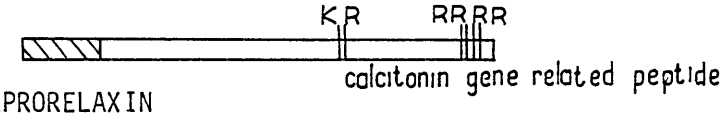
PREPROOPIOMELANOCORTIN — Nakanishi et al.1979.



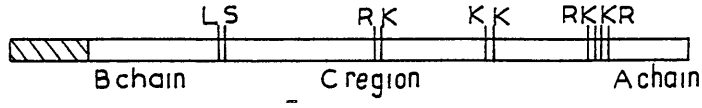
PREPROCALCITONIN — Amara et al. 1982.



PREPROCALCITONIN GENE-RELATED PEPTIDE — Amara et al. 1982.



PREPRORELAXIN — Niall et al. 1982.



Abbreviations: amino acids at known cleavage sites as follows; R = arginine, K = lysine, A = alanine, L = leucine, D = aspartic acid, V = valine, Q = glutamine, G = glycine and S = serine. Upward lines indicate known cleavage sites and downward lines represent potential cleavage sites. AVP = arginine vasopressin, ACTH = adrenocorticotropic hormone, MSH = melanotropin, CLIP = corticotropin-like intermediate lobe peptide, END = endorphin and CCP = calcitonin COOH-terminal cleavage peptide. Modified from Mains et al. 1983.

Not all cleavages occur at basic residues, the B and C chains within rat preprorelaxin are cleaved with chymotryptic-like specificity at a leucine-serine bond (Niall et al., 1982) and sequential aminodipeptidase cleavages release melittin from its precursor (Kreil et al., 1982). Within preproenkephalin the opioid peptides BAM 12P and BAM 22P are released at the carboxyl (COOH)-terminus by cleavage at glutamic acid-tryptophan and glycine-glycine residues respectively (Mizuno et al., 1980a and 1980b).

Recently it has become apparent that cleavages are tissue specific, with the same precursor giving rise to different products, for example POMC is processed principally to ACTH and β-LPH in anterior lobe pituitary cells whilst the major products in the intermediate lobe are α-MSH and β-END (Smyth and Zakarian, 1980).

The family of opioid peptides provides an excellent illustration of differential tissue processing and the diversity of products available from precursor molecules.

1.3 An historical perspective for the endogenous opioid peptides

The juice extracted from the seed capsules of the poppy Papaver somniferum is known as opium and comprises two classes of alkaloids, the phenanthrenes and the benzylisoquinolines. The major constituent responsible for its narcotic and analgesic effects in vivo is the phenanthrene morphine, representing approximately 10% of opium. Synthesis of morphine analogues provided information on the structural requirements for pharmacological action and the observation followed that the levorotatory form of a racemic mixture of enantiomers was responsible for analgesic action. Moreover, pharmacological action was

related to specific structural changes and certain modifications could produce partial or pure antagonists, for example the antagonist naloxone is produced by the substitution of the methyl group on the tertiary nitrogen of oxymorphone for an N-allyl group. These structural and stereospecific requirements, in conjunction with the existence of antagonists which could block morphine induced analgesia, led to the postulation that opiates must bind to specific receptors in order to elicit the chemical or physical effects leading to their observed actions.

The criterion of stereospecificity was used to demonstrate the existence of putative opioid receptors by displacing radiolabeled opiates bound to brain cell membranes with unlabeled opiates, but not their inactive enantiomers (Simon et al., 1973 ; Pert and Snyder, 1973 ; Terenius, 1973). A direct correlation between opiate binding and pharmacological action was also demonstrated (Pert and Snyder, 1973 ; Snyder et al., 1975).

In vitro bioassay systems were developed where the electrically stimulated contractions of isolated tissue preparations could be inhibited by opiate agonists and this effect reversed by opiate antagonists. These bioassay systems, utilizing guinea pig ileum (Kosterlitz and Waterfield, 1976) and mouse vas deferens (Henderson et al., 1972), also demonstrated direct correlation between the ability of low concentrations of opiate agonists to inhibit the contraction of these tissues and their analgesic potency in vivo, giving additional evidence that opiate action was receptor mediated. Moreover, the estimations of agonist and antagonist potency in both in vitro binding

studies and the guinea pig ileum bioassay were in good agreement (Creese and Snyder, 1975).

The discovery of opioid receptors in a wide range of vertebrate systems suggested the presence of an endogenous ligand which would bind to this receptor, producing a physiological response. Electrical stimulation of the central gray region of rat brain had been shown to produce long-lasting analgesia (Reynolds, 1969; Mayer and Liebeskind, 1974) and this analgesia could be partially reversed by naloxone (Akil et al., 1976). The rational for this observation was that electrical stimulation provoked the release of endogenous "opiate-like" substances which produce analgesia. Aqueous extracts of brain tissue were found to contain factors that imitated the effect of morphine on the in vitro bioassay (Hughes, 1975a) and in competitive binding studies (Terenius and Wählström, 1974). A factor was also reported present in extracts of bovine pituitary which had opioid receptor activity, but different properties to the substance(s) present in brain (Teschemacher et al., 1975). Then, in 1975b, Hughes et al. characterised and purified two pentapeptides from pig brain which were potent agonists at the opioid receptor and had the structures Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu. These were named Methionine^5-enkephalin and Leucine^5-enkephalin.

1.4 Biosynthesis of the endogenous opioids

1.4.1 Pro-opiomelanocortin - a common precursor for β-endorphin and adrenocorticotrophic hormone but not for Methionine^5-enkephalin

ME was found to be homologous with residues 61-65 of the peptide hormone β-LPH isolated from sheep pituitary by Li and Chung, (1976b).

Therefore, the question arose as to whether β-LPH was a precursor for ME. A peptide β-END, which had the sequence β-LPH-(61-91) and therefore ME at its NH_2-terminus, was isolated from camel pituitary (Li and Chung, 1976a) and shown to be a potent opioid agonist in receptor binding assays and the guinea pig ileum bioassay, in contrast to β-LPH which was inactive in both systems (Cox et al., 1976). Incubation of β-LPH with aqueous extracts of rat brain was shown to generate opioid activity thought to be related to the production of β-LPH fragments with opioid agonist activity (Lazarus et al., 1976). The opioid peptides α-endorphin and γ-endorphin, β-LPH-(61-76) and β-LPH-(61-77) respectivity, were purified from porcine hypothalamus-neurohypophysis (Ling et al., 1976) and the synthetic peptide β-LPH-(61-69) was shown to have opioid activity (Guillemin et al., 1976). An approximately 30000 Dalton protein containing the sequences of both ACTH and β-LPH was identified in mouse pituitary tumour cells and rat pituitary gland (Mains et al., 1977 ; Roberts and Herbert, 1977) and it was thought that there could be a primary biosynthetic pathway for opioid peptides from this large precursor, for example, from β-LPH, through β-END to ME.

Further studies, however, began to question this hypothesis. An estimation of β-END and enkephalin immunoreactivities (IR) in specific regions of rat brain showed that β-END-IR was located in the diencephalon but not in the hippocampus, cerebral cortex, cerebellum or striatum whereas enkephalin-IR was present predominantly in the striatum and diencephalon (Rossier et al., 1977). This was also shown to be the case in human brain (Gramsch et al., 1979). Immunocytochemical mapping visualised β-END immunoreactive structures in rat brain which were present in anatomically separable nerve fibres and neurones to those containing the enkephalins (Bloom et al., 1978).

In the hypothalamus, which contains both β-END and ME, the ME immunopositive cells could not be visualised within the hypothalamic β-LPH/β-END group. Unilateral lesions of the basal hypothalamus destroyed β-LPH/β-END cells without appreciably lowering ME levels suggesting the β-LPH/β-END and enkephalin systems were morphologically separate (Watson et al., 1978).

The ACTH/β-LPH precursor, POMC, was purified to homogeneity from camel pituitary gland (Kimura et al., 1979) and the primary amino acid sequence of bovine pituitary POMC deduced from cloned cDNA (Nakanishi et al., 1979). These sequences finally demonstrated that this precursor did not contain any copies of LE, indicating another biosynthetic route for, at least, this enkephalin. In addition, β-END had been found to be a potent opioid agonist in several <u>in vitro</u> systems suggesting a receptor mediated role for this product without further processing (Lord et al., 1977), an hypothesis supported by the absence of a recognized site for proteolytic cleavage which would release ME from β-END.

Nevertheless, the postulation that the enkephalins were indeed biosynthesised via a large precursor was supported by <u>in vitro</u> incorporation studies with [^{3}H]-tyrosine. After the labelling period incorporation of radioactivity into striatal proteins proceded linearly with time, with a lag phase of 1-2 hours before detection of radiolabelled enkephalins. Incorporation could be inhibited by cycloheximide, administered during the labelling and lag phases, indicating that biosynthesis of the enkephalins occured via a ribosomal process, with the lag phase suggestive of processing from a larger precursor (McKnight et al., 1979).

Putative opioid precursors were also detected after gel filtration chromatography of striatal extracts from guinea pig, rat, mouse and bovine tissues which did not contain POMC, β-LPH, β-END or α-endorphin (Lewis et al., 1978 ; Beaumont et al., 1979). Since opioid precursors themselves proved inactive on opioid assay systems the enzyme trypsin, which cleaves at the COOH-terminal side of basic residues, was employed, to digest the gel filtration eluates in order to liberate internal opioid sequences.

1.4.2 Enkephalin biosynthesis in the adrenal medulla

Studies using immunofluorescence techniques visualised ME-like immunoreactivity in the gland cells of guinea pig adrenal medulla (Schultzberg et al., 1978a and 1978b). Several groups reported the presence of enkephalin-like peptides and putative enkephalin precursors in bovine adrenal medulla (Costa et al., 1979 ; Lewis et al., 1979) and demonstrated that opioid material was stored and co-secreted with catecholamines from adrenal chromaffin granules (Viveros et al., 1979). Chromatography of bovine adrenal medulla, or chromaffin granule acid extracts, gave comparable results to those obtained with striatal extracts. Several species in the molecular weight range up to 20000 liberated peptides with opioid activity after digestion with trypsin. The absence of POMC and related peptides in the bovine adrenal medulla (Lewis et al., 1979) and the high concentration of opioids and opioid-containing peptides present made this an ideal tissue with which to study enkephalin biosynthesis.

Evidence for the hypothesis that ME and LE were synthesised via a larger precursor came from the following series of experiments. Pulse-chase studies demonstrated that $[^{35}S]$-methionine incubated with

cultured bovine chromaffin cells was incorporated into an approximately 22000 Dalton enkephalin-containing peptide before being detected in either free ME or a related smaller peptide (Rossier et al., 1980b).

Denervation of the rat adrenal gland was found to stimulate a large increase in the concentration of enkephalin and enkephalin-containing peptides in this tissue. The initial increase, occuring only in the region of a > 20000 Dalton species, was replaced over a time course of 96 hours by increasing concentrations of intermediate size enkephalin-containing peptides and finally free enkephalin (Lewis et al., 1981).

As previously described, many biologically active peptide sequences are located between pairs of basic residues within their precursors. Where this arrangement occurs these peptides can be liberated by treating the precursor with trypsin, which cleaves to the carboxyl side of basic residues, followed by carboxypeptidase B, which specifically removes the remaining basic amino acids from the COOH-terminus. Putative precursors can then be detected by assaying for releasable biological activity. This approach was used to identify putative opioid precursors. Gel filtration chromatography of bovine chromaffin granule extracts, followed by trypsin and carboxypeptidase B digestion, yielded opioid activity over the molecular weight range up to 50000. HPLC of the enzymatic digest from the high molecular weight region showed peptides eluting in positions corresponding to synthetic enkephalins, with an approximate ratio of 7:1 for ME : LE (Lewis et al., 1980b).

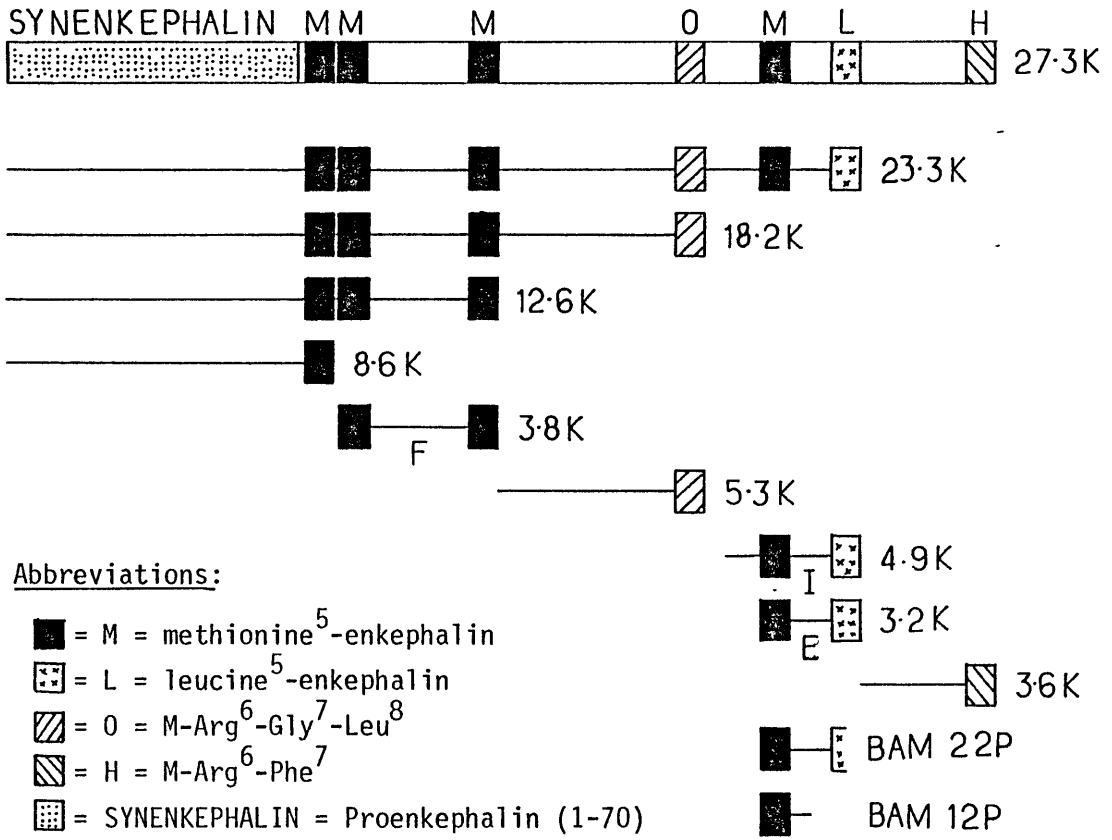
The strategy adopted, most successfully by Sidney Udenfriend's group, was to purify the putative intermediates in the biosynthesis of

enkephalin in order to build up a structure for the precursor (Udenfriend and Kilpatrick, 1983). The peptides were first isolated from an initial crude separation of bovine adrenal medulla chromaffin granule extract (Lewis et al., 1979) and then sequenced. In addition to both ME and LE several COOH-terminally extended small enkephalin congeners were purified. Two of these, the heptapeptide ME-Arg^{6}-Phe^{7} (Stern et al., 1979) and the octapeptide ME-Arg^{6}-Gly^{7}-Leu^{8} (Kilpatrick et al., 1981b) suggested that the enkephalin precursor may produce several biologically active products including the hepta- and octapeptides.

The structure of the higher molecular weight enkephalin-containing peptides and their interrelationship is shown schematically in Fig. 2. Peptides F (3800 Dalton) and I (4700 Dalton) were the first intermediates to be purified, the former proving that more than one copy of the same biologically active peptide, ME, could exist within one precursor whilst the presence of both ME and LE in the latter indicated a common precursor for both pentapeptides (Kimura et al., 1980 ; Jones et al., 1980 ; Stern et al., 1981).

These intermediates had several other interesting features. Peptide E (peptide I-(15-39) ; 3200 Dalton), with an enkephalin sequence at each terminus, was found to be an extremely potent opioid agonist on the guinea pig ileum (Kilpatrick et al., 1981a). A 5300 Dalton peptide (Jones et al., 1982a) contained the sequence -Asn-Ser-Ser- known to act as an attachment site for asparagine-linked oligosaccharide chains (Marshall et al., 1972). The 5300 Dalton species and a 18200 Dalton species containing this glycosylation site migrated with anomalously high molecular weights of 11000 Dalton and 22000 Dalton respectively on SDS-PAGE, a phenomenon associated with

FIG. 2. PROENKEPHALIN AND THE ENKEPHALIN-CONTAINING PEPTIDES IN THE ADRENAL MEDULLA



Noda et al.1982.

Patey et al.1984.

Kilpatrick et al.1982a

Jones et al.1982b.

Jones et al.1982b; Lewis et al.1980a

Kimura et al.1980.

Jones et al.1982a.

Stern et al.1981.

Kilpatrick et al.1981a

Stern et al.1981.

Mizuno et al.1980a.

Mizuno et al.1980b.

Modified from Patey et al.1984.

glycoproteins (Kilpatrick et al., 1982a). However Kilpatrick et al., (1983) have found no evidence for glycosylation of these peptides. The 18200 Dalton and a 12600 Dalton species both contained multiple copies of ME, the 18200 Dalton proving a common precusor for ME and the octapeptide (Lewis et al., 1980a; Jones et al., 1982b) while peptide B was the only species found containing ME-Arg^6-Phe^7.

The related opioid peptides BAM 12P, BAM 20P and BAM 22P were purified from acid extracts of crude adrenal medulla and found to correspond to peptide I-(15-27), I-(15-35) and I-(15-37) respectively (Mizuno et al., 1980a and 1980b). As previously described, BAM 12P and BAM 22P would need uncommon cleavages at the COOH-terminus to be released in vivo. More recently Patey et al., (1984), using an immunoblot technique in conjunction with specific radioimmunoassay, have detected a 23300 Dalton species in bovine adrenal medulla which terminates with LE.

Opioid biosynthesis has also been studied at the nucleotide level. Cell free translation of human phaeo-chromocytoma mRNA synthesised putative opioid precursors which liberated authentic ME following sequential digestion with trypsin and carboxypeptidase B (Giraud and Eiden, 1981). Using a similar approach, Dandekar and Sabol (1982a), showed that cell-free synthesis of ME-containing proteins could also be directed by bovine adrenal medulla mRNA. In this case the largest enkephalin-containing gene product had an apparent molecular weight of 31000 $\pm$ 1000 on SDS-PAGE.

An oligodeoxynucleotide probe of defined sequence complementary to the codons specifying the amino acid sequence NH_2-Trp-Trp-Met-Asp-Tyr-Gln-COOH, found within peptide E, was synthesised by Gubler et

al., (1981). This probe was found to hybridise to bovine adrenal medullary mRNA and could then be used to prime the reverse transcriptase directed production of cDNA with the correct nucleotide sequence to code for peptide E. The transcribed cDNA and the probe both hybridised to mRNA which was long enough to code for the proposed size of the enkephalin precursor ($\approx$1500 nucleotides). Similar results were obtained with both bovine adrenal medulla and human phaeo-chromocytoma, using oligodeoxynucleotide probes coding for ME. These probes also hybridised with POMC mRNA from mouse pituitary tumour cells and bovine pituitary. However the mRNA detected in adrenal tissues did not hybridise with a cDNA sequence complementary to mRNA coding for β-LPH, demonstrating that the adrenal mRNA was not related to POMC (Comb et al., 1982a).

The predicted amino acid sequence of bovine adrenal medulla proenkephalin, the putative precursor for both ME and LE, was simultaneously reported by two groups using the recombinant DNA approach. Gubler et al., (1982) used their peptide E derived probe to identify positive clones whose nucleotide sequences were then aligned with those coding for known enkephalin-containing peptides. The cDNA sequence formed was almost complete but terminated within known protein sequences. Noda et al., (1982) reported the complete mRNA sequence coding for preproenkephalin. The deduced amino acid structure (29786 Dalton) contained a 24 amino acid signal peptide and the sequences of all known adrenal enkephalin-containing species, Fig. 2. Shortly afterwards preproenkephalin was also cloned from human pheochromocytoma (Comb et al., 1982b).

1.4.3 Enkephalin biosynthesis in the CNS

The hypothesis that enkephalin biosynthesis in the CNS follows a similar pathway to that in the adrenal medulla is supported by evidence from biochemical and immunohistochemical investigations. Many groups have used the approach of raising specific antisera to adrenal peptides, known to be derived from proenkephalin, in order to identify the presence of immunologically related peptides in the brain and to compare their distribution in different brain regions. Most of these investigations have used gel filtration and/or HPLC to further characterise observed immunoreactivity, using HPLC retention time as the criterion for the immunoreactivity being analogous to the authentic peptide.

ME-Arg^6-Phe^7-IR was found to be present in striatal extracts from human, rat, cattle and guinea pig (Rossier et al., 1980a; Kojima et al., 1982). The hexapeptide ME-Arg^6 was also present in these tissues, as well as in porcine hypothalamus (Huang et al., 1979). The distribution of ME-Arg^6-Phe^7-IR in rat brain extracts was found to parallel that of ME-IR, with the highest levels in globus pallidus, intermediate levels in caudate putamen and hypothalamus and low levels in cortex and cerebellum (Boarder et al., 1982 ; Giraud et al., 1983). Confirmatory reports have shown that the molar ratio of ME-IR, LE-IR, ME-Arg^6-Phe^7-IR and ME-Arg^6-Gly^7-Leu^8-IR in guinea pig, rat and golden hamster brain is similar to their ratio within proenkephalin (Ikeda et al., 1982).

Ikeda et al., (1983) have also reported a parallel distribution for these four opioid peptides in human and bovine brain, with regional levels of the same order described by Giraud et al. (1983). Pittius et

al. (1983) found that BAM 12P-IR distribution in human brain was similar to that of ME-Arg^6-Phe^7-IR and ME-Arg^6-Gly^7-Leu^8-IR, but that this peptide was present in much lower concentrations than the hepta- and octapeptides. Baird et al. (1982) found BAM 12P-IR in extracts of bovine adrenal and hypothalamus, but not in these tissues from rat. In contrast to the adrenal, where 90% of BAM 12-IR was in a high molecular weight form, hypothalamic BAM 12P-IR corresponded to authentic BAM 12P. The distribution of BAM 22P-IR and peptide F-IR in the brain was similar to that of BAM 12P-IR. The molecular weight profiles of BAM 22P-IR and peptide F-IR from the brain and adrenal also showed that these peptides occur predominantly in a high molecular weight form in the adrenal gland (Höllt et al., 1982a).

A ME-Arg^6-Gly^7-Leu^8 immunoreactive species, with a molecular weight of approximately 8000 Dalton, has been detected in both rat brain and bovine adrenal chromaffin granules (Lindberg et al., 1983). This species has similar chromatographic characteristics to the 5300 Dalton adrenal intermediate previously isolated by Jones et al.,(1982a). Recently a novel amidated octapeptide, which can be released from proenkephalin only by a cleavage at a single basic arginine residue, has been purified from bovine caudate nucleus and named metorphamide. Metorphamide-IR has been located in extracts of rat and guinea pig brain and human adrenal medulla (Weber et al., 1983a; Sonders et al., 1984).

An antiserum directed towards the NH_2-terminal region of proenkephalin has been raised against the 8.6 kDalton enkephalin precursor from bovine adrenal medulla (Liston et al., 1983). This antiserum recognises multiple forms of ME-containing peptides in the bovine adrenal medulla. However, in bovine caudate nucleus the

immunoreactive species did not contain ME and, when purified, was found to be identical to proenkephalin-(1-70).This species has been called synenkephalin (Liston et al., 1984c). Synenkephalin-IR and ME-IR have a similiar regional distribution in bovine brain. The ratio of synenkephalin-IR to ME-IR estimated after trypsin and carboxypeptidase B digestion was found to be close to the value of 1:6 found for these peptides within the sequence of adrenal proenkephalin (Liston and Rossier, 1984a).

It was thought that, if proenkephalin was a precursor for ME and LE in the CNS, then higher molecular weight forms of these peptides, similiar to those purified from adrenal medulla, may exist in the brain. In many distribution studies the antisera that were used failed to cross-react with any higher molecular weight forms of the antigen in the brain (Boarder et al., 1982 ; Ikeda et al., 1982 ; Ikeda et al., 1983 ; Pittius et al., 1983). However, groups using the sequential trypsin and carboxypeptidase B digest to liberate enkephalin prior to radioreceptor-, radioimmuno- or bio-assays reported the presence of putative opioid precursors with estimated molecular weights of up to > 60000 in striatal extracts from cow, guinea pig and rat (Lewis et al., 1978; Beaumont et al., 1980; Kojima et al., 1982; Giraud et al., 1984b) Liston and Rossier (1984a) compared the synenkephalin-IR to ME-IR ratios in different bovine brain regions before and after enzymatic digestion and found that this ratio increased after digestion, most markedly in hypothalamus and caudate nucleus, indicating the release of ME from larger peptides.

Antisera raised against known adrenal proenkephalin peptides have been used immunohistochemically to study, more discreetly, the topography of immunoreactive species potentially derived from

proenkephalin. Comparative distribution studies in the brain have shown that regions of immunopositive staining in response to antisera raised against proenkephalin-(1-77), BAM 22P, ME-Arg^6-Gly^7-Leu^8 and LE coincide with regions containing ME-IR (Weber et al., 1982; Khachaturian et al., 1983). This immunoreactivity was colocalised in both perikarya and processes in rat and bovine brain (Watson et al., 1982 ; Liston et al., 1983). The intensity of immunopositive staining in different brain regions followed the order of magnitude previously observed for enkephalin concentrations in the same tissues, with particularly strong staining in the striatum and hypothalamus (Bloch et al., 1983).

Release of opioid peptides, immunologically similar to those derived from adrenal proenkephalin, has also been demonstrated from brain tissues. The peptides ME, LE, ME-Arg^6-Phe^7 and ME-Arg^6-Gly^7-Leu^8 can be released in vitro from rat striatal slices (Patey et al., 1983a). Synenkephalin was co-released with ME in vitro from bovine globus pallidus and neurohypophysis via a calcium dependent mechanism. The molar ratio of synenkephalin to ME, identical in both tissue and perfusate, was close to their stoichiometry within adrenal proenkephalin (Liston and Rossier, 1984b). Simultaneous release of ME-Arg^6-Gly^7-Leu^8-IR with an 8000 Dalton ME-Arg^6-Gly^7-Leu^8 immunoreactive species has been demonstrated from medulla pons and hypothalamus (Lindberg et al., 1984).

The most convincing evidence that the same proenkephalin gene is expressed in both adrenal medulla and brain was initially obtained from cell-free translation and cross-hybridisation experiments. Using a cell-free translation system Dandekar and Sabol (1982b) identified a putative primary gene product from bovine striatum which had a

molecular weight of 31000 ± 1000 and was similar to that produced from adrenal medulla using the same technique. This protein liberated ME and LE in a ratio of 4.7:1 after trypsin and carboxypeptidase B treatment. Antiserum directed against ME-Arg^{6}-Phe^{7} cross-reacted with a 30000 ± 500 Dalton protein which was consistently synthesised by translation of mRNA extracted from bovine and guinea pig striata, rat brain and bovine adrenal medulla. This protein also liberated ME sequences after digestion (Sabol et al., 1983). Cross-hybridisation experiments using cloned cDNA from human pheochromocytoma detected proenkephalin mRNA in rat striatum, hypothalamus, cortex, cerebellum, hippocampus, midbrain and brain stem as well as rat and bovine adrenal. The mRNA ratio between these regions paralleled that of ME (Tang et al., 1983 ; Legon et al., 1982).

During the preparation of this thesis the cDNA sequence of rat brain preproenkephalin has been reported (Yoshikawa et al., 1984; Howells et al., 1984). This is the first time that preproenkephalin has been cloned from nervous tissue. Rat preproenkephalin displays a high degree of homology with the bovine and human forms. The enkephalin, heptapeptide, octapeptide and peptide E sequences are all conserved as are the paired basic residues, the glycosylation site at position 152-154 and the cysteine residues located in the synenkephalin region. The nucleotide sequence of the cloned rat preproenkephalin gene has also been determined and found to be similar in structural organisation to the human gene (Rosen et al., 1984).

1.4.4 Biosynthesis of prodynorphin

Reports of a pituitary opioid peptide designated dynorphin, with properties differing from those of β-END (Cox et al., 1975 ; Goldstein

et al., 1981) were largely eclipsed by the discovery of the opioid properties of β-END and the enkephalins. This peptide, dynorphin-(1-17) (DYN 1-17), isolated and sequenced from porcine pituitary (Goldstein et al., 1981) and porcine duodenum (Tachibana et al., 1982), contained a LE sequence at its NH_2-terminus. Following this several LE-extended opioid peptides were purified from porcine hypothalamus, the nonapeptide α-neoendorphin, the octapeptide β-neoendorphin (α-neoendorphin-1-8) and dynorphin-(1-8) (DYN 1-8) (Kangawa et al., 1979; Kangawa et al., 1981 ; Minamino et al., 1981). Dynorphin-(1-24) and dynorphin-(1-32) peptides, isolated from porcine pituitary, contained DYN-(1-17) followed by a basic pair of residues and then a LE sequence. DYN-(1-17) and dynorphin-(20-32) were designated dynorphin-A (DYN-A) and dynorphin-B (DYN-B) respectively (Fischli et al., 1982a and 1982b). DYN-B had been simultaneously isolated from bovine posterior pituitary and named rimorphin (Kilpatrick et al., 1982b).

Kakidani et al. (1982) cloned cDNAs coding for the α-neoendorphin and dynorphin precursor. The deduced amino acid sequence of preprodynorphin contained a twenty amino acid signal peptide and the sequences of all LE-containing peptides isolated from mammalian tissue and not contained within proenkephalin. The cDNA sequence encoding the human prodynorphin sequence has also been reported (Horikawa et al., 1983).

Recent reports have included the sequence of the opioid peptide leumorphin, the COOH-terminal twenty-nine residues of prodynorphin, detected in porcine neurointermediate lobe (Nakao et al., 1983) and the detection of a 6000 Dalton LE-containing opioid peptide present in rat adenohypophysis which contains both DYN-A and DYN-B immunoreactivities (Seizinger et al., 1981; Seizinger et al., 1984).

1.4.5. Summary

Numerous peptides containing at the NH_2-terminus the conserved enkephalin sequence, Tyr-Gly-Gly-Phe-$^{Met}_{Leu}$, necessary for opioid activity have now been isolated. These peptides are all derived from one of three precursor molecules, POMC (Nakanishi et al., 1979), proenkephalin (also known as proenkephalin A) (Noda et al., 1982) and prodynorphin (also known as proenkephalin B) (Kakidani et al., 1982). In addition to the similarities already mentioned between prohormones, the opioid peptide precursors are all approximately the same length with an NH_2-terminal region containing a high concentration of cysteine residues.

These precursors and some of their products are shown schematically in Figs.1 and 2. Briefly, the POMC molecule contains one copy of the opioid peptide β-END located at the COOH-terminus, but POMC can be processed into many other biologically active peptides. In contrast, all the known biologically active peptides derived from proenkephalin and prodynorphin are opioid in nature. If proenkephalin were to be cleaved completely at all internal basic paired residues it would liberate four copies of ME and one copy each of LE, the octapeptide ME-Arg^6-Gly^7-Leu^8 and the heptapeptide ME-Arg^6-Phe^7. However larger intermediate peptides have been isolated, some of which have opioid activity and include peptide E, peptide F, BAM 12P and BAM 22P. Finally, prodynorphin contains three LE sequences flanked by basic paired residues but gives rise to LE extented peptides, including α-neoendorphin, β-neoendorphin, DYN-A and DYN-B (also known as rimorphin).

1.5 Other aspects of opioid biochemistry

1.5.1. Distribution

The gene products from POMC, proenkephalin and prodynorphin have a different distribution within the mammalian CNS and peripheral tissues. The peptides derived from POMC have four major locations, the anterior and intermediate lobes of the pituitary, the medial basal hypothalamus, with fibres spreading into the limbic system and the brain stem and, finally, a less prominant group of cells within the nucleus commissuralis and the nucleus of the solitary tract (Akil et al., 1984).

The proenkephalin related peptides are found peripherally in several structures, most notably the adrenal medulla and the myenteric plexus of the gastrointestinal tract. In the CNS the neuronal pathways are widespread, with the highest concentrations of peptides in striatum and hypothalamus. In contrast, the dynorphin related peptides occur principally in posterior pituitary, hypothalamus, brain stem and submucous plexus of the gastrointestinal tract. Within the hypothalamus they are located in several cell groups including the vasopressin producing cells of the magnocellular neurosecretory nuclei (Cuello, 1983 ; North and Egan, 1983 ; Watson et al., 1983).

1.5.2 Multiple opioid receptors

Early evidence for the hypothesis that the physiological response elicited by alkaloid drugs was receptor mediated included the observation that structural modification of opiates could transform an agonist into an antagonist or an agonist-antagonist. This suggested the

presence of specific receptors which could bind all three classes of drug. Houde and Wallenstein (1956) showed that administration of morphine/nalorphine mixtures to humans produced biphasic dose-response curves with nalorphine either antagonising the analgesic effect of morphine or producing analgesia in its own right. In an extension of the receptor concept, Martin (1967), postulated that this dual action of nalorphine was a result of its interaction with pharmacologically differentiable populations of opioid receptors. Martin et al., (1976) demonstrated that in the chronic spinal dog three different syndromes could be produced by morphine congeners and suggested that these syndromes were evoked by agonist action on different receptors. These receptors were designated μ, k and σ and their prototype agonists were morphine, ketocyclazocine and N-allylnormetazocine respectively. The idea of receptor heterogeneity was reinforced by the fact that the prototype k agonist did not substitute for morphine in relieving abstinence in the morphine dependent monkey, an observation in agreement with this compound being selective for the k but not the μ receptor (Martin et al., 1976).

1.5.3 Multiple endogenous opioid receptor ligands

Following the discovery of the endogenous opioid peptides it was of interest to compare the potency of opioids and opiates in several *in vitro* systems to evaluate whether their action was indicative of multiple receptor populations. Lord et al., (1977) showed that ME and LE were more potent in the mouse vas deferens bioassay than in the guinea pig ileum bioassay, in contrast to normorphine which was more potent in the latter tissue. It was concluded that the receptor populations in these two models were indeed heterogeneous, with the guinea pig ileum containing mainly μ receptors, mediating the action of

morphine and the mouse vas deferens containing mainly δ receptors, interacting preferentially with the opioid peptides. The action of both enkephalin and normorphine in the guinea pig ileum was equally well antagonised by naloxone, suggesting both compounds were acting on the same receptor in this tissue, whilst in the mouse vas deferens, whereas the same concentration of naloxone antagonised normorphine, a ten fold increase in naloxone was required to antagonise enkephalin, promoting the idea that they act at a different receptor in this tissue. A third bioassay system, the rabbit vas deferens, has been shown to respond exclusively to k agonists, indicating this preparation contains a homogeneous population of k-receptors (Oka et al., 1981).

The guinea pig ileum, mouse vas deferens and rabbit vas deferens preparations have been used in conjunction with radioreceptor binding studies to classify known endogenous opioid peptides on the basis of their receptor subtypes. The major problem with the binding studies has been to selectively label each receptor subtype. (D-Ala^2, MePhe^4, gly-ol^5) enkephalin has proved a selective agonist for the μ receptor (Kosterlitz and Paterson, 1981) while (D-Thr^2-Thr^6)-LE is selective for the δ receptor (Zajac et al., 1983). At the present time there are no selective agonists or antagonists available for the k-subtype.

The endogenous opioid peptides display receptor selectivity and have varying potencies and pharmacological profiles at each receptor type. The opioid peptide β-END derived from POMC was found to be equally potent at both the μ and δ receptors, but was inactive in the k specific rabbit vas deferens bioassay (Lord et al., 1977; McKnight et al., 1982). Of the opioid peptides contained within proenkephalin, the enkephalins and the related smaller peptides exhibit preference for the δ-receptor. This is most marked for LE, followed by ME, with

ME-Arg^6-Phe^7 almost equiactive at both μ and δ sites (Lord et al., 1977; McKnight et al., 1983). Both enkephalins are inactive in the rabbit vas deferens bioassay (McKnight et al., 1982). In binding assays ME-Arg^6-Phe^7 and ME-Arg^6-Gly^7-Leu^8 show the same affinity for δ and μ binding sites and also have low potencies at the k site (Magnan et al., 1982). The receptor selectivity of the larger proenkephalin derived opioid peptides, peptides E and F and BAM 22P, has not been well established. However they all produce in vivo naloxone reversible analgesia when injected into mouse brain, a response associated with action at the μ receptor (Höllt et al., 1982b). The amidated octapeptide metorphamide is the only endogenous opioid peptide so far isolated which acts preferentially at the μ receptor (Weber et al., 1983a). The dynorphin precursor also gives rise to opioid peptides with differential preference for opioid receptors. The trend is for this family to be increasingly potent and selective for the k receptor the longer the COOH-terminal extension from the LE sequence, the most potent being the larger peptides DYN-(1-13) and DYN-(1-17) followed by DYN-(1-8), DYN-(1-9) and α-neoendorphin (Corbett et al. 1982).

In general the higher molecular weight opioid peptides are more potent on the in vivo and in vitro systems. This is probably due to the rapid proteolysis of smaller peptides, since the incorporation of a "cocktail" of peptidase inhibitors in bioassay and binding systems potentiates the action of smaller peptides without having an effect on the potency of stable enkephalin analogues or β-END (McKnight et al., 1983). This greater stability, with respect to degradation, of the larger peptides is also reflected in tissue response. The metabolically labile peptides usually have a rapid onset and short duration of action whereas the stable peptides have a slower onset and a longer-lasting activity, as demonstrated by the pattern of activity of DYN-(1-8)

versus DYN-A on the guinea pig ileum and by enkephalin versus β-END on the mouse vas deferens (Corbett et al., 1982; Lord et al., 1977). Since rapid inactivation is one prerequisite for a neurotransmitter role it could be that the more metabolically labile opioids are candidates for a neurotransmitter role, whereas more stable peptides act as neuromodulators, or hormonally.

1.6 Concluding remarks

When this project began the biosynthesis of the enkephalins in the adrenal medulla was being unravelled, but it was still unclear whether biosynthesis in the CNS followed a similar pathway. Then the cDNA sequences for POMC, proenkephalin and prodynorphin were cloned and the deduced primary amino acid structures of these precursors were found to contain the sequences of all isolated opioid peptides. Although these precursors were cloned from non-neural tissues there was much evidence to support the hypothesis that they also act as precursors in the brain. However until the presence of these precursors is confirmed at the gene, mRNA and protein levels it cannot be definitely stated that all neural opioids have this origin or that opioid peptide sequences encoded within these genes are the same in every tissue or species. None of these genes, for example, could code for the 90000 Dalton putative proenkephalin species reported by Beaumont et al. (1980).

Information concerning the distribution of opioid peptide species, within both neural and non-neural tissues, is beginning to suggest that tissue specific processing, in combination with receptor distribution and the nature of receptor-ligand interactions, has the potential to produce flexibility of opioid activity in response to stimuli. It is therefore important to analyse the opioid precursor-product

relationships in different tissues and between different species. For example, while there had been reports of high molecular weight opioid precursors in striatal tissue (Beaumont et al., 1980 ; Kojima et al., 1982), other groups found no evidence for the existence of these peptides in this tissue and have proposed that neuronal proenkephalin, in constrast to adrenal proenkephalin, is processed rapidly to the penta-, hepta- and octapeptides (Liston et al., 1983 ; Ikeda et al., 1982 and 1983).

It was therefore of interest to investigate and characterise more fully the proenkephalin species present in striata in order to provide additional information on the biosynthetic route of the enkephalins in the striatum and to try and resolve the controversy over the presence of high molecular weight proenkephalin species and, therefore, the degree of processing within the striatum.

CHAPTER TWO

MATERIALS AND METHODS

Chapter two

Materials

Reagents were purchased as follows:

ME, LE and ME-Arg^{6}-Phe^{7} were from Cambridge Research Biochemicals

^{125}I -sodium iodide (17.4 Ci/mg, 100mCi/ml) was from NEN

chloramine-T and sodium metabisulphite were from Merek Darmstadt

Antisera were a generous gift from Dr J. Rossier

IgGsorb was from The Enzyme Company

95% oxygen/5% carbon dioxide was from British Oxygen

naloxone was a generous gift from ICI

Porapak-Q was from Waters Associates

Sephadex G-200, Sephacryl-300, Polybuffer exchanger 94, Polybuffer 74 and blue dextran were from Pharmacia Fine Chemicals

DE 52 anion exchanger was from Whatman

AG 501-X8(D) mixed bed ion exchanger, acrylamide, ammonium persulphate, Amido Black and nitrocellulose membrane (0.45 um) were from Bio-Rad

triethylamine, sodium dodecyl sulphate and B-mercaptoethanol were from Fluka AG

N,N'-methylene bisacrylamide was from Eastman Kodak

Ultradex and Ampholine carrier ampholytes were from LKB

peroxidase labelled sheep anti-rabbit IgG was from the Institut Pasteur

glutaraldehyde and formaldehyde were from BDH

bovine serum albumin, ovalbumin, soybean trypsin inhibitor, aldolase, carbonic anhydrase, cytochrome c, myoglobin, catalase, thyroglobulin, chymotrypsin, aprotinin, L-leucyl-L-leucine, trypsin (DPCC treated), carboxypeptidase B, N-benzoyl-L-arginine ethyl ester, hippuryl-L-arginine, hippuryl-L-phenylalanine, hydrogen peroxide, ammonium

sulphate, trichloroacetic acid, N,N,N',N'-tetramethyl- ethylenediamine (TEMED), bromophenol blue, Coomassie Brilliant Blue type-R-250, dithiothreitol, silver nitrate, 4-chloro-1-napthol and Folin-Ciocalteu's Phenol reagent were from the Sigma Chemical Company Staphylococcus aureus (V8 strain) protease was from Miles Laboratories

All other reagents were of analytical grade.

Chapter two

Methods

2.1 Tissue extraction

Guinea pigs (Dunkin-Hartley, 350-400 g, 10-12 weeks) and rats (Sprague-Dawley, 150 g, 6-8 weeks) were sacrificed by cervical dislocation. The brains were removed and the striata dissected out and frozen in liquid nitrogen within 2-3 min of death. Guinea pig adrenal glands were dissected out immediately after removal of the brain and frozen at the same time as the striata. Bovine striata were obtained fresh from the slaughter house and frozen within 30 min of death on solid CO_2. Tissues were either used directly or stored at -70°C.

Tissues were weighed, then homogenised from frozen using 10 s bursts of a polytron Ultra-Turrax at 1000 rpm, in either :

a) 50mM Tris-HCl buffer, pH 8.0 at 20°C, containing 8M urea ; 10% w/v at 20°C or

b) ice-cold 1M acetic acid-HCl pH 1.9 ; 10% w/v.

The Tris-urea homogenates were centrifuged at 200000 g_{av} for 90 min at 10°C and the acidified acetic acid homogenates at 50000 g_{av} for 60 min at 4°C. The supernatants were removed for gel filtration chromatography.

2.2 Radioimmunoassay

2.2.1 Iodination of the opioid peptides

ME, LE and ME-Arg^6-Phe^7 were iodinated by the chloramine-T method

(Hunter et al., 1962). The peptide (5 µg) was dissolved in 5 µl of distilled water in a 1.5 ml volume Eppendorf tube. The following reagents were added in succession : 10 µl of 0.5M sodium phosphate buffer pH 7.4, 5 µl of [^{125}I] sodium iodide (100 mCi/ml in 0.1mM sodium hydroxide) and 5 µl of a 2 mg/ml solution of chloramine-T in the phosphate buffer. Following a 30 s incubation at room temperature the reaction was stopped by addition of 10 µl of a 5 mg/ml solution of sodium metabisulphite in the phosphate buffer. The iodinated products were immediately separated by HPLC on a reverse phase Ultrasphere Octyl column (Altex; 0.45 x 25 cm) eluted by a 30 min continuous gradient of 0-50% (v/v) acetonitrile in 0.25 M triethylamine formate pH 3.0, at a flow rate of 1 ml/min.

The iodinated products were detected using an on-line scintillation gamma counter (Nardeux SMIG E724) to monitor the column eluate and 1 ml fractions were collected. Fractions corresponding to the mono-iodinated product were pooled, evaporated to dryness in a speed-vac concentrator (Savant) and resuspended in 50% methanol. The resuspension volume was chosen to give a final value of approximately 2 x 10^5 cpm/µl and the [^{125}I] tracer was routinely diluted 1:2000 in RIA buffer prior to use. The [^{125}I] tracers were stored at -20°C and had a shelf life of 2-4 months.

2.2.2 ME radioimmunoassay

The buffer used for the ME radioimmunoassay (RIA) was phosphate buffered saline (PBS) composed of 20mM sodium phosphate buffer pH 7.4 ; 153mM sodium chloride ; 3mM sodium azide and contained 100 mg/l of bovine serum albumin (PBSA buffer). The final RIA incubation volume of 300 µl contained 100 µl of standards or unknown samples containing

1-350 fmol of ME, 100 µl of [^{125}I]-ME diluted in PBSA buffer to give a total count of approximately 10000 cpm/incubation and 100 µl of the antiserum diluted 1:20000 in PBSA buffer. The antiserum was raised in a male New Zealand white rabbit immunised with ME conjugated to haemocyanin with carbodiimide. The final dilution of the ME antiserum in the incubation was 1:60000 (Patey et al., 1983b). Prior to RIA, ME standards were assembled in the same buffer as the unknown samples in order to keep the incubation medium constant within an individual assay. The incubation was carried out at 4°C for 16 h. On completion of the incubation the antigen-antibody complex was precipitated during a 20 min incubation at 4°C with 1.0 ml of a suspension of heat-killed, formalin fixed Staphylococcus aureus bacteria (IgGsorb) diluted 1:400 in PBS. After precipitation samples were centrifuged at 4000 g_{av} for 15 min, the supernatant removed by aspiration and the pellet counted in an LKB solid scintillation gamma counter.

2.2.3 LE Radioimmunoassay

The LE RIA was performed in the same phosphate buffers (PBS and PBSA) used for the ME RIA. The RIA was performed under non-equilibrium conditions (Patey et al., 1984). Standards or unknown samples containing 1-350 fmol of LE in 100 µl of an appropriate buffer were preincubated with 100 µl of LE antiserum, diluted 1:64000 with PBSA, for 16 h at 4°C. Then 100 µl of [^{125}I]-LE, diluted in PBSA to give a total count of 10000 cpm/incubation, was added and the incubation continued for 2 h at 4°C. The final incubation volume contained LE antiserum at a 1:192000 dilution. The antiserum was raised in a female white rabbit immunised with LE conjugated to ovalbumin with carbodiimide (Gros et al., 1978). The incubation was terminated as described for the ME RIA.

2.2.4 ME-Arg^6-Phe^7 radioimmunoassay

Prior to analysis by RIA, standards and unknown samples containing 5-2500 fmol of ME-Arg^6-Phe^7 were assembled in the same buffer and oxidised by incubation with an equal volume of 0.03% (v/v) hydrogen peroxide for 1 h at 37°C.

The ME-Arg^6-Phe^7 RIA was performed under non-equilibrium conditions. The RIA buffer was 0.1M Tris-HCl pH 8.4 containing 3mM sodium azide and 100 mg/l bovine serum albumin. Following oxidation, samples were lyophilised in a speed-vac concentrator, resuspended in 100 µl of RIA buffer and preincubated with 100 µl of the ME-Arg^6-Phe^7 antiserum, diluted 1:2000 in RIA buffer, for 16 h at 4°C. The antiserum was raised in rabbits immunised with ME-Arg^6-Phe^7 conjugated to bovine serum albumin with glutaraldehyde (Giraud et al., 1983). [^{125}I]-ME-Arg^6-Phe^7, diluted in RIA buffer in order to give a total count of 10000 cpm/incubation, was added and the incubation continued for 3 h at 4°C. The incubation was terminated as described for the ME RIA.

RIA results were calculated using an automatic fitting programme internal to the LKB multigamma solid scintillation counter. Using this programme a standard displacement curve is constructed from given standard data points using a spline function interpolation. The programme then calculates values for unknown samples by comparsion of the data with the standard curve. All calculations are made using the log. of sample concentration.

2.3 Mouse vas deferens bioassay

Male T.O. albino mice (6-8 weeks) were killed by cervical dislocation and the vasa deferentia removed. Each vas deferens was mounted in a 1.0 ml organ bath and perfused with oxygenated (95% O_2, 5% CO_2) Krebs solution at 37°C. The tissues were stimulated (S88 stimulator ; Grass Instruments) via two platinum electrodes, placed at the upper and lower ends of the bath, with trains consisting of 6 pulses of 30 V and 0.5 ms duration at intervals of 40 ms. Pulse trains were repeated every 10 s. The electrically induced tissue contractions were measured by an isometric transducer (UF1, Lectromed) connected to a pen recorder (MX 212 ; Devices). The composition of the Krebs was (mM) ; sodium chloride 133, potassium chloride 4.7, calcium chloride 2.52, sodium hydrogen phosphate 1.38, sodium hydrogen carbonate 27.4 and glucose 11. The dipeptide L-leucyl-L-leucine (2 mM) was included in the Krebs solution during later assays (McKnight et al., 1983).

Synthetic ME was diluted in Krebs solution to a final concentration of 17.5μM (10 ng/μl) or 1.75μM (1 ng/μl) and used to construct a dose-response curve over the range 1-100nM. Tissue perfusion was stopped prior to the addition of test peptides which were then injected directly into the bath, in a maximum volume of 50 μl, using a Hamilton syringe. ME was added in single doses and once the maximum response was reached for each dose the tissue was washed by perfusion with Krebs. Unknown samples were added to the bath in Krebs solution, in the same manner as the synthetic peptide and were classified as containing bioassayable opioid activity if an inhibitory response to the electrically evoked tissue twitch was reversed by 900nM naloxone. The opioid peptide content of an unknown sample was estimated in ME equivalents by comparing the % twitch inhibition produced by the

test substance with control dose-response curves for, ME constructed before and after sample application.

2.4 Removal of salts and concentration of peptide fractions by Porapak-Q

The procedure was based on the method of Vogel and Altstein (1977). Columns (0.8 X 2.5 cm) were prepared by packing 350 mg of Porapak-Q, suspended 10% w/v in methanol, into disposable econocolumns (Biorad). Columns were pretreated with alternate washes of 10 ml methanol and 10 ml water for three cycles and residual methanol removed with 30 ml water prior to sample application in aqueous solution. Columns were then washed with 30 ml water and eluted with two washes of 3.5 ml methanol. Routinely, methanol eluates were collected in 50 ml pear-shaped flasks and the solvent removed by rotary evaporation at 40°C.

2.5 Enzymatic digestion

2.5.1 Trypsin and carboxypeptidase B

a. Prior to radioimmunoassay

Samples required for analysis by RIA were digested with trypsin at a final concentration of 50 µg/ml by incubation for 2 h at 37°C in an appropriate buffer. The enzymatic reaction was stopped by heat inactivation at 100°C for 20 min, followed by centrifugation at 4000 g_{av} for 5 min at 4°C. Carboxypeptidase B was then added directly to the samples to a final concentration of 5 µg/ml (see individual methods) and the samples were digested by incubation for 60 min at 37°C. The

enzymatic reaction was stopped by heat inactivation at 100°C for 15 min followed by centrifugation at 4000 g_{av} for 5 min at 4°C. Aliquots were removed for RIA.

b. Prior to radioimmunoassay and/or mouse vas deferens bioassay

When samples were to be analysed by either mouse vas deferens (MVD) bioassay alone or by both MVD bioassay and RIA a modified digestion procedure was employed. Samples were digested with trypsin at a final concentration of 50 µg/ml by incubation for 2 h at 37°C in an appropriate buffer system. The samples were then concentrated over Porapak-Q to remove salts and trypsin. The methanol elute was evaporated to dryness and samples were resuspended in 2 ml of 50mM Tris-HCl buffer pH 8.0 at 37°C and digested with carboxypeptidase B at a final concentration of 5 µg/ml for 60 min at 37°C. Carboxypeptidase B activity was stopped by heat inactivation at 100°C for 15 min followed by reapplication of the sample to Porapak-Q columns. After elution the methanol fractions were divided into suitable aliquots for MVD bioassay and RIA and the solvent removed by rotary evaporation at 40°C.

2.5.2 Staphylococcus aureus (strain V8) protease

Samples were digested by Staphylococcus aureus (strain V8) protease (S. aureus protease) at a final concentration of 40 µg/ml in 50mM Tris-HCl buffer pH 7.8, by incubation for 16 h at 37°C. Enzymatic activity was stopped by heat inactivation for 20 min at 100°C.

2.6. Spectrophotometric determination of enzyme activity

2.6.1 Trypsin

N-Benzoyl-L-arginine release from the synthetic substrate N-benzoyl-L-arginine ethyl ester (BAEE), by trypsin catalysed hydrolysis was monitored in a Cecil CE292 spectrophotometer by observing the increase in optical density at 253 nm with time (Schwert and Takenaka, 1954). The reaction was initiated by the addition of 0.2 ml of trypsin solution to 3 ml of a 1mM solution of BAEE in a quartz cuvette, light path 1 cm, giving a final trypsin concentration of 3.125 µg/ml in the cuvette. The addition of the enzyme solution was taken as time zero and the increase in optical density was measured at 10 s intervals.

The rate of spontaneous substrate hydrolysis under the same conditions was measured by the addition of 0.2 ml of buffer to 3 ml of 1mM BAEE solution. Unless otherwise specified, assays were conducted at 23°C, with both enzyme and substrate in 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$.

2.6.2 Carboxypeptidase B

Hippuric acid release from the synthetic substrate hippuryl-L-arginine, by carboxypeptidase B catalysed hydrolysis, was monitored in a Cecil CE292 spectrophotometer by observing the increase in optical density at 254 nm with time (Wolff et al., 1962). The reaction was initiated by the addition of 100 µl of carboxypeptidase B solution to 3 ml of a 0.5mM solution of hippuryl-L-arginine in a quartz cuvette, light path 1 cm, giving a final carboxypeptidase B

concentration of 0.48 µg/ml in the cuvette. The addition of the enzyme was taken as time zero and the increase in optical density was measured at 10 s intervals. Assays were conducted at 37°C in a variety of buffer systems. The rate of spontaneous substrate hydrolysis under the same conditions was measured by the addition of 100 µl of buffer to 3 ml of 0.5mM hippuryl-L-arginine solution.

Carboxypeptidase B was tested for carboxypeptidase A activity by monitoring spectrophotometrically at 254 nm the ability of the enzyme to catalyse hippuric acid release from the carboxypeptidase A substrate hippuryl-L-phenylalanine (Folk and Schirmer, 1963). The reaction was initiated by the addition of 100 µl of carboxypeptidase B solution to 3 ml of 0.1mM hippuryl-L-phenylalanine in a quartz cuvette, light path 1 cm, giving final enzyme concentrations of 0.5, 5 or 50 µg/ml in the cuvette. The addition of enzyme was taken as time zero and the change in optical density was measured at 30 s intervals. Assays were conducted at 23°C in 50mM Tris-HCl buffer pH 7.9 at 37°C. The rate of spontaneous substrate hydrolysis under the assay conditions was measured by the addition of 100 µl of buffer to 3 ml of 0.1mM hippuryl-L-phenylalanine solution.

2.7 Reverse phase HPLC

Reverse phase HPLC was performed at 25°C using an Ultrasphere octyl column (Altex; 0.45 x 25 cm) equilibrated with a running buffer of 20mM ammonium acetate pH 4.0 containing 20% (v/v) acetonitrile. Prior to reverse phase HPLC, synthetic standard peptides (ME and LE) and lyophilised samples were incubated for 1 h at 37°C in 100 µl 1.5% (v/v) hydrogen peroxide. Standards and samples were then lyophilised and resuspended in HPLC running buffer. Aliquots (50 µl) were injected on

to the column and eluted isocratically in the running buffer at a flow rate of 1 ml/min. Fractions (500 µl) were collected and suitable aliquots lyophilised prior to resuspension in RIA buffer and assay for ME-IR and LE-IR.

The reverse phase HPLC retention times of the synthetic peptides ME and LE, under these conditions, were established both before and after incubation of the peptides with hydrogen peroxide. Standards were monitored by u.v. absorbance at 280 nm. Following calibration with synthetic peptides, the column was washed with three 30 min continuous gradients of 20-50% (v/v) acetonitrile in 20mM ammonium acetate pH 4.0, before reequilibration with running buffer and then sample application.

2.8 Ultrafiltration

An Amicon ultrafiltration unit was routinely used to both concentrate samples and/or change the sample buffer composition. The membranes used were low binding YM10 or YM2 which retain proteins of molecular weight > 10000 and > 2000 respectively. A nitrogen pressure of 60 psi was used to filter the samples through the membrane. Buffer composition was changed by alternately concentrating and diluting the sample in the required medium.

2.9 Gel filtration chromatography

2.9.1 Sephadex G-200

A Sephadex G-200 column (2.5 x 76 cm) was equilibrated at a flow rate of 12 ml/h in 1M acetic acid pH 2.1 at 4°C. Striatal tissue was prepared by acetic acid extraction followed by protein precipitation

with ammonium sulphate (15% saturation for 3 h at 4°C). The ammonium sulphate precipitate was pelleted by centrifugation at 50000 g_{av} for 20 min at 4°C and the supernatant decanted. The pellet was resuspended in 1M acetic acid pH 2.1 containing 8M urea, by sonication on ice and then diluted to a final concentration of 6M urea with the column medium. Samples (8 ml corresponding to 1 g of original tissue weight) were applied directly to the column and eluted with 1M acetic acid pH 2.1. The column was calibrated with the following standard proteins chromatographed under the same conditions ; aldolase (160 kDalton) bovine serum albumin (69 kDalton) and ovalbumin (43 kDalton).

Column fractions were collected and suitable aliquots analysed for opioid peptide activity using the MVD bioassay, following sequential digestion with trypsin and carboxypeptidase B. Prior to digestion the acetic acid was removed by freeze drying and the lyophilised protein resuspended in 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2 mM $CaCl_2$.

2.9.2 Sephacryl-300

A Sephacryl-300 column (2.6 x 86 cm) was equilibrated at 20°C at a flow rate of 13.5 ml/h, in a running buffer of 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea. Tissues were prepared by either 8M urea or acetic acid extraction and samples (6 ml) were applied directly to the column and eluted with running buffer. The column was calibrated with the following standard proteins chromatographed under the same conditions ; blue dextran, bovine serum albumin (69 kDalton), ovalbumin (43 kDalton), soybean trypsin inhibitor (21 kDalton), cytochrome c (13 kDalton) and enkephalin (0.55 kDalton).

Column fractions were collected and suitable aliquots analysed for opioid peptide activity using the MVD bioassay and/or specific RIA for ME and LE. For this purpose samples were diluted 1:4 with 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$, to give a final urea concentration of 2M and digested with trypsin followed by carboxypeptidase B. Aliquots analysed for opioid peptide activity before enzymatic digestion were diluted ten fold in RIA buffer, giving a final concentration of 800mM urea, prior to direct RIA for ME and LE.

2.10 Ion-exchange chromatography

A DE52 anion exchange column (2.6 x 12 cm) was equilibrated in a running buffer of 50mM Tris-HCl pH 8.8 at 4°C containing 4M urea, at a flow rate of 1 ml/min at 4°C. Fractions corresponding to a high molecular weight peak, separated by Sephadex G-200 chromatography, were pooled and protein precipitated with ammonium sulphate (15% saturation for 3 h at 4°C). The ammonium sulphate precipitate was pelleted by centrifugation at 50000 g_{av} for 20 min at 4°C, the supernatant decanted and the pellet resuspended in 50mM Tris-HCl buffer pH 8.8 at 4°C containing 8M urea, by sonication on ice. The sample was then diluted to a final concentration of 4M urea with 50mM Tris-HCl pH 8.8 at 4°C and applied directly to the column. Non-adsorbed material, monitored by optical density at 280 nm, was eluted with running buffer at a flow rate of 1 ml/min at 4°C. The adsorbed material was eluted by a continuous ionic strength gradient from 0 to 0.25M NaCl in 7.8 column volumes of running buffer at 4°C. The column was finally washed with 200mM HCl containing 8M urea at a flow rate of 1 ml/min at 4°C.

Fractions were collected and analysed for opioid peptide activity using the MVD bioassay, following sequential digestion with trypsin and

carboxypeptidase B. For this purpose samples were diluted to a final urea concentration of 2M with 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$. Acid wash fractions were adjusted to pH 7.9 at 37°C using 0.1M NaOH prior to digestion.

2.11 Isoelectricfocusing

The experimental procedure was as described in the LKB application note 198. Isoelectricfocusing (IEF) utilized a flat bed of granulated dextran gel (Ultradex) as the stabilisation medium and was conducted using an LKB multiphor unit and associated gel casting equipment. The initial gel bed volume of 100 ml consisted of 4% (w/v) Ultradex slurried in 50mM Tris-HCl buffer pH 8.0 at 20°C containing the sample, 2% (v/v) Ampholine carrier ampholytes of the appropriate pH range and either 6M or 2M urea.

After gel casting excess water was removed from the slurry by evaporation in a light air stream until the weight loss equalled the evaporation limit of the gel. This limit was taken as 75 % of the weight of water which could be removed before the gel cracked. This value was dependent on the urea molarity. The weight of water which could be removed before cracking was determined experimentally for each urea concentration used and the evaporation limit under these conditions calculated.

After evaporation, the gel was subjected to IEF at 8 W constant power, with the current and voltage limited to 20 mA and 1.19 kV respectively, for 16 h at 10°C.

IEF of calibration proteins was conducted under standard conditions in 4% (w/v) Ultradex slurried in 50mM Tris-HCl buffer pH 8.0 at 20°C containing 6M urea, 2% (v/v) Ampholine pH range 3.5-10 and 200 mg each of the following proteins; myoglobin, catalase, ovalbumin, thyroglobulin and chymotrypsin.

Following IEF a paper print was taken to visualise the protein zones. A dry filter paper was placed gently on the surface of the gel for 30-60 s, transfered gel side down on to a glass plate and then dried quickly in a hot air stream. The adsorbed protein was fixed by three washes of 10% (w/v) trichloroacetic acid for 15 min each. The paper was then stained in 0.2% (w/v) Coomassie Brilliant Blue type-R-250 dissolved in 1 l of destaining solution (methanol : acetic acid : water in a 1:1:5 volume ratio) for 10 min before destaining with frequent changes of destaining solution. Immediately after the print was taken the gel protein zones were immobilised, by pressing a fractionating grid on to the gel and the pH gradient along the length of the gel was determined using a surface electrode (Pye Unicam). Proteins were eluted from the gel bed using a filtering apparatus consisting of a column (1.2 x 4 cm), with a nylon gauze support at one end, slotted into a centrifuge tube. Each protein zone was removed from the grid separately and placed on to the gauze in the column. The gel was suspended in 2 ml of urea, of the same molarity used in the original slurry and the eluate was allowed to drip slowly through the gauze before centrifugation at 12000 g_{av} for 5 min to dehydrate the gel. This elution procedure was then repeated with an additional 1 ml of urea solution giving a final sample volume of 3.5 ml.

2.11.1 The effect of Ampholine on opioid peptide assay

A gel slurry of 4% (w/v) Ultradex in 50mM Tris-HCl buffer, pH 8.0 at 20°C, containing 6M urea and 2% (v/v) ampholine pH range 3.5-10 was subjected to IEF under standard conditions. Zones eluted from the cathodic and anodic ends of the pH gradient were pooled and had a pH of 9.3 and 6.2 respectively. These eluates were used to investigate the effect of residual Ampholine on enzyme activities during digestion. The fractions were diluted to a final urea concentration of 2M using 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$, the pH adjusted to pH 7.9 at 37°C and aliquots retained to solubilize the synthetic enzyme substrates. The residual eluate was digested with trypsin and carboxypeptidase B, as previously described and aliquots removed from each enzyme incubation to assay for catalytic activity. Sampling time was 2 h for trypsin and 30 min for carboxypeptidase B.

2.11.2 Mixed bed ion exchange chromatography

Columns (1.0 x 25 cm) were packed with AG 501-X8(D) mixed bed ion exchange resin in 2M urea. Columns were pretreated with 10 mg of bovine serum albumin in 3.5 ml of 2M urea. Samples were eluted from IEF gels containing 2M urea, as previously described, applied directly to the AG-501-X8(D) column and eluted with 2M urea. The absorbance of the column eluate was monitored at 280 nm.

2.12 Chromatofocusing

A column of polybuffer exchanger (PBE) 94 was equilibrated with 25mM imidazole-HCl buffer pH 7.2, containing 6M urea. Samples were equilibrated in the same buffer by alternate concentration and dilution

in an Amicon ultrafiltration unit. The final sample volume of 15 ml was applied to the column. The pH gradient was developed by elution with 10 column volumes of 12.5% polybuffer 74 (PB74)-HCl pH 3.8 containing 6M urea. After completion of the pH gradient the column was washed with a solution containing 6M urea and 1M NaCl.

Fractions were collected and suitable aliquots analysed for opioid peptide content by ME RIA following sequential digestion with trypsin and carboxypeptidase B. For this purpose samples were diluted 1:3 with 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$, to give a final concentration of 2M urea and a final pH of between 7.4-7.9. Enzymatic activity was stopped by heat inactivation, except in the case of the NaCl containing fractions, which were concentrated over Porapak-Q after trypsin digestion, to remove both enzyme and salt before addition of carboxypeptidase B.

2.13 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) utilized slab gels prepared as described by O'Farrell (1975) for the second dimension, with the inclusion of 5M urea in both the 12% resolving and 4 % stacking gels. The following solutions were used for SDS-PAGE ;

Resolving gel buffer : 0.75M Tris-HCl pH 8.8 containing 0.2% (w/v) SDS and 10M urea.

Stacking gel buffer : 0.5M Tris-HCl pH 6.8 containing 0.4% (w/v) SDS.

Running buffer : 0.025M Tris/ 0.195M glycine pH 8.8 containing 0.1% (w/v) SDS.

Sample buffer : 0.0625M Tris-HCl pH 6.8 containing 5M urea, 4.6% (w/v) SDS and 5% (v/v) β-mercaptoethanol.

Modified sample buffer : 0.125M Tris-HCl pH 6.8 containing 9.2% (w/v) SDS and 10% (v/v) β-mercaptoethanol.
30% acrylamide containing 28.4% (w/v) acrylamide and 1.6% (w/v) N,N'-methylene-bisacrylamide.
10M urea.

The acrylamide solution was filtered through a 0.6 µm Millipore filter after preparation. The running buffer and acrylamide were stored at 4°C, whilst the remaining solutions were kept at room temperature.

The resolving gel was prepared by mixing resolving gel buffer with 30% acrylamide and water in a 5:4:1 volume ratio. The solution was filtered through a 6 µm Millipore filter, degased under vacuum and then polymerisation initiated by the addition of 0.02% (v/v) of 10% ammonium persulphate and 0.045% (v/v) N,N,N',N'- tetramethylethylenediamine (TEMED). The gel was poured immediately using slab gel casting equipment (LKB). With this method approximately 25 ml of resolving gel solution produced a resolving gel of 1.5 mm thickness and 115 mm length with a final concentration of 0.375M Tris-HCl buffer at pH 8.8 containing 12% acrylamide, 0.1% SDS and 5M urea. The surface of the gel was overlaid with water and polymerisation was completed within 1 h.

The water and unpolymerised gel were replaced with resolving gel buffer diluted two fold in water and the gel stored overnight at 20°C before addition of the stacking gel. The stacking gel was prepared by mixing stacking gel buffer with 10M urea, 30% acrylamide and water in a 15:30:8:7 volume ratio. The solution was filtered through a 6 µm Millipore filter, degased under vacuum and polymerisation initiated by the addition of 0.17% (v/v) of 10% ammonium persulphate and 0.05% (v/v) of TEMED. The running buffer overlay was removed from the resolving gel

and the stacking gel poured until the solution reached the top of the glass plates. A comb was placed in the top of the stacking gel to provide loading spaces of 150 µl capacity for a ten space comb and 100 µl capacity for a fifteen space comb. The stacking gel was 1.5 mm thick and 50 mm long with a final concentration of 0.25M Tris-HCl buffer pH 6.8 containing 4% acrylamide, 0.1% SDS and 5M urea.

Sample preparation prior to SDS-PAGE

a. Standard proteins

The following protein standards ; ovalbumin (43 kDalton), carbonic anhydrase (30 kDalton), soybean trypsin inhibitor (21 kDalton), cytochrome c (13 kDalton) and aprotinin (6 kDalton), were solubilized in sample buffer to achieve a final concentration of 50 µg/ml for each protein. Aliquots of this stock solution (50 µl ; 2.5 µg/protein) were included routinely during SDS-PAGE.

b. Unknown proteins

Lyophilised protein was resuspended in 1 volume of 10M urea and an aliquot removed for determination of opioid peptide content. The residual volume was mixed with an equal volume of modified sample buffer.

Prior to SDS-PAGE standards and unknown samples were heat treated for 15 min at 100°C and then centrifuged at 10000 g_{av} for 5 min. The supernatant was removed, mixed with 2.5% (v/v) β-mercaptoethanol and 2.5% (v/v) of 0.25% (w/v) bromophenol blue in sample buffer and

recentrifuged at 10000 g_{av} for 3 min. The supernatant was loaded on to the gel.

Gel lanes not occupied by standards or unknown samples were loaded with sample buffer prepared in the same manner. All lanes contained an equal loading volume to ensure an even electrophoretic front.

Gels were assembled in an LKB electrophoresis cell and SDS-PAGE conducted at 15°C under the following electrical conditions : a constant current of 20 mA for the stacking gel and 40 mA for the resolving gel with the voltage and power limited to 500 V and 10 W respectively. Electrophoresis was typically completed within 6-7 h.

2.14 Electrophoretic transfer blotting

Electrophoretic transfer of proteins from SDS-PAGE gels to a nitrocellulose membrane (pore size 0.45 um) was achieved by the "western" blot technique described by Towbin et al., (1979) using a Bio-Rad Trans-Blot cell. The electrode buffer was 25mM Tris /192 mM glycine pH 8.8 containing 20% (v/v) methanol. Following SDS-PAGE the gel corresponding to the standard and sample lanes required for protein transfer by blotting were removed and placed on a sponge pad soaked in electrode buffer. Nitrocellulose was cut to the correct size and pretreated by a two minutes immersion in water. The membrane was placed over the gel, the lane orientation carefully marked and a second sponge pad, soaked in electrode buffer, placed over the membrane. This formed a "sandwich" of gel and nitrocellulose between the two pads which was then secured in a hinged holder and slotted into the trans-blot cell, with the nitrocellulose facing the anode. The nitrocellulose was handled with tweezers and the gel and membrane kept moist during

assembly. The transfer was conducted at 9 V/cm for 3 h at 4°C. After electrophoretic transfer the nitrocellulose paper was rinsed for 10 min at room temperature in 50mM Tris-HCl buffer pH 7.4 at 20°C containing 200mM NaCl. The protein standard lane was removed and proteins visualised by staining with Amido Black, whilst the sample lanes were subjected to immunoblot.

2.15 Protein visualisation

2.15.1 Silver staining

Following SDS-PAGE protein bands were visualised using the silver staining method of Morrissey (1981).

All solutions were prepared as 100 ml in ultrapure water except for 10% (v/v) glutaraldehyde which was 50 ml in the same medium. The procedure was performed with gentle agitation throughout and gels were handled with rinsed gloves.

The gel was prefixed by soaking in methanol : acetic acid : water in a 5:1:4 volume ratio for 30 min followed by methanol : acetic acid : water in a 5:7:88 volume ratio for 30 min. The gel was fixed by soaking in 10% (v/v) glutaraldehyde for 30 min and then washed thoroughly by soaking overnight in 1 l of ultrapure water followed by a 30 min rinse in water. After washing the gel was soaked in 5 µg/ml dithiothreitol solution for 30 min followed by 0.1% (w/v) silver nitrate solution for 30 min. The gel was rinsed rapidly, once with water and then twice with developer (50 µl of 37% formaldehyde in 100 ml of 3% (w/v) sodium carbonate) and then soaked in developer. When the required level of staining was attained the reaction was stopped by addition of 5 ml of

2.3M citric acid followed by agitation for 10 min. The gel was then washed in several changes of water for 30 min, soaked for 10 min in 0.03% (w/v) sodium carbonate and heat sealed in a polythene bag. Gels were stored at 4°C.

2.15.2 Protein staining with Amido Black

This was performed according to the method of Schaffner et al., (1973). The nitrocellulose tracks to be developed were placed in 0.1% (w/v) Amido Black in methanol : acetic acid : water in a 45:10:45 volume ratio and the protein bands stained for 2-3 min. The nitrocellulose was then washed in water to remove excess staining solution before destaining in several changes of methanol : acetic acid : water in a 90:2:8 volume ratio. The nitrocellulose was destained for 4 min, to prevent deformation of the membrane, then washed in water and dried between sheets of filter paper.

2.16 Immunoblot

After electrophoretic transfer by western blot, protein bands bound to the nitrocellulose membrane were developed using the immunoblot technique described by Hawkes et al., (1982). The nitrocellulose membrane was soaked for 45-60 min in 50mM Tris-HCl pH 7.4 at 20°C containing 200mM NaCl and 3% (w/v) bovine serum albumin (TBSA buffer) and then sealed in a polythene bag with the primary serum (rabbit anti-ME), diluted 1:500 in TBSA. The membrane and primary antiserum were incubated for 12-16 h at 4°C and all subsequent procedures were performed at this temperature.

The membrane was rinsed for 30 min in 3-6 changes of 50mM Tris-HCl buffer pH 7.4 at 20°C containing 200mM NaCl (TBS) and for 10 min in TBSA. Rinsing was followed by incubation with the second serum, sheep anti-rabbit IgG coupled to horseradish peroxidase, diluted 1:200 in TBSA. The membrane was then rinsed in high salt (50mM Tris-HCl buffer pH 7.4 at 20°C containing 500mM NaCl) for 10 min, followed by 20 min in 2-4 changes of TBS and then placed in developer. The developing solution was 3 mg/ml of 4-chloro-1-naphthol in methanol diluted in a 1:5 volume ratio with TBS and containing 0.01% (v/v) hydrogen peroxide. Bands became visible within 15 min at 20°C and the reaction was stopped by placing the membrane in distilled water. All stages were performed with gentle agitation. The nitrocellulose was finally dried between sheets of filter paper.

2.16.1 Enzymatic treatment of immunoblot

Nitrocellulose membrane lanes visualised by immunoblot were excised longitudinally from the membrane and sliced horizontally into 2 mm strips, using a McIlwain tissue chopper, along the entire length of the nitrocellulose. The strips were incubated with trypsin, 100 µg/ml in 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$, for 16 h at 37°C followed by carboxypeptidase B, 0.1 µg/ml in the same buffer, for 1 h at 37°C. Enzyme activity was stopped after each incubation by heat inactivation for 20 min at 100°C.

2.17 Protein estimation

Protein estimations were conducted by the method of Lowry et al., (1951) using bovine serum albumin as the reference protein.

CHAPTER THREE

VALIDATION OF METHODOLOGY

Chapter three

Validation of methodology

3.1 Spectrophotometric determination of enzyme activity

The first step in the detection of putative opioid peptide precursors was to liberate their internal biologically active opioid peptide sequences by digesting samples with trypsin followed by carboxypeptidase B. These digestions were performed in several different modified buffers and it was therefore necessary to estimate the degree of enzyme activity under all experimental conditions.

Activity was measured as the rate of enzyme catalysed hydrolysis of an appropriate synthetic substrate. This rate was calculated from the linear portion of the curve obtained by plotting increase in optical density due to hydrolysis against time. Spontaneous hydrolysis was observed only for the trypsin substrate and this rate of reaction was routinely subtracted from enzymatic rates.

The rate of substrate hydrolysis by freshly prepared enzyme, at 23°C in 50mM Tris-HCl buffer, pH 7.9 at 37°C, containing 2mM $CaCl_2$, was taken as the maxmium rate of enzyme activity and this control was included routinely. The rate of substrate hydrolysis under all other conditions tested was expressed as a percentage of this maximum rate.

In order to mimic sample digestion conditions enzymes were pretreated, prior to assay, as follows ;

a. Trypsin : trypsin was preincubated in the buffer under investigation for 2 h at 37°C at a concentration of 50 µg/ml. Aliquots were then

removed to assay for residual trypsin activity.

b.Carboxypeptidase B : trypsin was preincubated as for the trypsin assay and then heat inactivated for 20 min at 100°C. After cooling, carboxypeptidase B (final concentration 15 µg/ml) was added and the enzyme solution preincubated for a further 60 min at 37°C. Aliquots were then removed to assay for residual carboxypeptidase B activity.

Before each assay the appropriate synthetic substrate was freshly prepared in the same buffer as the enzyme.

3.1.1 The effect of incubation period and the presence of calcium ions on trypsin activity

In the presence of 2mM $CaCl_2$ trypsin activity decreased by 15% over a 24 h incubation period, whereas in the absence of 2mM $CaCl_2$ enzyme activity decreased almost 100% over the same time period and to 43.7% of the maximum rate after a 2 h incubation, Table I. This demonstrated that 2mM $CaCl_2$ was required to maintain enzyme activity and that, with this inclusion, the incubation period could be extended to at least 24 h without a debilitating loss of activity.

3.1.2 The effect of urea on trypsin activity

Trypsin was preincubated in 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$ and an increasing concentration of urea (0-8M). Aliquots were assayed for residual enzyme activity. As shown in Fig. 3, when the urea concentration was increased trypsin activity decreased, reaching 50% of its maximum rate in 4M urea and being totally inhibited in 8M urea. From these results it was established that the presence of 2M urea did not substantially affect the ability of trypsin to hydrolyse a synthetic substrate. It was decided that this concentration

TABLE 1

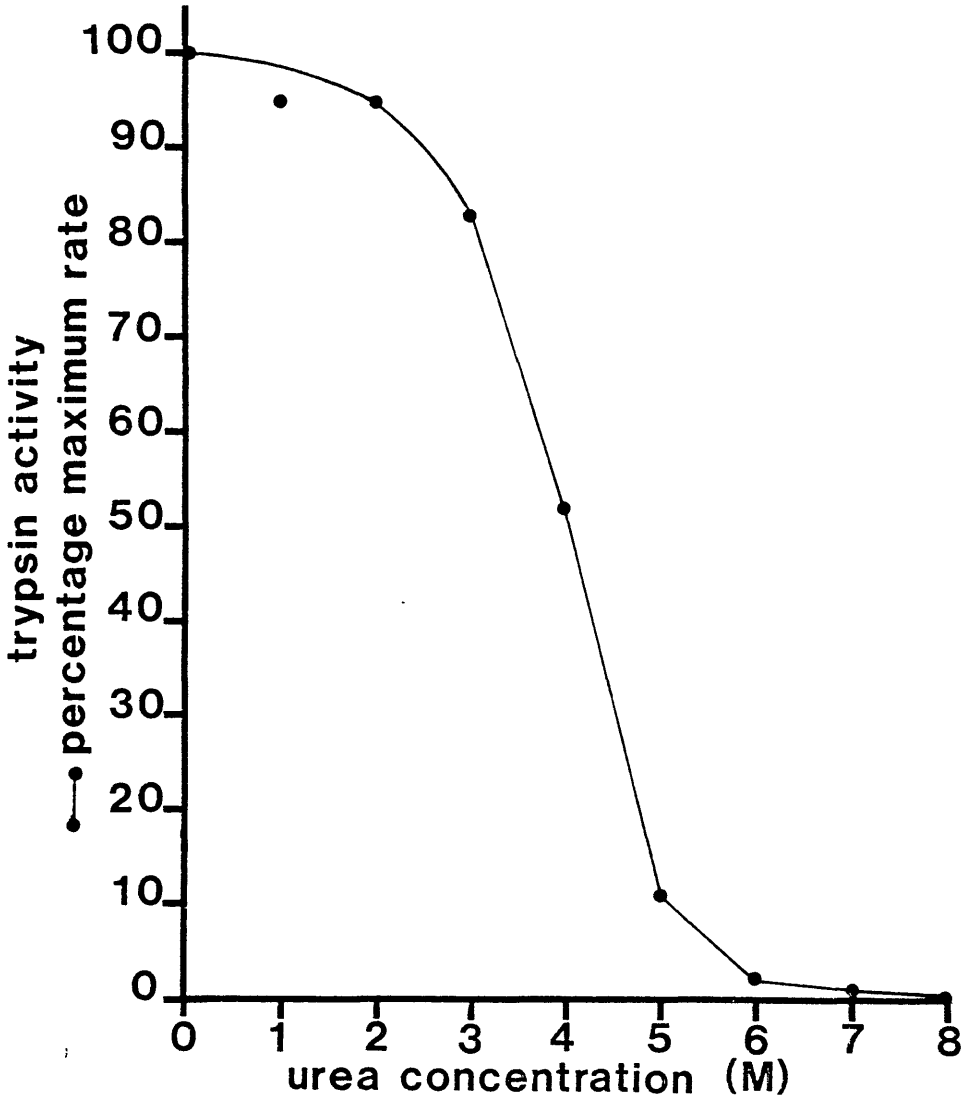
THE EFFECT OF INCUBATION PERIOD AND CALCIUM ION CONCENTRATION ON TRYPSIN ACTIVITY.

	INCUBATION PERIOD IN HOURS	TRYPSIN ACTIVITY % MAXIMUM RATE
Presence of 2mM $CaCl_2$	2	94
	24	85
Absence of 2mM $CaCl_2$	2	44
	24	3.6

Trypsin was preincubated in 50mM Tris-HCl buffer pH 7.9 at 37°C in the presence and absence of 2mM $CaCl_2$. Aliquots were removed after 2 h and 24 h and assayed for residual enzyme activity.

FIG. 3

THE EFFECT OF INCREASING UREA CONCENTRATION ON TRYPSIN ACTIVITY.



Trypsin (50 µg / ml) was preincubated for 2h at 37°C in 50mM Tris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$ and an increasing concentration of urea (0-8 M). Aliquots (0.2 ml) were removed and assayed for residual trypsin activity.

of urea was acceptable during trypsin digestion of unknown samples. Therefore, samples were routinely diluted to this concentration with 50 mMTris-HCl buffer pH 7.9 at 37°C containing 2mM $CaCl_2$, prior to digestion.

3.1.3 The effect of buffer composition on trypsin activity

The solutions listed in Table 2 were all diluted 1:3 with 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$ to a final urea concentration of 2M. Trypsin was preincubated in each modified buffer, followed by assay for residual enzyme activity. The level of trypsin activity was acceptable in all buffer systems except those containing residual Ampholine from IEF, where enzyme activity was reduced by 60%. The concentration of trypsin used to digest IEF eluates was increased two fold, to a final concentration of 100 µg/ml, to compensate for this reduction in activity.

3.1.4 Methods to remove trypsin activity

All the procedures described in Table 3 were effective in stopping trypsin activity. Concentration over Porapak-Q was efficient in removing the enzyme without prior precipitation with trichloroacetic acid. The Porapak-Q removal procedure was investigated further as described in Table 4. Trypsin was found to elute during the washing stages routinely employed with Porapak-Q. Following trypsin digestion of unknown samples, enzyme activity was stopped by heat inactivation prior to RIA or by removal over Porapak-Q prior to RIA and/or MVD bioassay.

TABLE 2

THE EFFECT OF BUFFER COMPOSITION ON TRYPSIN ACTIVITY

BUFFER COMPONENTS	TRYPSIN ACTIVITY % MAXIMUM RATE
12.5% polybuffer 74-HCl pH 3.8 / 6M urea	100
25 mM imidazole-HCl pH 7.2 / 6M urea	93.4
1M NaCl / 6M urea	87.4
Ampholine pH 9.3 / 6M urea	40.8
Ampholine pH 6.2 / 6M urea	38.0

Buffer components were all diluted with 50mM Tris-HCl pH 7.9 at 37°C, containing 2mM $CaCl_2$, to a final concentration of 2M urea. Samples were then preincubated with trypsin followed by assay for residual enzyme activity. Ampholine solutions were obtained by elution from an IEF gel as previously described.

TABLE 3

METHODS TO REMOVE TRYPSIN ACTIVITY

PROCEDURE	TRYPSIN ACTIVITY % MAXIMUM RATE
Heat inactivation for 20 min at 100°C	1.2
Concentration over Porapak-Q	2.4
Precipitation with 5% TCA followed by concentration over Porapak-Q	1.7
Trypsin inhibitor	0.6

Trypsin was preincubated in 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$. Aliquots were removed and treated as follows:

a) heat inactivation for 20 min at 100°C.

b) concentration over Porapak-Q.

c) precipitation with 5% TCA followed by centrifugation at $50000g_{av}$ for 15 min at 4°C and concentration of the supernatant over Porapak-Q.

d) addition of trypsin inhibitor in a ratio of 1.1:1 (w/w) with trypsin.

Following concentration over Porapak-Q samples were resuspended in their original volume with the Tris-HCl buffer.

Sample aliquots were assayed for residual enzyme activity.
TCA = trichloroacetic acid

TABLE 4

REMOVAL OF TRYPSIN ACTIVITY BY PORAPAK-Q

PORAPAK-Q FRACTION	TRYPSIN ACTIVITY % MAXIMUM RATE
Initial aqueous elute	90.0
1st wash- 10ml water	5.0
2nd wash- 10ml water	0.4
3rd wash- 10ml water	0.7
Final elution- 10ml methanol	0.6

Trypsin was preincubated in 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$ and concentrated over Porapak-Q. Column eluates and washes were collected, dried by rotary evaporation at 40°C, resuspended in 200µl of the Tris-HCl buffer and assayed for residual enzyme activity.

3.1.5 The effect of buffer composition on carboxypeptidase B activity

Carboxypeptidase B activity in the range of buffers used experimentally was estimated as described in Table 5. Enzyme activity was reduced by 50-55% in the presence of 2M urea. The inclusion of 4.2% polybuffer 74 or 8.3mM imidazole, in addition to the 2M urea, did not cause any further reduction in activity. However, the presence of 0.33M NaCl in the Tris-urea buffer lowered the level of carboxypeptidase B to 10% of its maximum rate.

During the sequential digestion of unknown samples the level of carboxypeptidase B activity in the incubation buffers used was judged to be acceptable with the exception of those containing NaCl. In this case samples were desalted over Porapak-Q prior to digestion with carboxypeptidase B.

In the presence of residual Ampholine carboxypeptidase B activity approached the maximum rate. Therefore, concentration over Porapak-Q prior to carboxypeptidase B digestion, was efficient in removing from IEF gel eluates any substance(s) which may have interfered with enzyme activity.

Carboxypeptidase B did not catalyse the hydrolysis of a synthetic substrate for carboxypeptidase A at enzyme concentrations up to ten fold higher (50 µg/ml) than the maximum enzyme concentration (5 µg/ml) used to digest unknown samples. Carboxypeptidase B, at this concentration, appeared free from contaminating carboxypeptidase A activity.

TABLE 5

THE EFFECT OF BUFFER COMPOSITION ON CARBOXYPEPTIDASE B ACTIVITY

BUFFER COMPOSITION	CARBOXYPEPTIDASE B % MAXIMUM RATE
50mM Tris-HCl pH 7.9 at 37°C-2mM $CaCl_2$.	100
50mM Tris-HCl pH 7.9 at 37°C-1.5mM $CaCl_2$-2M urea.	43
33mM Tris-HCl pH 7.4 at 37°C-1.3mM $CaCl_2$-2M urea-4.2% polybuffer 74	43
33mM Tris-HCl-8.3mM imidazole-HCl pH 7.9 at 37°C-1.3mM $CaCl_2$-2Murea.	45
33mM Tris-HCl pH 7.9 at 37°C-1.3mM $CaCl_2$-2M urea-0.33M NaCl.	10
50mM Tris-HCl pH 7.9 at 37°C-2mM $CaCl_2$- residual Ampholine pH 9.3	97
50mM Tris-HCl pH 7.9 at 37°C-2mM $CaCl_2$- residual Ampholine pH 6.2.	99

All buffers were preincubated with trypsin and carboxypeptidase B prior to assay for residual carboxypeptidase B activity. Ampholine solutions were obtained by elution from an IEF gel as previously described. Following preincubation with trypsin, Ampholine solutions were concentrated over Porapak-Q and resuspended in 50mM Tris-HCl pH 7.9 at 37°C prior to preincubation with carboxypeptidase B (15 µg/ml, 1h at 37°C) and assay for residual enzyme activity.

3.2 Radioimmunoassay of opioid peptides

3.2.1 Iodination of the opioid peptides

ME, LE and ME-Arg^6-Phe^7 were iodinated by the chloramine-T method (Hunter et al., 1962). The separation of reaction products following iodination of LE is shown in Fig. 4. This type of profile was also obtained for HPLC separations following iodination of ME and ME-Arg^6-Phe^7. Using this system the non-iodinated peptide eluted first, followed by a major peak, representing the mono-iodinated peptide and, finally, a much smaller peak, probably representing the di-iodinated form of the peptide. Fractions corresponding to the mono-iodinated product were pooled, evaporated to dryness and resuspended in 50% methanol prior to use, as previously described.

3.2.2 Radioimmunoassay displacement curves

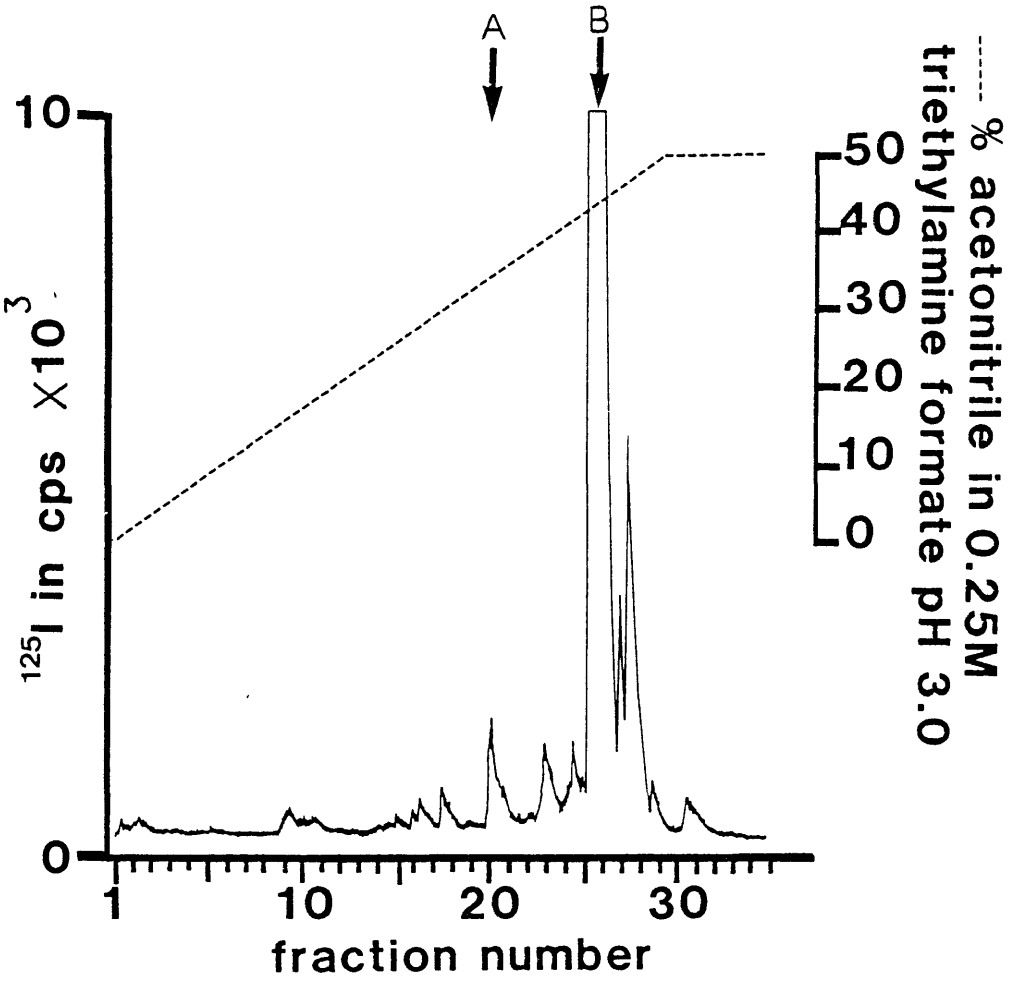
ME RIA

Under the assay conditions the ME RIA had a sensitivity of 1 fmol of ME and an IC_{50} of 10 fmol of ME. The antiserum typically bound 20% of the trace after incubation. Displacement curves were parallel for the range of sample buffers used, which replaced RIA buffer with up to 2M urea and/or 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$, Fig. 5.

The displacement curves obtained for LE and ME against the ME antiserum were not parallel under the same assay conditions, with a cross-reactivity for LE of 10-20%, expressed on a molar basis, over the range 10-350 fmol, Fig. 6. Cross-reactivities with other ME related

FIG. 4 .

PURIFICATION OF ^{125}I-LE BY REVERSE PHASE HPLC

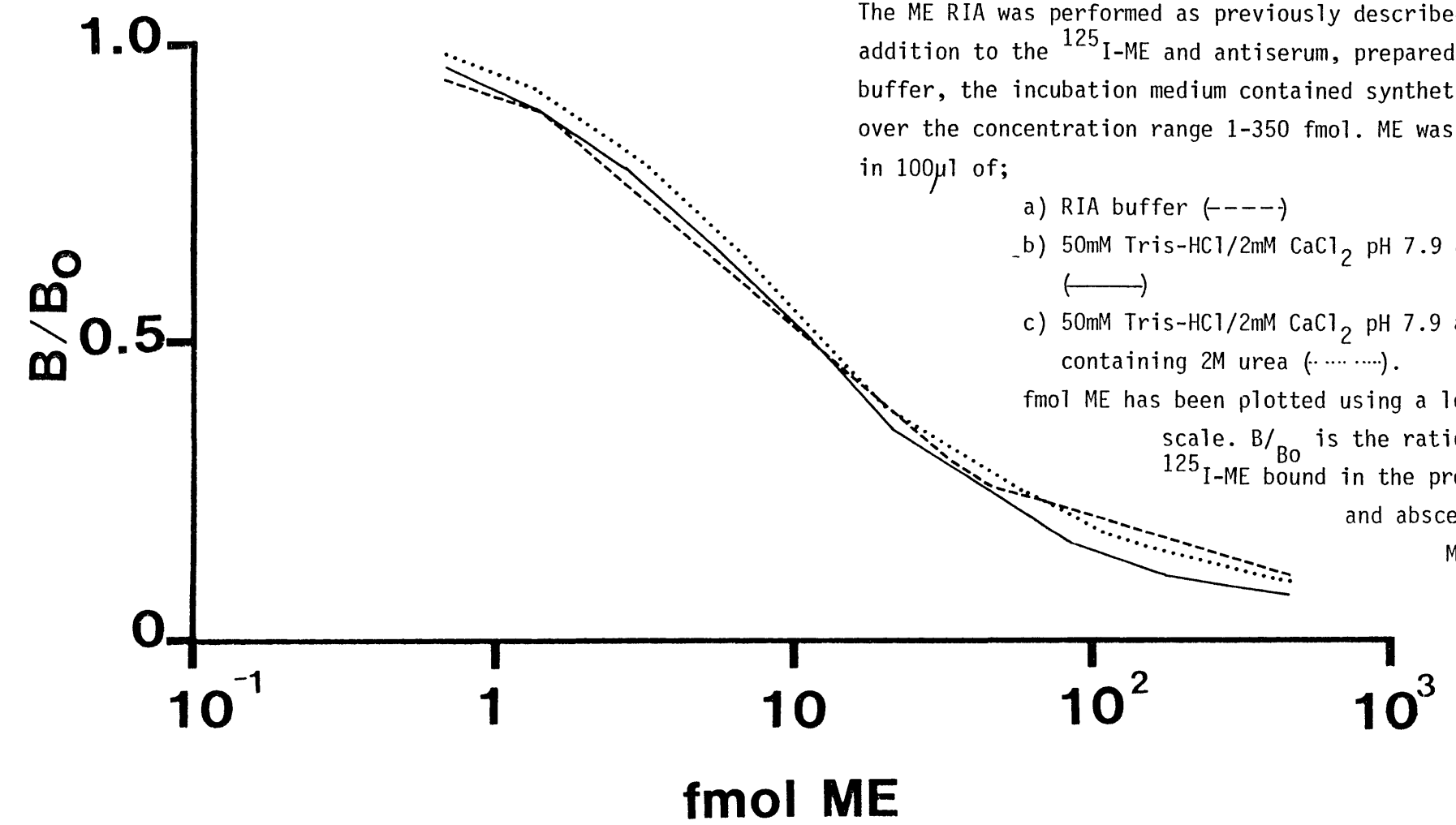


Following iodination of LE by the chloramine-T method the reaction products were separated by reverse phase HPLC (Ultraspher octyl column, Altex; 0.45 x 25 cm). Products were eluted with a continuous gradient of 0-50% acetonitrile in 0.25 M triethylamine formate pH 3.0, at a flow rate of 1 ml/min.

Elution gradient (----); ^{125}I-LE in cps X 10^{3}(——);

A = the elution position of non-iodinated LE and B = the major peak of iodinated peptide subsequently used for RIA.

FIG. 5 . EFFECT OF BUFFER COMPOSITION ON THE ME RIA DISPLACEMENT CURVE.



The ME RIA was performed as previously described. In addition to the ^{125}I-ME and antiserum, prepared in RIA buffer, the incubation medium contained synthetic ME over the concentration range 1-350 fmol. ME was added in 100µl of;

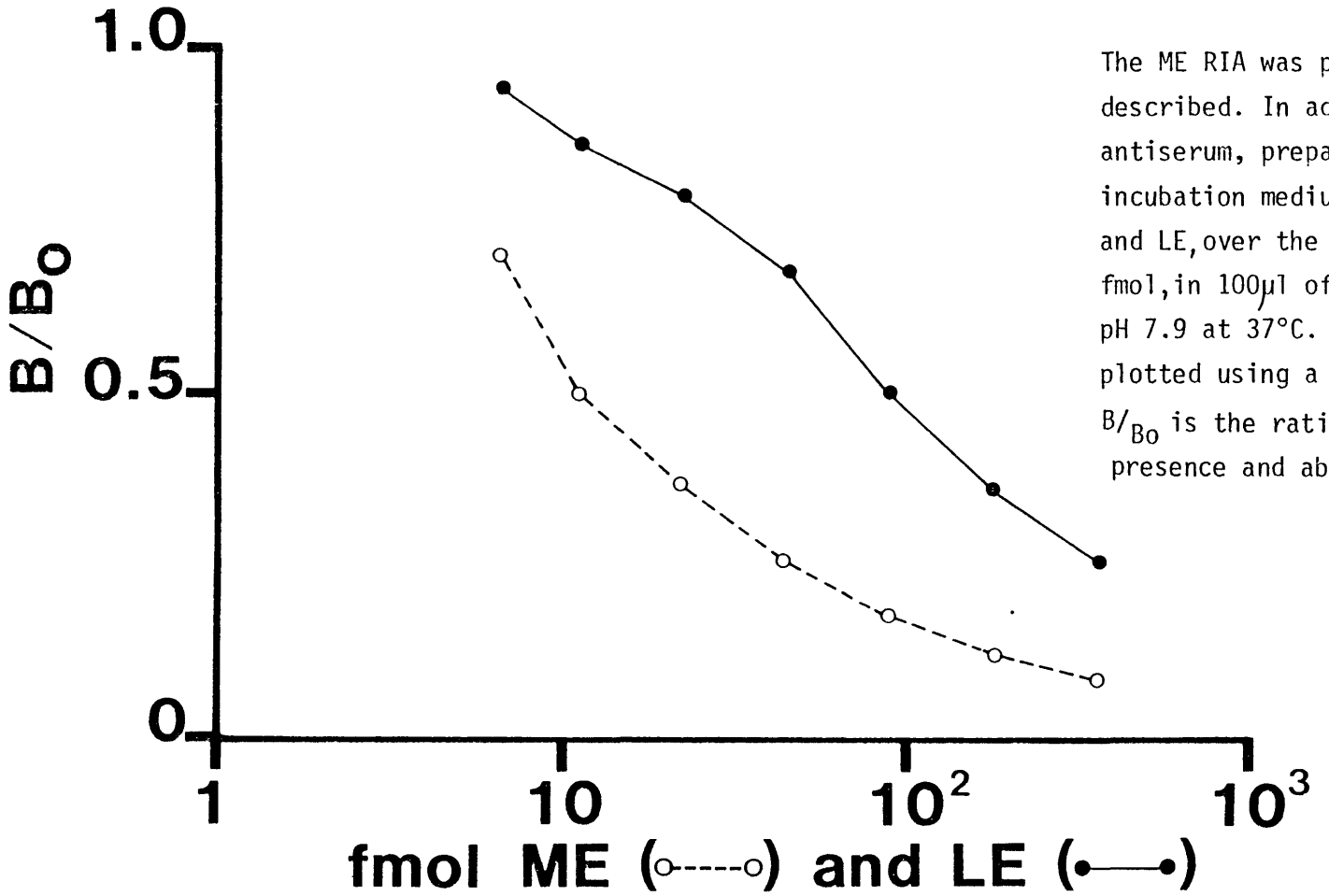
a) RIA buffer (----)

b) 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C (———)

c) 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C containing 2M urea (·····).

fmol ME has been plotted using a log. scale. $B/_{Bo}$ is the ratio of ^{125}I-ME bound in the presence and abscence of ME.

FIG. 6 . ME RIA DISPLACEMENT CURVE : CROSS-REACTIVITY WITH LE.



The ME RIA was performed as previously described. In addition to the ^{125}I-ME and antiserum, prepared in RIA buffer, the incubation medium contained synthetic ME and LE, over the concentration range 10-350 fmol, in 100µl of 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C. fmol ME and LE have been plotted using a log. scale.
$B/_{Bo}$ is the ratio of ^{125}I -ME bound in the presence and absence of synthetic ME and LE.

peptides were (on a molar basis); oxidised ME, 100%; ME-Arg^{6}, 4%; ME-Lys^{6}, 7%; ME-Arg^{6}-Phe^{7}, 4%; ME-Arg^{6}-Gly^{7}-Leu^{8}, 2%; Arg^{-1}-ME, 60%; the 8.6 kDalton adrenal proenkephalin, 28% (Liston and Rossier, 1984a).

The maximum number of samples within one RIA was 180. Standards processed at the beginning and end of such an RIA demonstrated high fidelity over the duration of processing, Fig. 7.

LE RIA

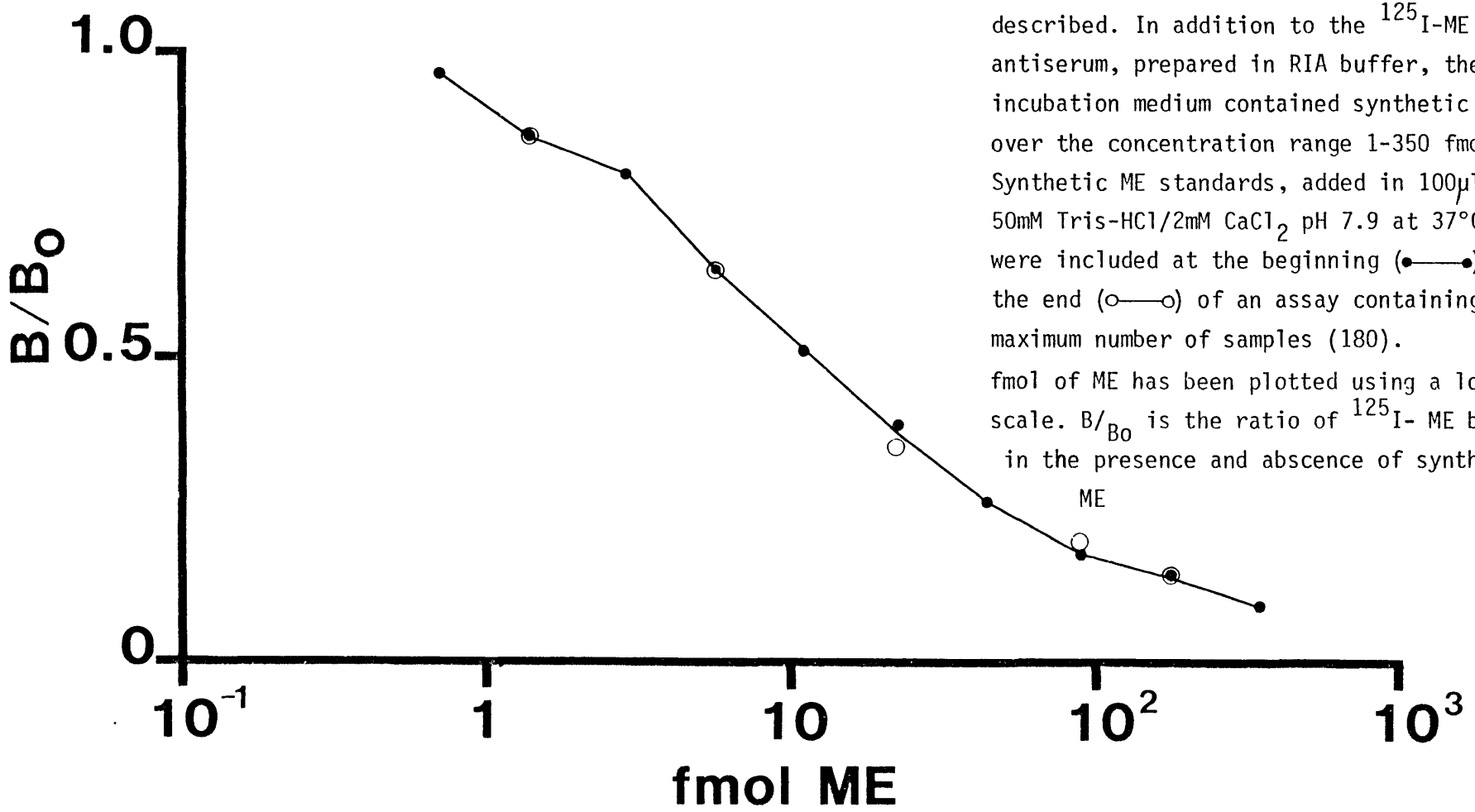
Under non-equilibrium RIA conditions the LE RIA had a sensitivity of 2 fmol of LE and an IC_{50} of 20 fmol of LE, Fig. 8. The antiserum typically bound 25% of the trace after incubation. Displacement curves were parallel for the range of sample buffers used, which replaced RIA buffer with up to 1M urea and/or 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$.

This antiserum demonstrated 15% cross-reactivity with ME, expressed on a molar basis, with parallel displacement curves for ME and LE over the range 10-350 fmol, Fig. 9. Cross-reactivities with other LE related peptides were (on a molar basis); ME-Arg^{6}, 2%; ME-Lys^{6} and ME-Arg^{6}-Arg^{7}, < 0.1%; ME-Arg^{6}-Phe^{7} and ME-Arg^{6}-Gly^{7}-Leu^{8}, < 1% (Giraud et al., 1983).

ME-Arg^{6}-Phe^{7} RIA

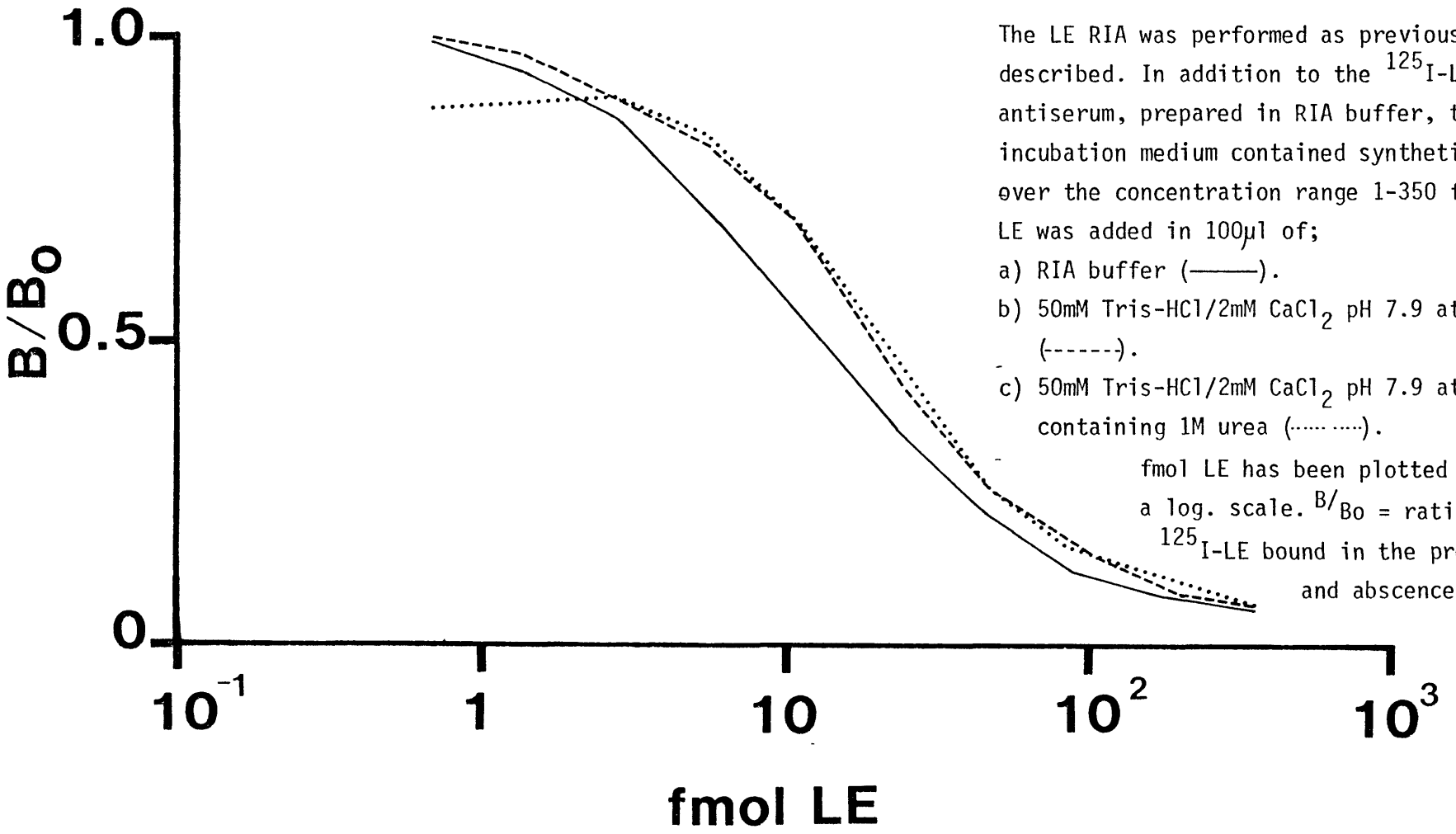
Under non-equilibrium RIA conditions the ME-Arg^{6}-Phe^{7} RIA had a sensitivity of 10 fmol of ME-Arg^{6}-Phe^{7} with an IC_{50} of 80 fmol of Me-Arg^{6}-Phe^{7}. The antiserum typically bound 10% of the trace after incubation, Fig. 10.

FIG. 7 . REPRODUCIBILITY OF DISPLACEMENT CURVES PROCESSED AT THE BEGINNING AND THE END OF THE ME RIA.



The ME RIA was performed as previously described. In addition to the ^{125}I-ME and antiserum, prepared in RIA buffer, the incubation medium contained synthetic ME over the concentration range 1-350 fmol. Synthetic ME standards, added in 100µl of 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C, were included at the beginning (•——•) and the end (○——○) of an assay containing the maximum number of samples (180).
fmol of ME has been plotted using a log. scale. $B/_{Bo}$ is the ratio of ^{125}I- ME bound in the presence and abscence of synthetic ME

FIG. 8 . EFFECT OF BUFFER COMPOSITION ON THE LE RIA DISPLACEMENT CURVE.



The LE RIA was performed as previously described. In addition to the ^{125}I-LE and antiserum, prepared in RIA buffer, the incubation medium contained synthetic LE over the concentration range 1-350 fmol. LE was added in 100µl of;

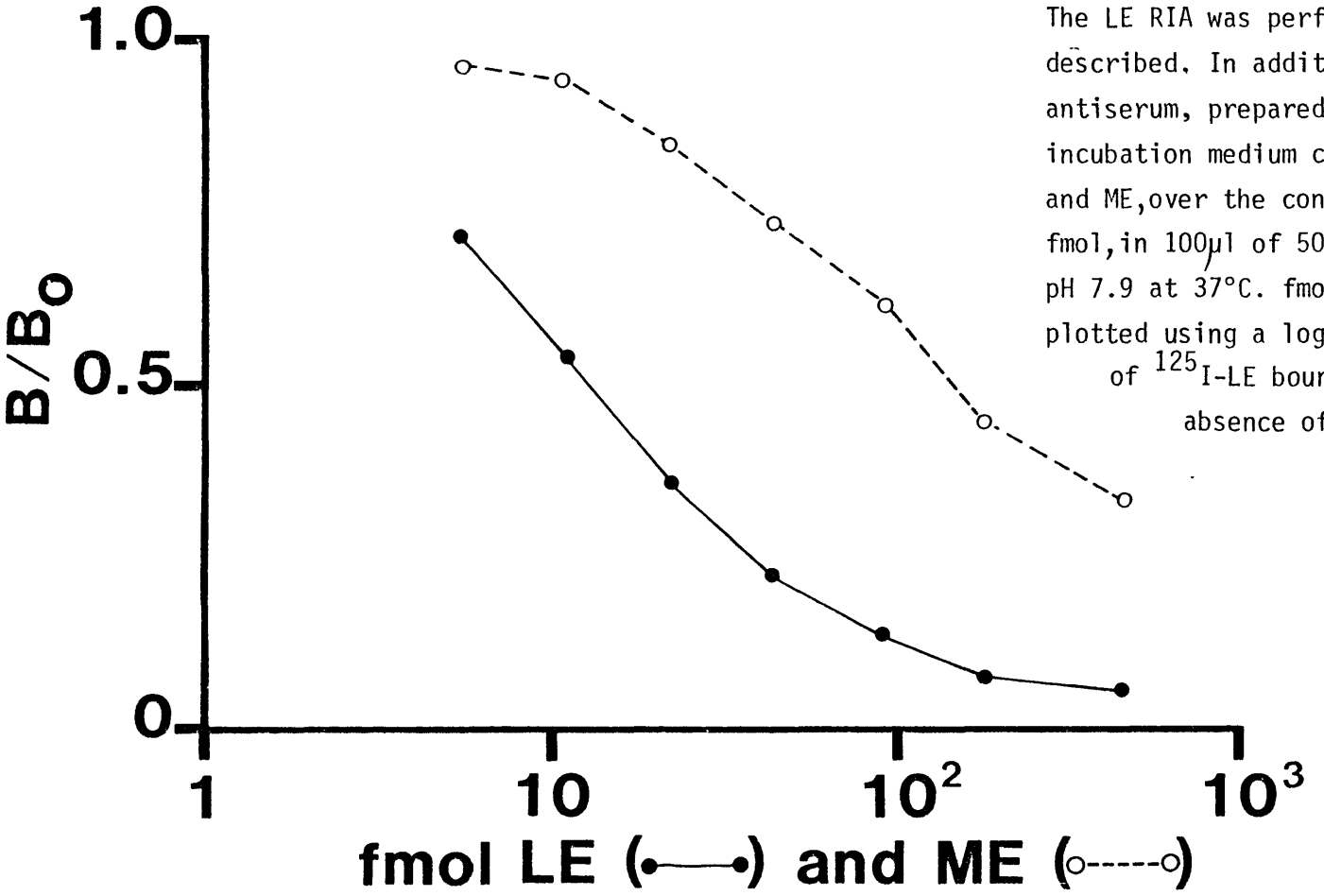
a) RIA buffer (———).

b) 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C (------).

c) 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C containing 1M urea (····· ·····).

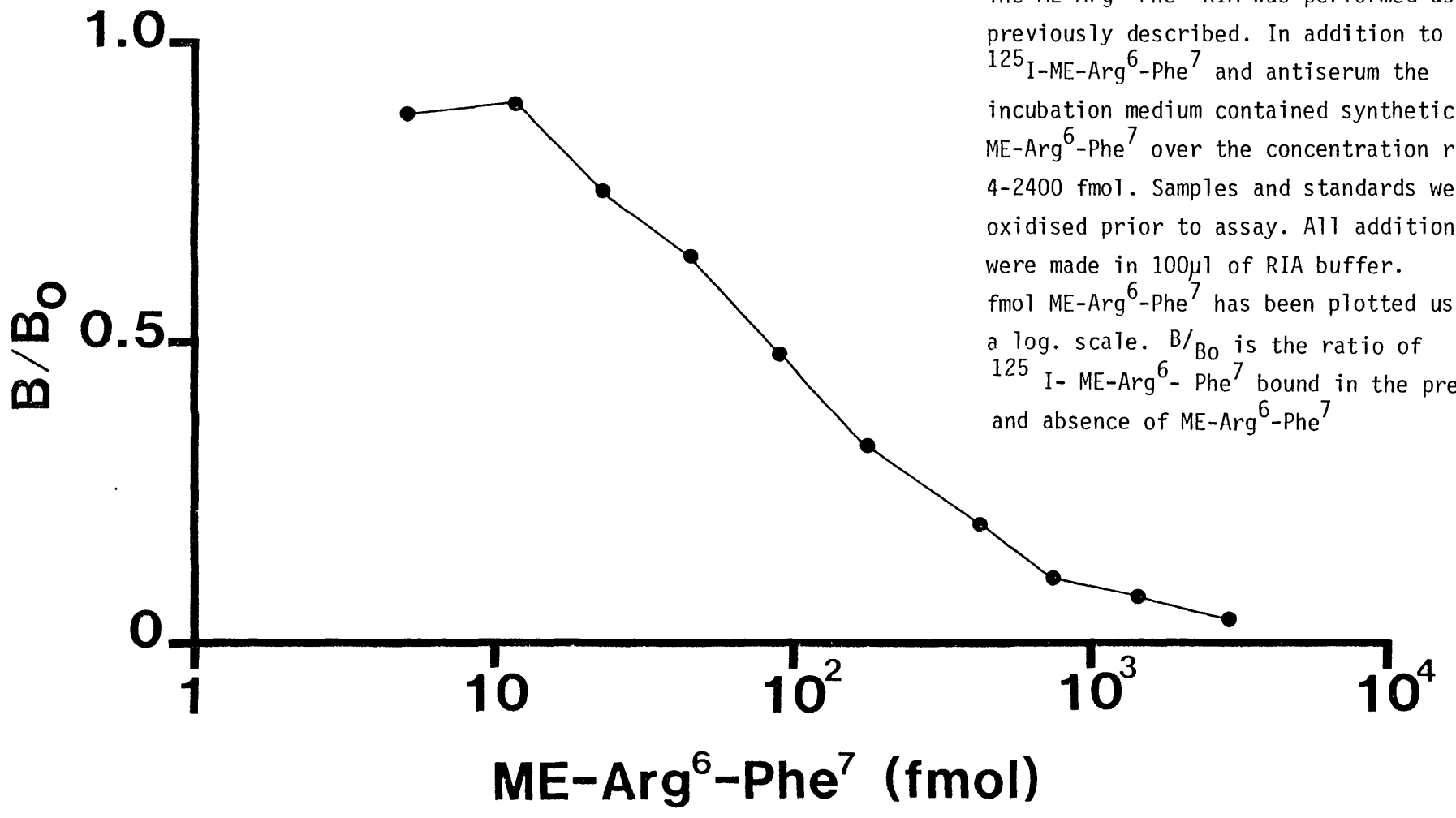
fmol LE has been plotted using a log. scale. $^{B}/_{Bo}$ = ratio of ^{125}I-LE bound in the presence and abscence of LE

FIG. 9 . LE RIA DISPLACEMENT CURVE : CROSS-REACTIVITY WITH ME.



The LE RIA was performed as previously described. In addition to the ^{125}I-LE and antiserum, prepared in RIA buffer, the incubation medium contained synthetic LE and ME, over the concentration range 10-350 fmol, in 100µl of 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C. fmol ME and LE have been plotted using a log. scale. B/B_o is the ratio of ^{125}I-LE bound in the presence and absence of synthetic LE and ME.

FIG. 10 . ME-ARG^6-PHE^7 RIA DISPLACEMENT CURVE.



The ME-Arg^6-Phe^7 RIA was performed as previously described. In addition to the ^{125}I-ME-Arg^6-Phe^7 and antiserum the incubation medium contained synthetic ME-Arg^6-Phe^7 over the concentration range 4-2400 fmol. Samples and standards were oxidised prior to assay. All additions were made in 100µl of RIA buffer. fmol ME-Arg^6-Phe^7 has been plotted using a log. scale. $B/_{Bo}$ is the ratio of ^{125}I- ME-Arg^6- Phe^7 bound in the presence and absence of ME-Arg^6-Phe^7

The ME-Arg^6-Phe^7 antiserum cross-reacted (on a molar basis) 100% with an oxidised form of ME-Arg^6-Phe^7 and less than 0.1% with ME, LE, ME-Arg^6, ME-Arg^6-Arg^7, ME-Lys^6, DYN-(1-17), α-neoendorphin, β-neoendorphin and BAM 22P (Giraud et al., 1983).

3.3 Mouse vas deferens bioassay

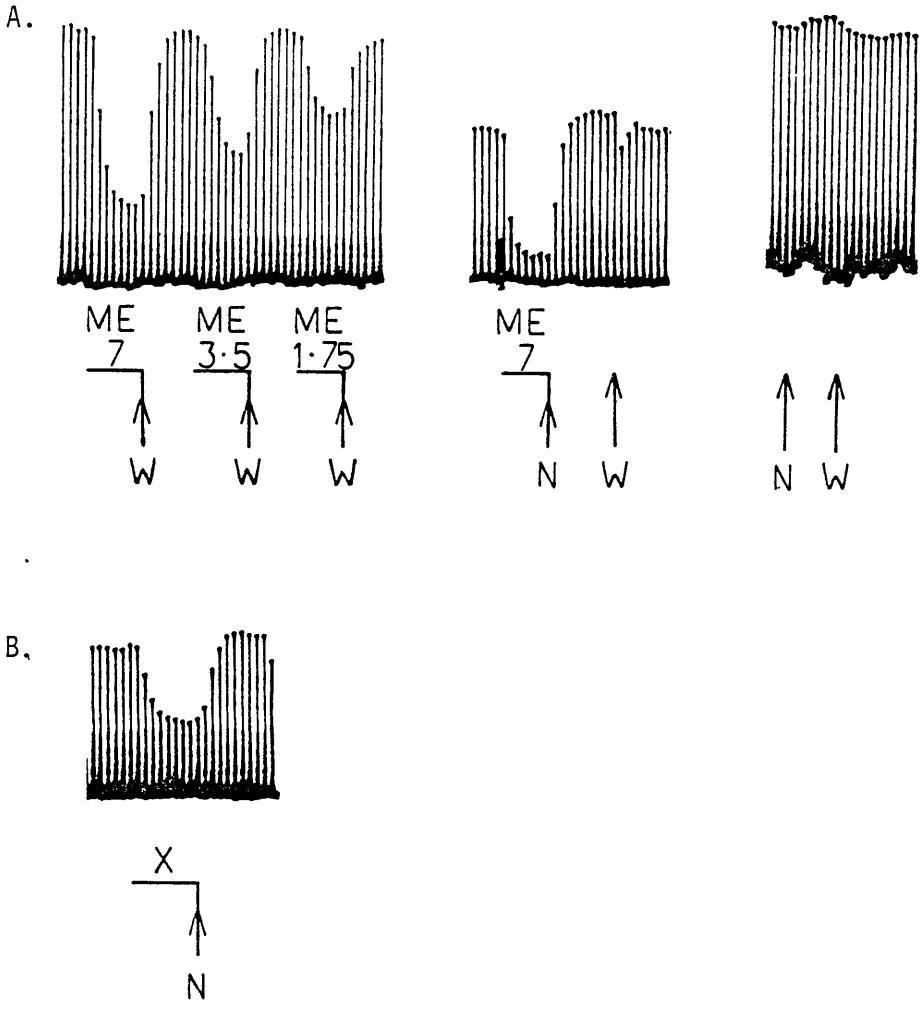
ME inhibited the electrically induced contractions of the mouse vas deferens in a dose-dependent manner and this inhibition was completely reversed by 900 nM naloxone, Fig. 11A. This figure also shows a typical twitch depression produced by the addition of an unknown test substance(s) containing opioid activity to the tissue bath, Fig. 11B. This inhibitory response was also blocked and reversed by 900 nM naloxone. Application of this concentration of naloxone alone had no observable effect on the tissue preparation, Fig. 11A.

Typical control dose-response curves to ME are shown in Fig. 12. The inclusion of 2mM L-leucyl-L-leucine in the Krebs solution lowered the IC_{50} value for ME; in this case the value was reduced five fold from 15.5 nM to 3.5 nM.

When control dose-response curves were repeated on the same tissue, during the course of an experiment, there was no significant alteration in the sensitivity of the tissue to ME, Table 6. The mean IC_{50} values obtained varied between tissues, but were always of the same order of magnitude.

FIG. 11 .

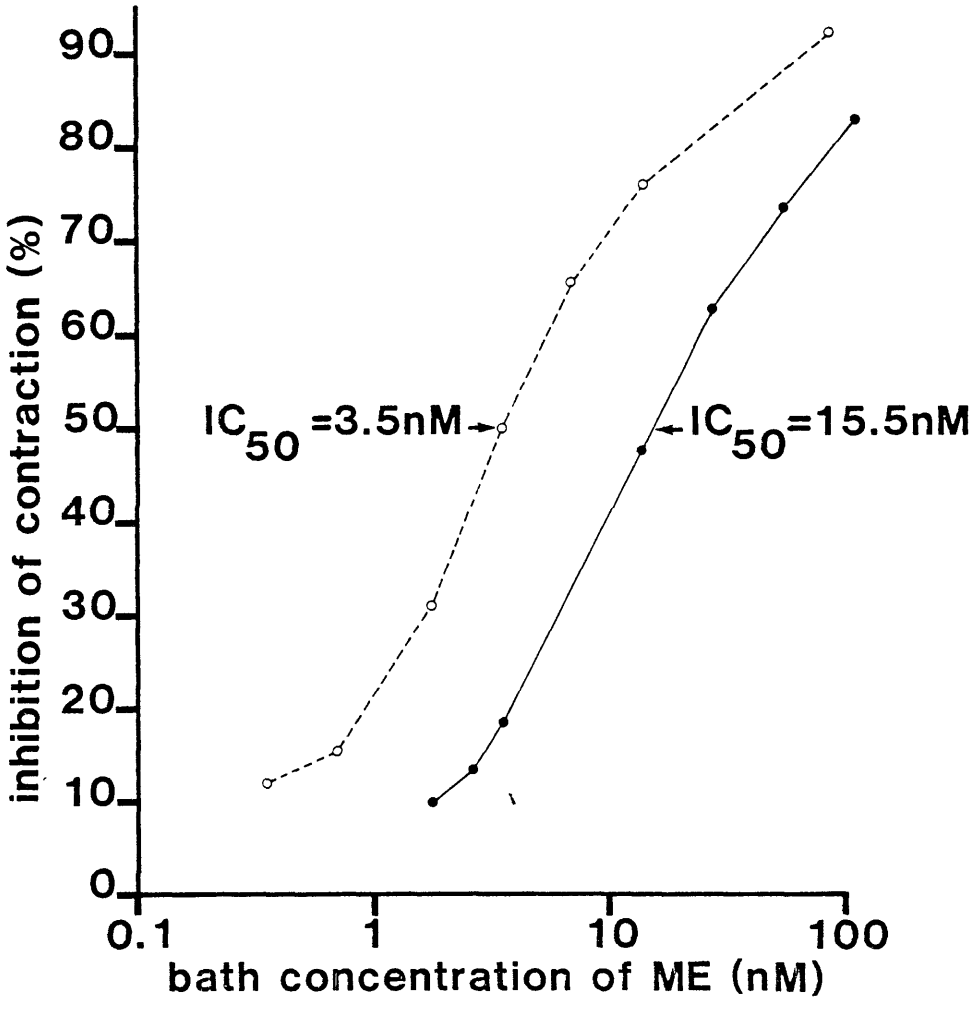
RESPONSE OF THE MVD BIOASSAY TO ME AND TEST SUBSTANCES.



The MVD bioassay was performed as previously described. Tissues were perfused with oxygenated Krebs solution containing 2mM L-leucyl-L-leucine. Trace A shows (right to left) graded control inhibitory responses to ME at bath concentrations of 1.75, 3.5 and 7.0 nM, the reversal of this inhibition by naloxone (N=900nM) and the effect of naloxone alone on the tissue preparation. Trace B shows the typical response to an unknown test substance (X) which was also reversed by naloxone. Ideally the test substance was added in order to produce a 50% depression of the twitch. W = washing by Krebs perfusion.

FIG.12

THE ABILITY OF ME TO INHIBIT THE ELECTRICALLY INDUCED CONTRACTIONS OF THE MOUSE VAS DEFERENS IN THE PRESENCE AND ABSENCE OF L-LEUCYL-L-LEUCINE.



The MVD bioassay was performed as previously described. Single doses of ME were injected into the organ bath to give a concentration range over 1-100 nM. Dose-response curves were constructed in the presence (o---o) and absence (•——•) of 2mM L-leucyl-L-leucine.

Bath concentration of ME (nM) has been plotted using a log. scale.

TABLE 6

THE EFFECT OF REPEATED ME DOSE-RESPONSE CURVES ON TISSUE SENSITIVITY

	MEAN IC_{50} ± SEM
KREBS SOLUTION	15.5 ± 2.95nM (n=10)
KREBS SOLUTION plus 2mM L-LEUCYL-L-LEUCINE	4.72 ± 1.27nM (n=11)

The M V D bioassay was performed as previously described. Tissues were perfused with oxygenated Krebs solution in the presence and absence of 2mM L-Leucyl-L-Leucine. Single doses of ME were injected into the bath in order to construct a dose-response curve over the range 1-100nM. Dose-response curves were repeated on the same tissue. The mean ± SEM value was calculated for a single tissue. n = number of dose-response curves / tissue.

3.4. Isoelectricfocusing

3.4.1 The effect of high urea concentration on IEF

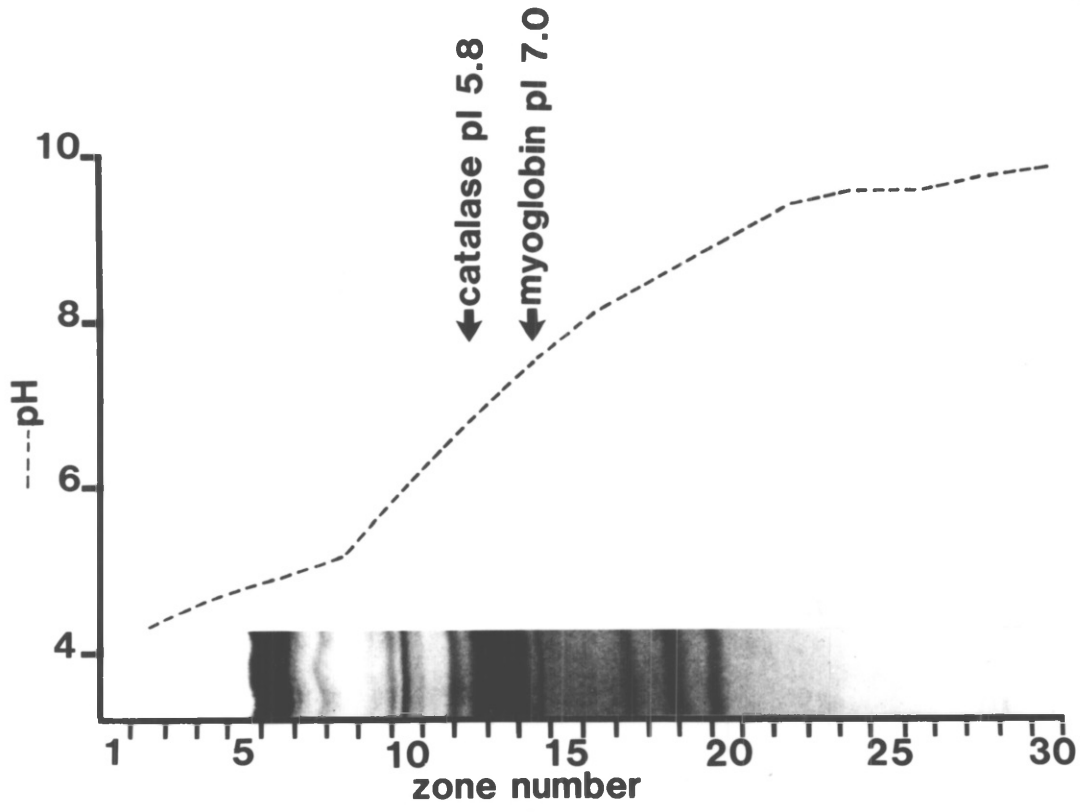
The inclusion of 6M urea and 50mM Tris-HCl pH 8.0 at 20°C in the 4% (w/v) Ultradex gels used for IEF was validated by electrofocusing known proteins as described in Fig. 13. Formation of the pH gradient was satisfactory at this concentration of salt and urea.

Visualisation of the protein standards, focused within the gel, by Coomassie blue staining of a paper print, indicated good resolution of these species at a protein concentration of 10 mg/ml of initial gel volume. This loading was comparable to the gel capacity of 5-10 mg/ml for complex protein mixtures. The protein standards were resolved into many bands demonstrating that these proteins were not chromatographically pure. Therefore pI values could only be estimated for myoglobin and catalase, which were present as strongly coloured bands before staining, at pH 7.9 and pH 6.8 respectively, as compared with their literature pI values of 7.0 and pH 5.8. This demonstrates that unknown samples subjected to IEF, in the presence of urea, may equilibrate at a pH which is different to their pI value.

3.4.2 Removal of Ampholine using mixed bed ion exchange chromatography

When samples containing a known amount of bioassayable opioid activity were passed over mixed bed ion exchanger the bioassayable opioid activity was no longer recoverable Table 7. In contrast, the recovery from columns pretreated with an excess of bovine serum albumin was 84%. The sample emerged as a single peak in the void volume (6 ml) of the column and no other peak emerged within the 40 ml column volume

FIG.13 THE EFFECT OF 6M UREA ON ISOELECTRICFOCUSING.



The following protein standards (200 mg/ protein) were slurried in a 4% (w/v) Ultradex gel containing 50mM Tris-HCl pH 8.0 at 20°C, 6M urea and 2% Ampholine pH 3.5-10: myoglobin, catalase, ovalbumin, thyroglobulin and chymotrypsin. IEF was conducted at 8W for 16h at 10°C; pH gradient (------); protein bands visualised by Coomassie blue staining.

TABLE 7

REMOVAL OF AMPHOLINE BY MIXED BED ION EXCHANGE CHROMATOGRAPHY

	AG-501-X8(D) RESIN			
	WITHOUT BSA PRETREATMENT		WITH BSA PRETREATMENT	
	PRE COLUMN	POST COLUMN	PRE COLUMN	POST COLUMN
Bioassayable opioid activity in pmol ME equivalents	40	not-detectable	40	33.6
Percentage recovery		0		84

Guinea pig striata were extracted in 5% acetic acid-HCl pH 1.9 prior to protein precipitation with 15% saturated ammonium sulphate.The precipitate was pelleted by centrifugation (50000 g_{av} for 20 min at 4°C) and then resuspended in 50mM Tris-HCl/2mM $CaCl_2$/8M urea pH 8.0 at 20°C by sonication on ice. Bioassayable opioid activity was then estimated using the MVD bioassay following digestion with trypsin and carboxypeptidase B as previously described. Aliquots corresponding to 40 pmol ME equivalents were then diluted to a final volume of 3.5 ml containing 12.5mM Tris, 2M urea and 2% (v/v) Ampholine pH 7-11. Samples were passed over AG-501-X8(D), in the presence and absence of a column pretreatment with 10 mg bovine serum albumin (BSA). The void volume peak was collected, adjusted to 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C, digested with trypsin and carboxypeptidase B and analysed for bioassayable opioid activity. The chosen concentration of Ampholine represented the maximum that could be eluted from an IEF gel.

monitored.

3.5 Discussion : detection of opioid-containing peptides

3.5.1 Enzymatic digestion

It is now generally accepted that biologically active peptides are derived from large precursor molecules by post-translational processing and that, in the majority of cases, cleavage is directed towards the paired basic residues bracketing the active sequences. Steiner, (1976) proposed a scheme for the processing of precursor to active peptide(s) which required trypsin-like activity to cleave the paired basic residues, followed by carboxypeptidase B-like activity to specifically remove the remaining COOH-terminal basic amino acid(s). This followed pioneering work which showed that insulin, C peptide and four basic amino acids could be released in a stoichiometric ratio from proinsulin by digestion with trypsin and carboxypeptidase B (Kemmler et al., 1971). This approach was used successfully to liberate opioid peptides from higher molecular weight forms which were themselves inactive as opioid ligands (Hughes et al., 1980).

This investigation has used this classical method of sequential digestion by trypsin and carboxypeptidase B, followed by *in vitro* assay for opioid activity, to detect putative opioid precursors. Since the two stage enzymatic treatment formed an essential step in the assay for opioid-containing peptides it was important to verify that the enzymes were active in the range of digestion buffers. Enzyme activity was assessed by the rate of catalysed hydrolysis of a synthetic substrate. Although the action of an enzyme on a synthetic substrate could not be extrapolated to judge its efficiency in digesting an unknown protein

complex, this strategy demonstrated whether an enzyme was capable of catalysis under certain conditions and provided guide lines for experimental design.

Factors such as the conformation and concentration of the different protein molecules within a particular substrate complex could affect the efficiency of the enzymes in liberating opioid peptides from their precursors (Geisow, 1978). Therefore, ideally, to ensure the maximum yield of opioid peptide products from a particular protein complex it would be necessary to determine precise digestion conditions, in terms of enzyme concentration, choice of buffer and incubation time, etc. for every type of substrate used experimentally. Even in such a thorough investigation one would need to assume the impossible, that every tissue extraction and subsequent procedure resulted in an identical protein pool. Therefore, it has to be accepted that this method of liberating opioid peptides cannot be considered reproducible or quantitative.

3.5.2 Trypsin activity

The inclusion of 2mM $CaCl_2$ in the incubation medium was essential for prolonged trypsin activity. This corroborates the observation that calcium ions retard the autolysis of trypsin. The calcium ions are thought to induce a conformation change which results in a more compact enzyme structure (Walsh and Wilcox, 1970).

Increasing concentrations of urea in the incubation medium decreased trypsin activity. In 8M urea the enzyme was completely inhibited. Urea is a denaturing agent which causes a reversible disruption of the non-covalent forces determining tertiary protein

structure and stabilises a more random coil formation for these molecules. Urea causes denaturation of the trypsin molecule, disrupting the correct conformation for catalytic activity and inactivating the enzyme. As the concentration of urea increases, the degree of denaturation increases and catalysis is reduced. Under conditions where a percentage of trypsin remains in the active form enzyme activity is irreversibly lost as the denatured form is digested (Delaage and Lazdunski, 1968). Urea could also denature the substrate, altering the conformation of the cleavage site, which could either enhance or reduce catalysis depending on the ability of the enzyme to interact with the new spatial arrangement.

The presence of Ampholine in the incubation mixture also reduced trypsin activity. Ampholine is a mixture of a large number of polyamino-polycarboxylic acids, with different pI's in the range 2.5-11. Presumably certain of these species bind to either the enzyme or substrate, causing a reduction in the affinity of the enzyme for the substrate. The mechanism for this inhibitory effect is not clearly understood, but is probably extremely complex since it involves many free reactants.

3.5.3 Carboxypeptidase B activity

The reduction of carboxypeptidase B activity by 50% in the presence of 2M urea was probably also due to denaturation of the enzyme. Urea has a more marked effect on this enzyme than on trypsin.

3.5.4 Enzyme specificity

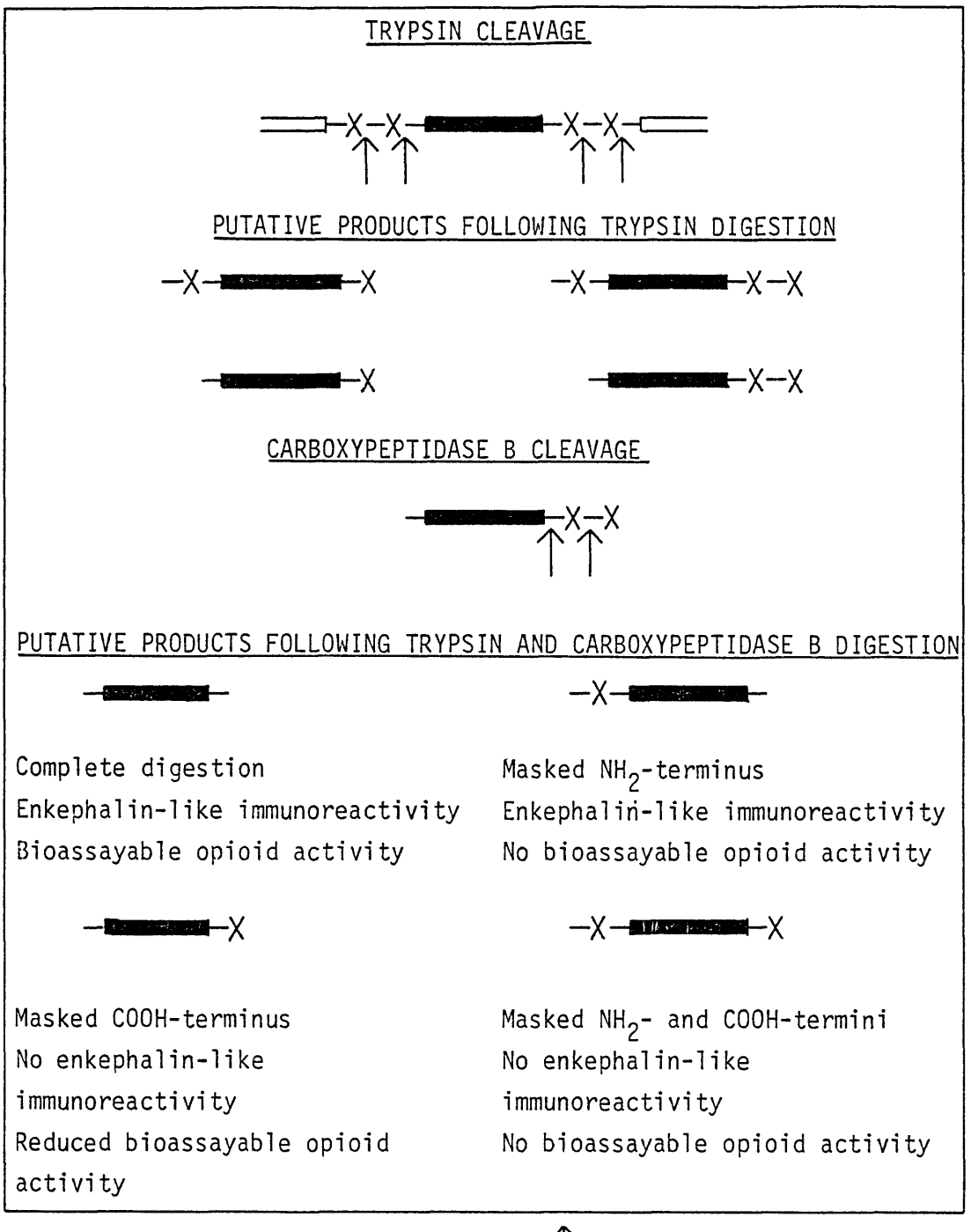
Trypsin hydrolyses peptides at bonds involving the carboxyl group

of L-arginine or L-lysine. The exact sequence of trypsin cleavages involving pairs of basic residues is unknown. Therefore, in a typical prohormone, where the biologically active peptide(s) is sandwiched between two pairs of basic residues, the enzyme can potentially cleave at four different sites Fig. 14. Following trypsin digestion, carboxypeptidase B specifically removes basic amino acids only from the COOH terminus of the peptide.

Trypsin and carboxypeptidase B treatment of proenkephalin is assumed to liberate all the internal ME and LE sequences. However, if one action of trypsin is to cleave between the pair of basic residues at the NH_2-terminus of certain molecules and both basic amino acids are not removed, the digestion procedure will not be efficient in exposing the NH_2-terminal enkephalin sequence essential for opioid activity on the MVD bioassay. This would result in an underestimation of the opioid activity within a particular sample. Under circumstances where trypsin did not remove both NH_2 basic amino acids but carboxypeptidase removed both COOH-terminal basic residues, the hexapeptide Arg^{-1}-ME would be the major product liberated by sequential digestion of proenkephalin. In contrast to the MVD bioassay, the ME RIA could still recognise the ME sequences since the COOH-terminally directed ME antiserum used in this study has 60 % cross-reactivity with Arg^{-1}-ME.

In the situation where trypsin cleaves both basic amino acids from the NH_2-terminus, leaving either one or two still present at the COOH-terminus, the extent of enkephalin release is determined by the action of carboxypeptidase B. If carboxypeptidase B does not complete the removal of both basic residues the resulting hexa- and heptapeptides retain bioassayable opioid activity, although in all cases the IC_{50} value is higher than for the enkephalins (McKnight et

FIG. 14. LIBERATION OF ENKEPHALIN FROM OPIOID CONTAINING PEPTIDES BY SEQUENTIAL DIGESTION WITH TRYPSIN AND CARBOXYPEPTIDASE B.



Abbreviations: X = basic amino acid; ↑ = possible cleavage site; ▬ = enkephalin sequence; ▭ = peptide spacer region.

This scheme outlines the possible cleavage products following digestion of an opioid-containing peptide with trypsin and carboxypeptidase B.

al., 1983). In this case the ME antiserum would not recognize the enkephalin related peptides as it shows only 4% cross-reactivity with ME-Arg^6 and 7% cross-reactivity with ME-Lys^6 (Liston and Rossier, 1984a).

Contamination of carboxypeptidase B by other peptidases could cause degradation of enkephalin, producing fragments displaying neither immunoreactivity nor bioassayable opioid activity. The carboxypeptidase B used in these experiments did not appear to contain carboxypeptidase A activity, under the experimental conditions, but other peptidase activity was not tested.

There is little precise information on the ratio of enkephalin congeners produced by sequential digestion with trypsin and carboxypeptidase B. Kilpatrick et al., (1982a) detected the presence of ME-Lys^6, ME-Arg^6, Lys^{-1}-ME-Lys^6 and Arg^{-1}-ME-Lys^6-Lys^7 in a tryptic digestion of the 18.2 kDalton adrenal proenkephalin species, suggesting that trypsin may leave a masked NH_2-terminus.

Other strategies have been developed to reduce the quantitation errors intrinsic to the sequential digestion. Several groups have identified enkephalin precursors by RIA following digestion with trypsin alone, using specific antiserum raised against ME-Arg^6 (Cupo et al., 1984; Giraud et al., 1984b). However, the assumption that this hexapeptide was obtained specifically and quantitatively from proenkephalin species by tryptic hydrolysis was not supported by Prado et al., (1983) who found that trypsin released ME-Arg^6 and ME-Arg^6-Arg^7 from the synthetic substrate BAM 22P in a ratio of 3:1 respectively.

Another approach has been to employ direct RIA using antisera which recognise higher molecular weight forms of the antigen. Anti-peptide F serum recognised proenkephalin intermediates in both brain and adrenal medulla (Hollt et al., 1982a). Similarly, an antiserum raised against the purified 8.6 kDalton precursor from bovine adrenal medulla, which recognises the proenkephalin-(1-70) determinant, detected all known precursor forms containing this sequence in this tissue (Liston et al., 1983). Recently Christie et al., (1984), using synthetic proenkephalin-(95-117) as the antigen, have produced an antiserum which recognises adrenal medullary proenkephalin species.

In this study, the use of both RIA and MVD bioassay to detect enkephalin-like immunoreactivity and bioassayable opioid activity, assay methods directed against both the NH_2- and COOH-termini of enkephalin, provided a guide to the efficiency of the digestion and confirmed that the immunoreactive material contained opioid activity.

3.5.5 Mouse vas deferens bioassay

In the MVD bioassay, opioid agonists depress the output of noradrenalin released by electrical stimulation, producing an inhibition of the tissue twitch (Henderson et al., 1972). McKnight et al., (1983) found that the potencies of rapidly degradable opioid peptides in isolated tissue preparations were increased following the application of peptidase inhibitors. This effect did not occur with synthetic metabolically stable opioid peptide analogues, suggesting potentiation was due to the inhibition of degrading enzymes. The most effective combination of inhibitors was found to be bestatin, thiorphan, captopril and L-leucyl-L-leucine. Bestatin inhibits the aminopeptidases which cleave the Tyr^1-Gly^2 bond of enkephalin, whilst

thiorphan and captopril are specific inhibitors of "enkephalinase" (endopeptidase 24.11.)and angiotensin-converting enzyme respectively, which cleave the Gly^3-Phe^4 bond within enkephalin (Schwartz, 1983). The mechanism of inhibition by L-leucyl-L-leucine is less clear but may be substrate competition as other dipeptides have been shown to inhibit degradation of ME and LE when added to brain homogenate and striatal slice incubations. Moreover, this inhibition decreased with time (Henderson et al., 1978).

In this study L-leucyl-L-leucine was incorporated into the Krebs solution in later experiments. The remaining inhibitors were unavailable in sufficient quantities. However L-leucyl-L-leucine (2mM) was shown to produce an increase in ME potency, on the MVD bioassay, of the same order as that produced by the complete inhibitor mixture ($ME\text{-}IC_{50}$ values; without inhibitors 17.7 $\pm$ 2.37nM, with L-leucyl-L-leucine 2.74 $\pm$ 0.35nM and with the complete inhibitor mixture 1.45 $\pm$ 0.18nM). Addition of the dipeptide in our system produced a reduction in the $ME\text{-}IC_{50}$ value commiserate with that found by McKnight et al., (1983).

Agonist activity has been defined as the concentration of test substance required to inhibit the tissue response by 50% (IC_{50}). Therefore, as far as possible, unknown samples were tested at concentrations corresponding to their IC_{50} values, where quantitation is most reliable. As previously stated, unknown samples were classified as containing opioid activity only if the inhibitory response was reversed by 900 nM naloxone. Samples analysed by MVD bioassay were assayed against a synthetic ME standard and the results have been expressed in pmol ME equivalents. Since LE is more potent than ME on the MVD bioassay (Lord et al., 1977) values expressed in this way would

be artificially high for samples containing LE. This discrepancy is reduced in the presence of inhibitors when LE and ME are equipotent (McKnight et al., 1983). These values also assumed that all the opioid activity present in test samples was due to enkephalin, if another opioid peptide(s) had been present estimations could be even more inaccurate.

Finally, the opioid activity within a sample would also have been underestimated by the MVD bioassay if a percentage of the ME present had been oxidised to the sulphoxide form as this is inactive on the MVD bioassay (J. Hughes, personal communication)

CHAPTER FOUR

GEL FILTRATION CHROMATOGRAPHY

OF TISSUE EXTRACTS

Chapter four

Gel filtration chromatography of tissue extracts

4.1 Introduction

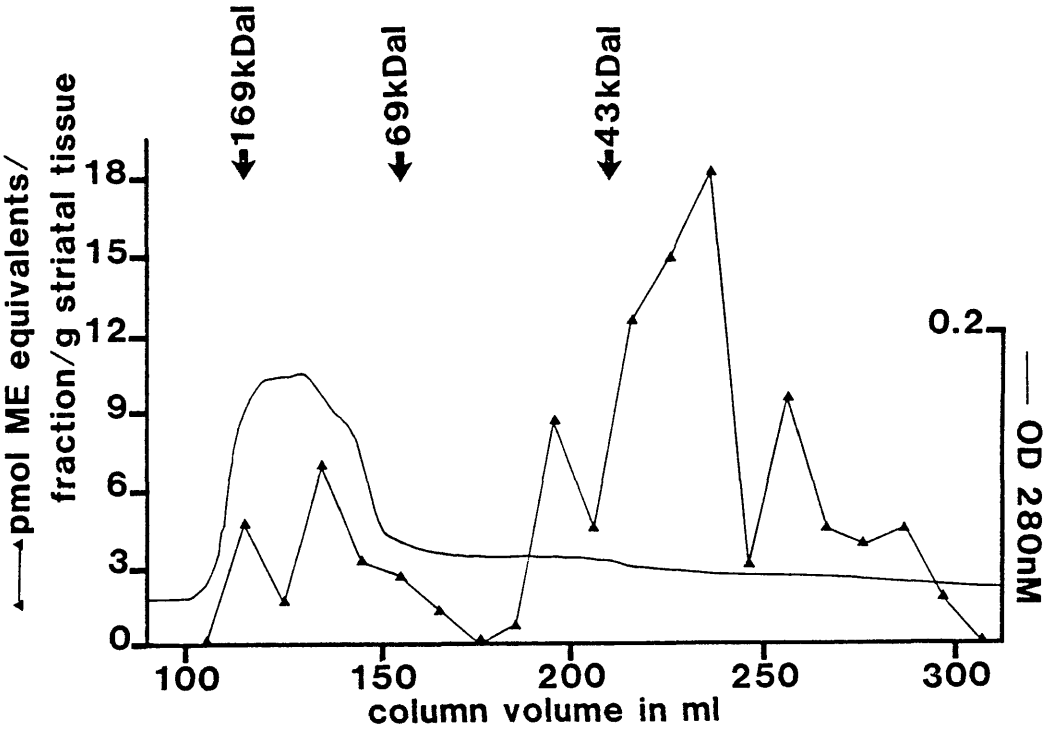
Initial studies using Sephadex G-200 gel filtration chromatography confirmed the presence of putative opioid precursors in guinea pig striatum. Sephacryl-300 chromatography was found to provide better resolution of these species and was employed to compare the opioid-containing peptides present in guinea pig, rat and bovine striata and guinea pig adrenal extracts. The effect of extraction procedure and post-mortem changes on the levels of putative opioid precursors in guinea pig striatum was also studied.

4.2 Preliminary experiments

4.2.1. Sephadex G-200 chromatography of guinea pig striatal extract

When a Sephadex G-200 separation of guinea pig striatal extract was analysed for opioid peptide content the profile of bioassayable opioid activity, liberated after sequential digestion with trypsin and carboxypeptidase B, was composed of two main regions ; a high molecular weight peak eluting over the apparent molecular weight (M_r) range 60000-90000 and a low molecular weight region eluting over the apparent M_r range 30000-50000, Fig. 15. The profile suggested that both these regions were composed of several species of opioid-containing peptides. Control incubations with boiled enzymes showed no bioassayable activity throughout the eluate.

FIG.15 . G-200 CHROMATOGRAPHY OF GUINEA PIG STRIATAL EXTRACT.



Guinea pig striatal tissue was extracted in 1M acetic acid-HCl pH 1.9 and protein was precipitated with 15% saturated $(NH_4)_2SO_4$. The precipitated material was resuspended in acidified urea and chromatographed over Sephadex G-200 (2.5 x 76 cm) in 1M acetic acid pH 2.1 at a flow rate of 12ml / h at 4°C. Eluate fractions were lyophilised by freeze-drying and resuspended in 50mM Tris-HCl / 2mM $CaCl_2$ pH 7.9 at 37°C. Samples were then digested with trypsin (50 µg / ml for 18h at 37°C) followed by centrifugation at 50000 g_{av} for 15 min at 4°C. Supernatants were retained for carboxypeptidase B treatment and pelleted material was resuspended in the Tris-HCl buffer and redigested with trypsin under the same conditions. Following the second trypsin digestion the supernatants were combined and the suspension passed over Porapak-Q prior to carboxypeptidase B digestion (10 µg / mlfor 1h at 37°C). Opioid peptide content was estimated using the MVD bioassay; pmol ME equivalents/ g striatal tissue (▲——▲);absorbance at 280 nm (———); molecular weight markers: aldolase (169 kDal.), bovine serum albumin (69 kDal.) and ovalbumin (43 kDal.).

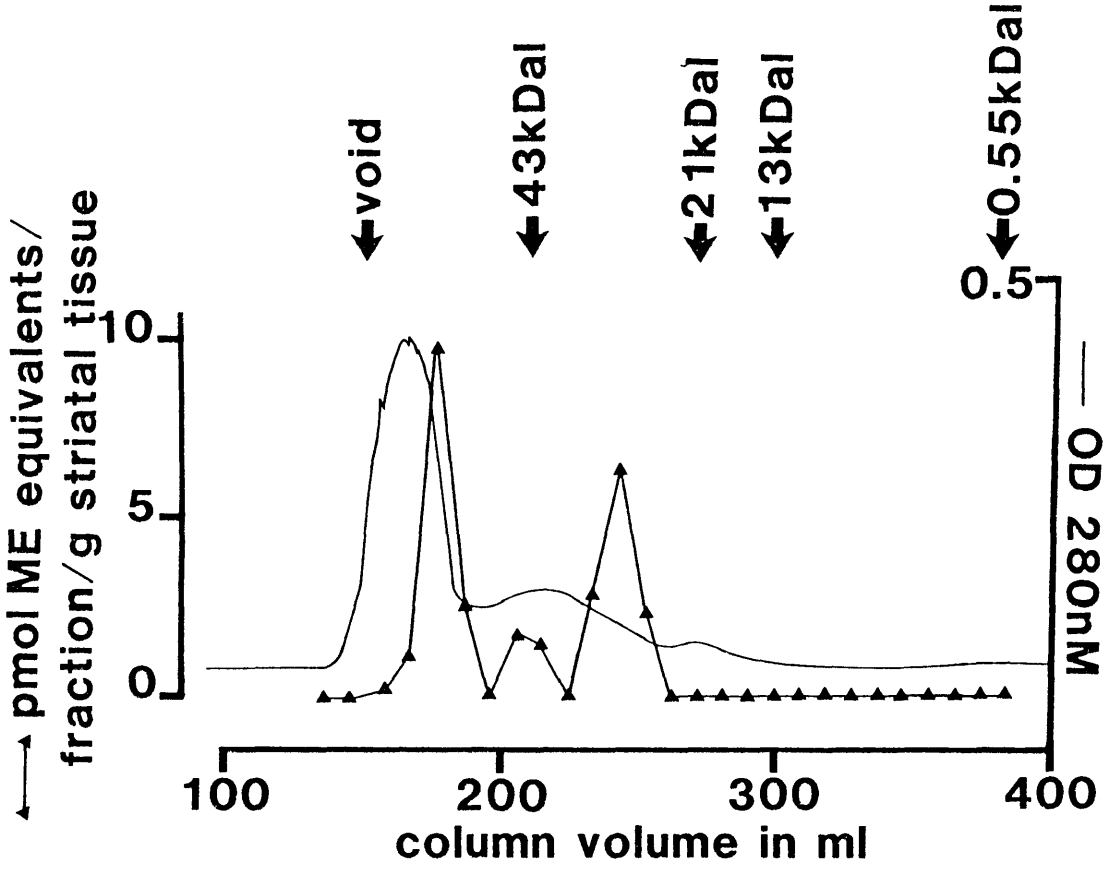
4.2.2 Sephacryl-300 chromatography of the high molecular weight peak separated over G-200

The high molecular peak of opioid-containing peptides (apparent M_r 60000-90000) separated by Sephadex G-200 gel filtration of guinea pig striatal tissue was rechromatographed over Sephacryl-300. Column fractions digested by trypsin and carboxypeptidase B yielded three regions of bioassayable opioid activity eluting with apparent M_r's of > 70000, 43000 and 29000, demonstrating that the 60-90 kDalton region from Sephadex G-200 chromatography contained more than one species of opioid-containing peptide, Fig. 16.

4.2.3 Discussion

Gel filtration chromatography was employed as the first stage in resolving opioid-containing peptides extracted from striatal tissue. Initial separations were effected over Sephadex G-200 using acetic acid as the extraction and elution medium. Ammonium sulphate precipitation was used to separate putative opioid precursors from the peptide fraction before application to the column (Chabrier et al., 1982). Digestion of column fractions followed by MVD bioassay revealed two regions of opioid-containing peptides in the apparent M_r ranges 90000-60000 and 50000-30000. Bioassayable opioid activity and u.v. absorbance followed similiar elution profiles indicating a poor degree of resolution and purification. Therefore, it was decided to investigate another gel filtration medium, Sephacryl-300, in order to improve the separation of opioid-containing peptides. As an initial experiment the high molecular weight region separated over G-200 was rechromatographed over Sephacryl-300, in a running buffer of 50 mM Tris-HCl pH 8.0 at 20°C containing 8 M urea.

FIG. 16 . SEPHACRYL-300 CHROMATOGRAPHY OF THE HIGH MOLECULAR WEIGHT REGION SEPARATED OVER SEPHADEX G-200



Guinea pig striatal extract was separated over Sephadex G-200 as described in Fig.15 . Fractions corresponding to the high M_r region (60-90 kDal.) were pooled and protein was precipitated with 15% saturated ammonium sulphate. The precipitate was resuspended in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and chromatographed over Sephacryl-300 in the same buffer, as described in Fig, . Eluted fractions were diluted to a final urea concentration of 2M with 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C and digested with trypsin followed by carboxypeptidase B. Opioid peptide content was estimated using the MVD bioassay. pmol ME equivalents / g striatal tissue (▲——▲); absorbance at 280 nm (———). Molecular weight markers: blue dextran (void), ovalbumin (43 kDal.), soy bean trypsin inhibitor (21 kDal.), cytochrome c (13 kDal.) and enkephalin (0.55 kDal.)

Sephacryl-300 chromatography resolved opioid-containing peptides contained within the G-200 high molecular weight region into three peaks, eluting with apparent M_r's of > 70000, 43000 and 29000, confirming the presence of several putative opioid precursors in this fraction. These apparent M_r's, estimated after Sephacryl-300, chromatography were lower than those previously observed following a Sephadex G-200 separation. There are several possible explanations for both the improved resolution and change in M_r obtained with Sephacryl-300. This column had a better theoretical resolving ability in the M_r range of interest (20000-60000) when run in 8M urea. The Sephacryl-300 column was 10 cm longer, with an increase of 18% in bed volume, as compared with the Sephadex G-200 column, which would improve resolution. Sephacryl-300 chromatography was conducted in the presence of 8 M urea, when proteins are judged to be in a denatured state resulting in the dissociation of proteins bound by non-covalent forces. This reduced the possibility that the opioid-containing peptides, eluting with high apparent M_r's, were agregates of smaller moieties. Apparent high M_r's obtained over Sephadex G-200 may have been due to aggregation in non-denaturing conditions.

These results indicated that, at least over the molecular weight region of interest, Sephacryl-300 provided a better resolution of opioid-containing species than Sephadex G-200. It was determined to investigate the complexity of the complete striatal tissue opioid precursor and peptide pool using this chromatographic technique. For this purpose a tissue extraction medium of 50mM Tris-HCl pH 8.0 at 20°C, containing 8M urea, was chosen. This medium ensured non-denaturing conditions throughout the initial stages of tissue preparation, minimizing non-covalent protein aggregation. Both tissue

extraction and Sephacryl-300 chromatography could be performed in the same medium, avoiding the possibility of precipitating material when changing buffering conditions. It was felt that an 8M urea extraction would give a maximum yield of opioid-containing material, as this is a very effective solubilising reagent.

4.3 Results

4.3.1 Sephacryl-300 chromatography of guinea pig striatal extract

When a Sephacryl-300 separation of guinea pig striata, extracted in 8M urea, was analysed for opioid peptide content by direct RIA both ME-like immunoreactivity (ME-IR) and LE-like immunoreactivity (LE-IR) were detected eluting almost exclusively as a single peak in the total column volume, Fig. 17A. The elution position corresponded to that of synthetic enkephalin and represented material with an apparent M_r of < 2000. The ratio of ME-IR to LE-IR in this peak was 4.8:1 as compared to the ratio of 4.3:1 for these peptides estimated from an aliquot prior to chromatography. The recoveries after Sephacryl-300 separation were 154% and 138% for ME-IR and LE-IR respectively, Table 8.

Following treatment by trypsin and carboxypeptidase B, fractions were analysed for opioid peptide content using both MVD bioassay and RIA for ME and LE. In addition to a large peak eluting in the total column volume (M_r < 2000) four distinct regions of bioassayable naloxone reversible opioid activity were detected, corresponding to opioid-containing peptides with apparent M_r's of > 70000 (Peak I), 66000 (Peak II), 29000 (Peak III) and 16000-6000 (Peak IV), Fig. 17B. A similiar profile was obtained when aliquots of the same fractions were assayed for ME-IR and LE-IR, with the exception that little, or no,

FIG.17

SEPHACRYL-300 CHROMATOGRAPHY OF GUINEA PIG STRIATAL EXTRACT

Guinea pig striata were extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and samples (5ml) were chromatographed over Sephacryl-300 (2.6 x 86 cm) in the same buffer. Elution was conducted at 13.5 ml / h at room temperature and fractions were analysed for opioid peptide content as follows;

FIG. A. Aliquots were assayed by direct RIA for ME-IR (●——●) and LE-IR (○——○). For this purpose samples were diluted ten fold in RIA buffer giving a final concentration of 270mM urea in the incubation volume (300µl).

FIG. B and C. Residual column fractions were diluted with 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$ to a final urea concentration of 2M and digested with trypsin followed by carboxypeptidase B. Digested fractions were concentrated over Porapak-Q. The resulting methanol eluates were divided into 2 fractions and evaporated to dryness under vacuum. Fractions were then either resuspended in Krebs solution and analysed for opioid activity using the MVD bioassay (▲——▲), FIG. B. or resuspended in RIA buffer and assayed by RIA for ME-IR (●——●) and LE-IR (○——○), FIG. C.

Bioassayable opioid activity is expressed in pmol ME equivalents / g striatal tissue. Immunoreactivity is expressed in pmol ME- or LE-IR / g striatal tissue. RIA data has been corrected with respect to antisera cross-reactivity.

The calibration of the Sephacryl-300 column is shown in FIG.17D. The molecular weight markers are; blue dextran (void),bovine serum albumin (68 kDal.), ovalbumin (43 kDal.), soy bean trypsin inhibitor (21 kDal.), cytochrome c (13 kDal.) and enkephalin (0.55 kDal.). Absorbance at 280 nm (———).

FIG.17 A.

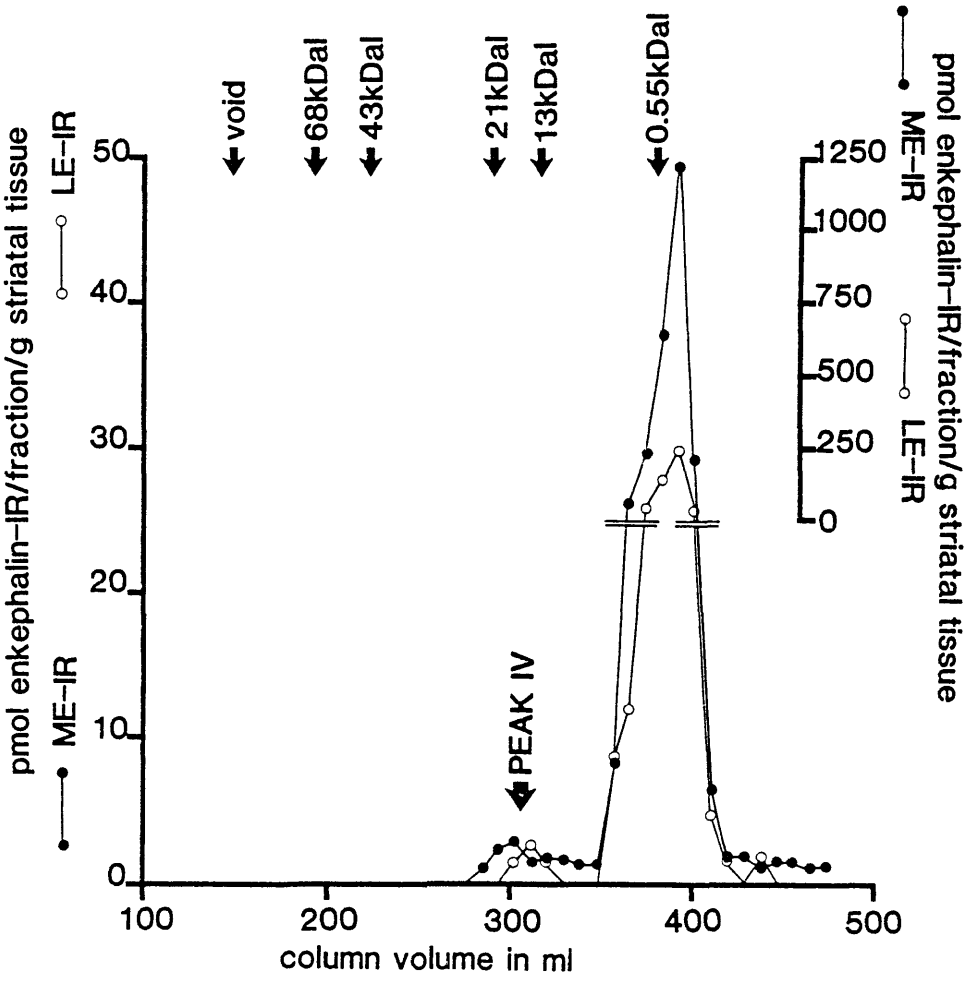

pmol enkephalin-IR/fraction/g striatal tissue
ME-IR
LE-IR
50
40
30
20
10
0
void
68kDal
43kDal
21kDal
13kDal
0.55kDal
PEAK IV
1250
1000
750
500
250
0
100
200
300
400
500
column volume in ml

FIG.17 B.

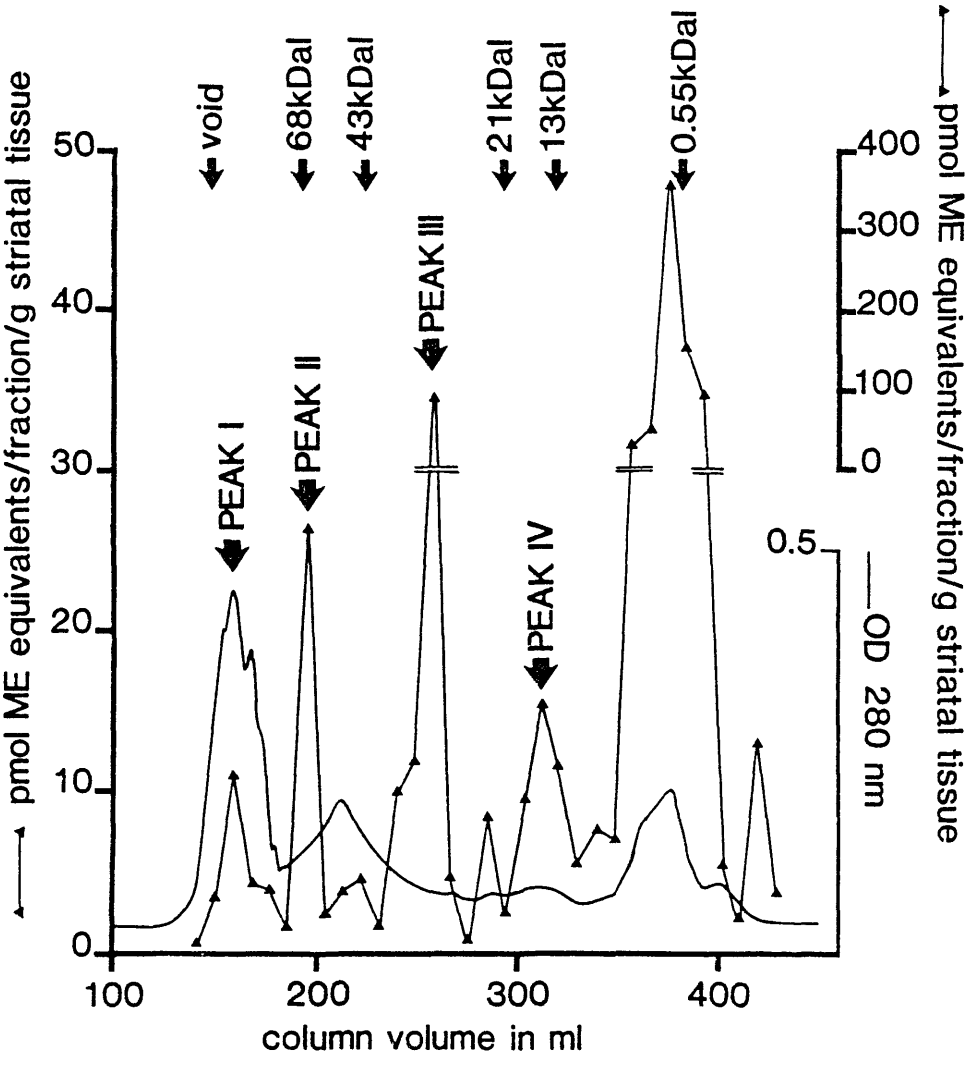

void
68kDal
43kDal
21kDal
13kDal
0.55kDal
PEAK I
PEAK II
PEAK III
PEAK IV
pmol ME equivalents/fraction/g striatal tissue
OD 280 nm
column volume in ml
0
10
20
30
40
50
100
200
300
400
0.5

FIG. 17 C.

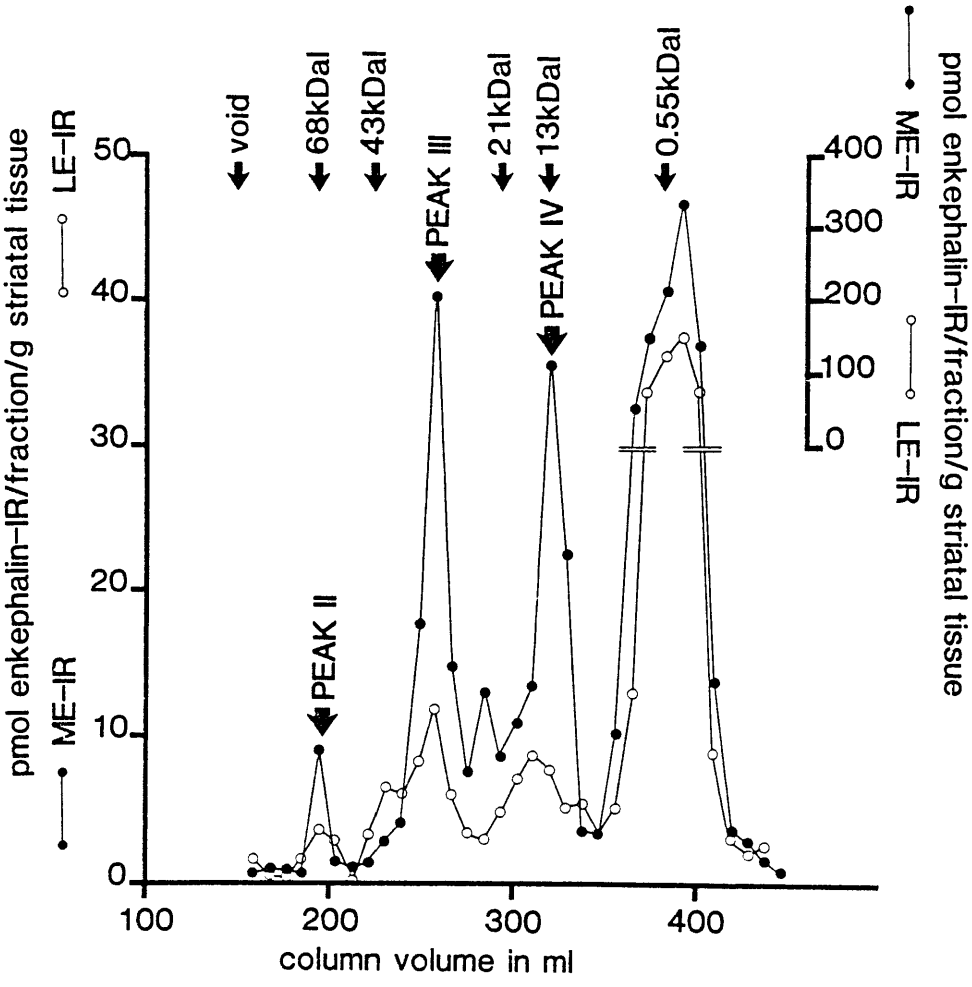
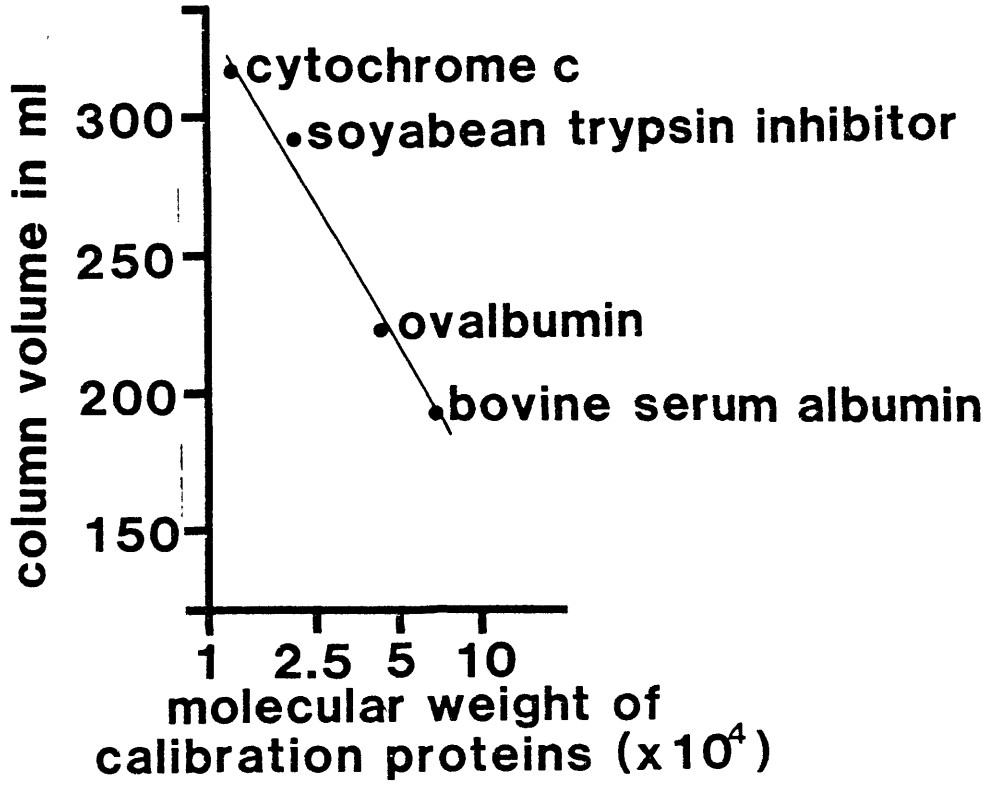

pmol enkephalin-IR/fraction/g striatal tissue
ME-IR
LE-IR
50
40
30
20
10
0
void
68kDal
43kDal
21kDal
13kDal
0.55kDal
PEAK II
PEAK III
PEAK IV
400
300
200
100
0
100
200
300
400
column volume in ml

FIG. 17 D.

CALIBRATION OF SEPHACRYL-300



A Sephacryl-300 column (2.6 x 86 cm) was equilibrated at a flow rate of 13.5 ml/h at 20°C in 50mM Tris-HCl buffer pH 8.0 at 20°C containing 8M urea and calibrated using the following standard proteins:

blue dextran, bovine serum albumin (68 kDal.), ovalbumin (43 kDal.), soyabean trypsin inhibitor (21 kDal.), cytochrome c (13 kDal.) and enkephalin (0.55 kDal.). Blue dextran eluted after 144ml (void volume) and enkephalin eluted after 381 ml (total column volume). M_r has been plotted using a log. scale.

TABLE 8

RECOVERY OF ME-IR AND LE-IR FOLLOWING SEPHACRYL-300 CHROMATOGRAPHY

IMMUNOREACTIVITY IN P MOL /g STRIATAL TISSUE	PRIOR TO S-300 CHROMATOGRAPHY	FOLLOWING S-300 CHROMATOGRAPHY
ME-IR	1568	2416
LE-IR	364	503
% recovery ME-IR		154
% recovery LE-IR		138
Ratio ME-IR:LE-IR	4.3:1	4.8:1

Guinea pig striata were extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and chromatographed over Sephacryl-300 in the same buffer. Aliquots were analysed by direct RIA for ME-IR and LE-IR before and after chromatography. For this purpose samples were diluted ten fold in RIA buffer to give a final urea concentration of 270mM in the RIA incubation volume (300µl). Following chromatography direct RIA detected immunoreactivity almost exclusively in the total column volume ($\leq$2 kDalton). The ratio ME-IR:LE-IR refers to immunoreactivity eluting in this peak.

immunoreactivity was observed in any of the fractions corresponding to Peak I, Fig. 17C. ME-IR and LE-IR were detected in every peak, with ME-IR as the major component at each molecular weight. The bioassayable opioid activity present as Peak I was not detected prior to digestion or after digestion with trypsin alone.

The relationship between bioassayable and immunoreactive opioid material, liberated after trypsin and carboxypeptidase B digestion of a Sephacryl-300 separation, is shown in Table 9. Higher values for bioassayable opioid activity were obtained for Peaks I and II whilst the reverse was true for Peaks III, IV and the total column volume peak. The total bioassayable opioid activity eluted from the column represented only 57% of the total immunoreactivity eluted under the same conditions. The predominant high molecular weight species eluting before the total column volume was Peak III which contained 12.4% of all bioassayable opioid activity and 7.9% of all immunoreactivity of the separation after digestion.

4.3.2 Sephacryl-300 chromatography of bovine and rat striatal extracts

Extracts of rat and bovine striata gave comparable results to those of guinea pig striata, when chromatographed over Sephacryl-300 under the same conditions, with detectable levels of ME-containing peptides eluting over the entire M_r range up to 70000, Fig. 18. In bovine striata the levels of ME-IR liberated after digestion of fractions eluting before the total column volume were considerably lower than in either guinea pig or rat (40 pmol ME-IR/g bovine striatal tissue in comparison to 170 pmol ME-IR/g rat striatal tissue and 210 pmol ME-IR/g guinea pig striatal tissue). However, although lower amounts of ME-containing peptides were detected in bovine tissue, the

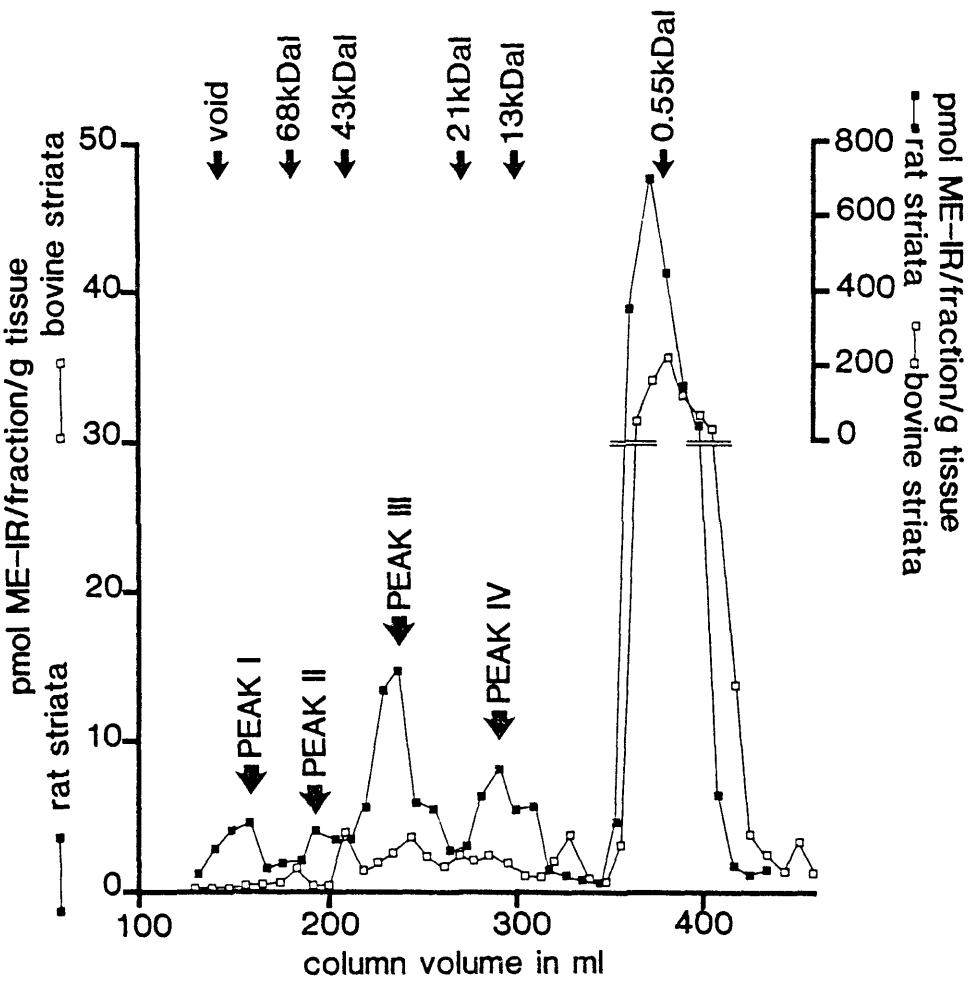
TABLE 9
COMPARISON OF OPIOID -LIKE ACTIVITY DETECTED BY M.V.D. BIOASSAY AND RIA FOLLOWING SEPHACRYL-300 CHROMATOGRAPHY

ASSAY	ACTIVITY	PEAK NUMBER					TOTAL COLUMN ACTIVITY
		I	II	III	IV	TOTAL COLUMN VOLUME	
M V D BIOASSAY	Bioassayable opioid activity in p mol	22.5	30.0	119.9	60.7	730	963
	% total bioassayable opioid activity	2.3	3.1	12.4	6.3	76	
RIA for both ME and LE	Total immunoreactivity in p mol	3.1	19.1	133.6	138.7	1387	1697
	% total immunoreactivity	0.18	1.14	7.9	8.2	82	
ME RIA	ME-IR in p mol	1.2	11.4	88.2	97.4	927	1138
	% total ME-IR	0.1	1.0	7.7	8.6	81	
LE RIA	LE-IR in p mol	1.89	7.72	45.3	41.2	460	559
	% total LE-IR	0.3	1.4	8.1	7.4	82	

Guinea pig striata were extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and chromatography over Sephacryl-300 in the same buffer. Fractions were analysed for bioassayable opioid activity, ME-IR and LE-IR following sequential digestion with trypsin and carboxypeptidase B. Bioassayable opioid activity and immunoreactivity have been adjusted /g striatal tissue. The percentage activity for each peak has been calculated as a function of the total bioassayable opioid activity or immunoreactivity eluted from the column.

FIG.18

SEPHACRYL-300 CHROMATOGRAPHY OF RAT AND BOVINE STRIATAL EXTRACTS.



Rat and bovine striatal tissues were extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and chromatographed over Sephacryl-300 in the same buffer, as described in FIG.17 . Aliquots were digested with trypsin followed by carboxypeptidase B and analysed for ME-IR; pmol ME-IR / g rat striatal tissue (■——■) and bovine striatal tissue (□——□).

proportion of immunoreactivity incorporated within higher molecular weight forms was of the same order in both rat and cow, 6% and 5% respectively. In comparison, guinea pig striata contained a higher percentage (approximately 19%) of ME-IR within ME-containing peptides.

In both the rat and cow, digestion of fractions eluting near the void volume of the column was found to generate bioassayable opioid activity, but, as with the guinea pig, little or no immunoreactivity, Table 10.

4.3.3 Sephacryl-300 chromatography of guinea pig striata frozen within 2 min of sacrifice and after a 30 min incubation at 25°C

When guinea pig striata were frozen within two min of death, extracted in Tris-8M urea and chromatographed over Sephacryl-300, the opioid-containing peptide profile was as previously described. Digestion of column fractions revealed the presence of four peaks of ME-IR and/or bioassayable opioid activity corresponding to Peaks I, II, III and IV, in addition to the total column volume peak.

In comparison, guinea pig striata frozen after a 30 min incubation at 25°C, extracted and chromatographed under the same conditions, demonstrated differences in the levels of bioassayable opioid activity and ME-IR liberated after digestion. There were no significant changes in the elution profile of the major opioid containing peptides present, Fig. 19. With respect to the minor opioid-containing species, the bioassayable activity detected in Peak I was reduced by 40% after a 30 min incubation at 25°C, Table 10. Similarly, this incubation reduced the levels of ME-IR and bioassayable opioid activity contained within Peak II, by 74% and 80% respectively (for bioassayable opioid activity

TABLE 10

BIOASSAYABLE OPIOID ACTIVITY IN PEAK I AFTER DIGEST

TISSUE PREPARATION	BIOASSAYABLE OPIOID ACTIVITY IN P MOL ME EQUIVALENTS/g TISSUE
Guinea pig striata : acetic acid extraction	7.0
Guinea pig striata	21.9
Rat striata	24.5
Bovine striata frozen within 30 min of death	3.1
Guinea pig striata frozen 30 min after death	12.8
Guinea pig adrenal	NOT-DETECTABLE

All tissues were frozen within 2 min of death and extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea unless otherwise stated. Extracts were chromatographed over Sephacryl-300 in the Tris-8M urea buffer. Fractions corresponding to Peak I (>70 kDal.) were pooled, digested with trypsin followed by carboxypeptidase B and analysed for bioassayable opioid activity using the M.V.D. bioassay.

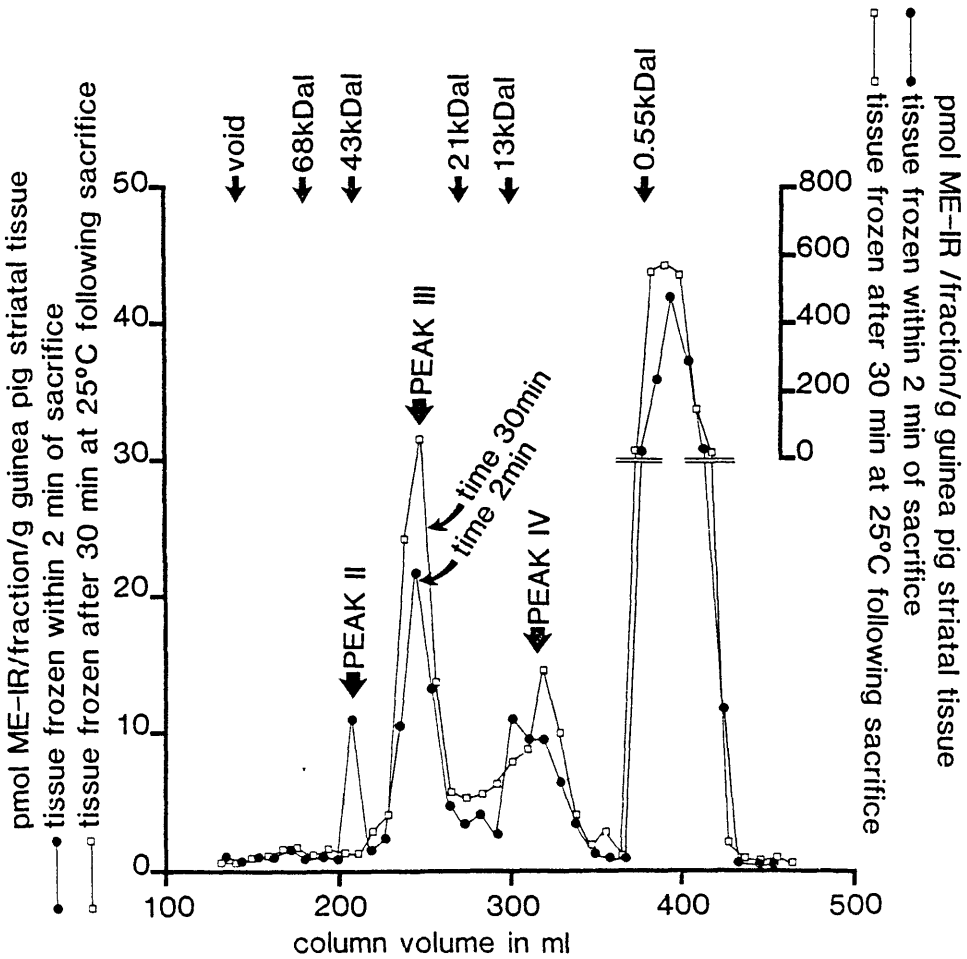
Bioassayable opioid activity has been adjusted / g tissue.

FIG. 19

SEPHACRYL-300 CHROMATOGRAPHY OF GUINEA PIG STRIATA FROZEN

a) WITHIN 2 MIN OF DEATH AND

b) AFTER A 30 MIN INCUBATION AT 25°C



Striata were removed alternately from the left and right hemispheres of ten guinea pigs and frozen within 2 min of death in liquid N_2. The remaining striata were incubated for 30 min at 25°C before freezing. Tissues were extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and chromatographed over Sephacryl-300 in the same buffer, as described in FIG. 17 . Aliquots were digested with trypsin followed by carboxypeptidase B and analysed for ME-IR; pmol of ME-IR / g striatal tissue frozen within 2 min of death (●——●) and after a 30 min incubation at 25°C (□——□).

this was a reduction from 16.6 pmol ME equivalents/g striatal tissue to 3.2 pmol ME equivalents/g striatal tissue; immunoreactivity data is shown in Fig. 19). In contrast, the ME-IR levels in Peaks III and IV increased to 148% (Peak III) and 128% (Peak IV) following a 30 min incubation before freezing. An increase was also observed in the ME-IR eluting in the total column volume. In this case the level rose by 162%.

4.3.4 Sephacryl-300 chromatography of guinea pig striata following acetic acid extraction

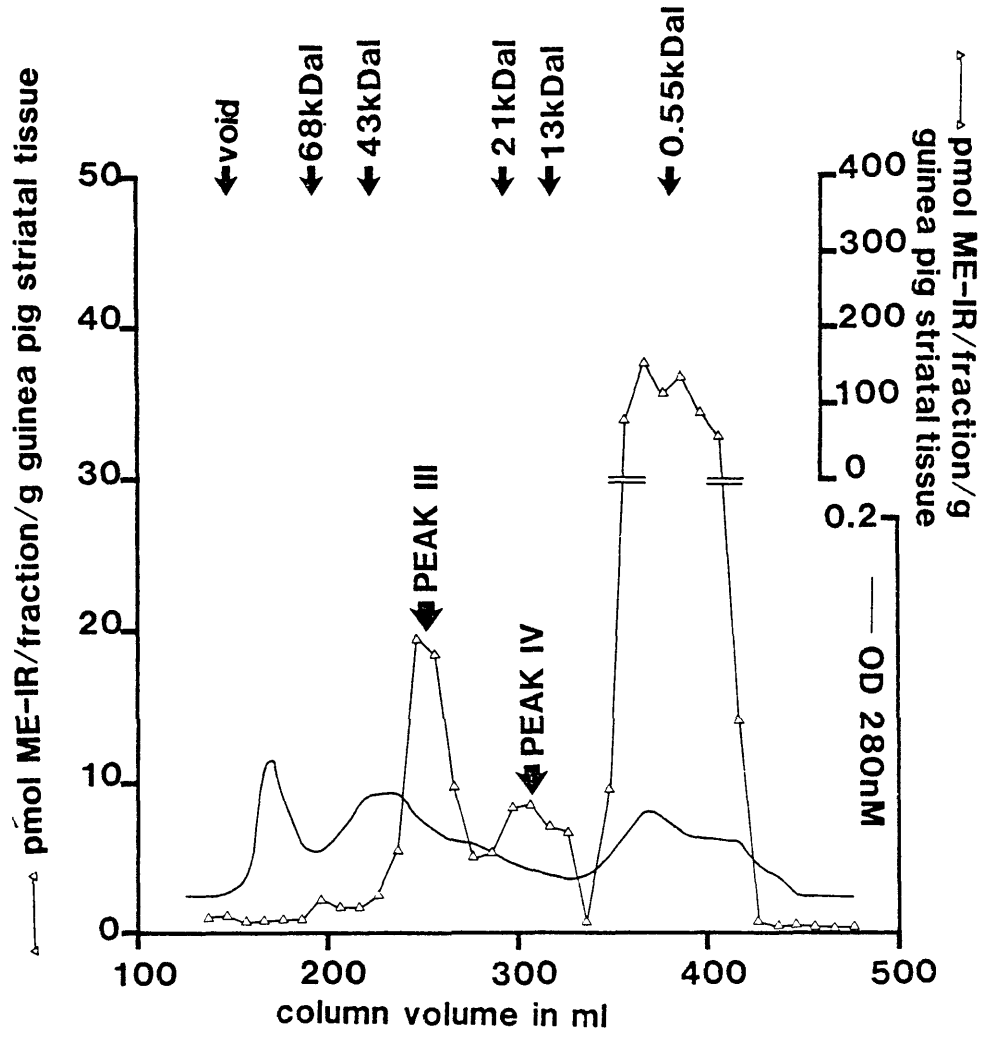
When guinea pig striata were extracted in 1M acetic acid-HCl pH 1.9, instead of Tris-8M urea and chromatographed over Sephacryl-300, under the same conditions as an 8M urea extract, the profile of ME-IR obtained after digestion showed decreased levels of putative opioid peptide precursors eluting > 30 kDalton, but a comparable distribution for those eluting < 30 kDalton, Fig. 20. Acetic acid extraction reduced the yield of opioid-containing peptides liberating bioassayable opioid activity from Peak I, Table 10. As previously observed Peak I contained little immunoreactivity, Fig. 20.

4.3.5 Sephacryl-300 chromatography of guinea pig adrenal extract

Guinea pig adrenal preparations showed considerable differences to those of guinea pig striata, following extraction and Sephacryl-300 chromatography in Tris-8 M urea, Fig. 21. After digestion, ME-IR was observed eluting over the entire M_r range up to 30000, with the major species, in this instance, eluting between 25-6 kDaltons. Putative opioid precursors with apparent M_r > 30000 were not detected, either by RIA or MVD bioassay. The level of ME-IR shown by direct ME RIA of the

FIG. 20

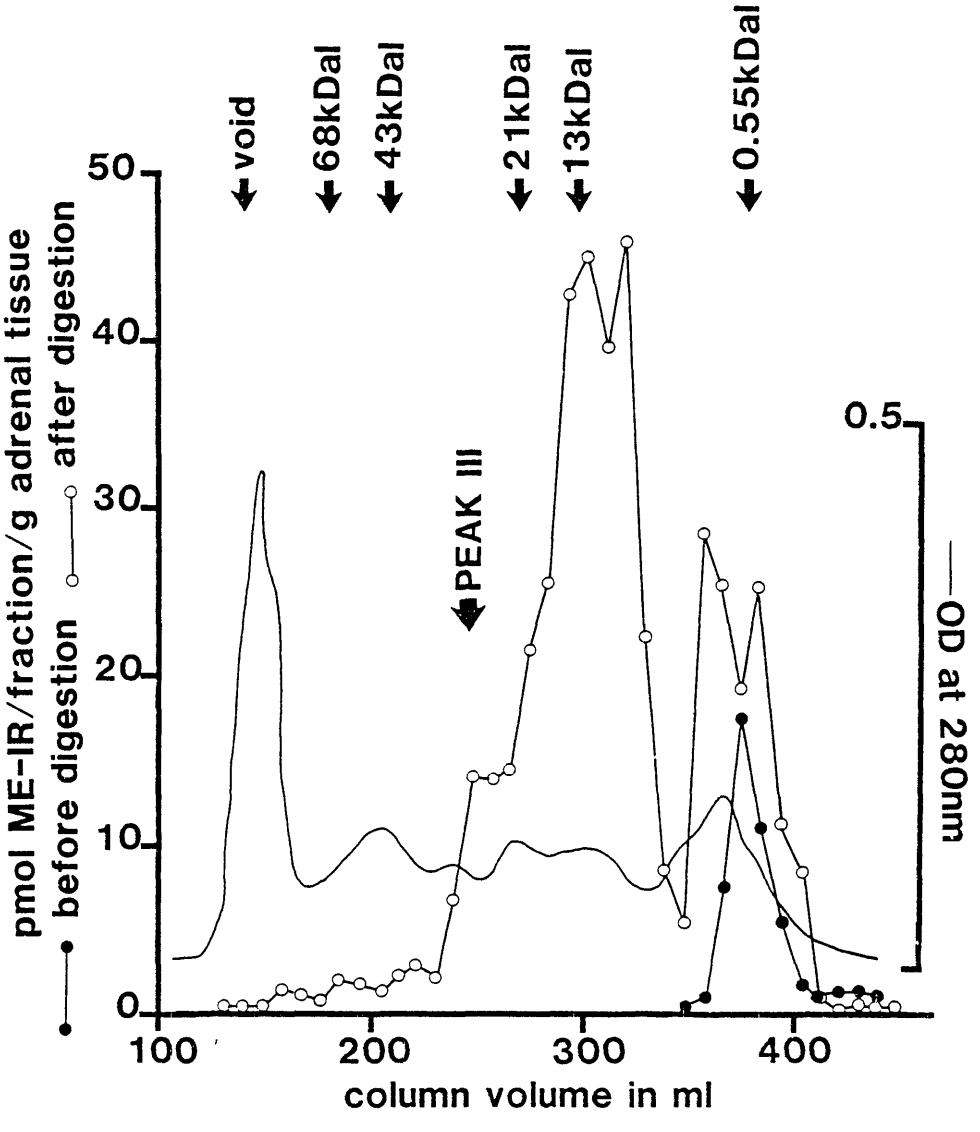
SEPHACRYL-300 CHROMATOGRAPHY OF GUINEA PIG STRIATAL TISSUE EXTRACTED IN ACETIC ACID.



Guinea pig striata were extracted in 1M acetic acid-HCl pH 1.9 (10% w/v) and the supernatant (6ml) applied to a Sephacryl-300 column. Elution was conducted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea, as described in FIG 17 . Aliquots were digested with trypsin followed by carboxypeptidase B and analysed for ME-IR; pmol of ME-IR / g striatal tissue (△——△); absorbance at 280 nm (———).

FIG. 21

SEPHACRYL-300 CHROMATOGRAPHY OF GUINEA PIG ADRENAL GLAND EXTRACT.



Guinea pig adrenal glands were extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and chromatographed over Sephacryl-300 in the same buffer, as described in FIG. 17 . Aliquots were heat treated at 100°C for 20 min, digested with trypsin followed by carboxypeptidase B and analysed for ME-IR. Fractions eluting < 2 kDal. were assayed directly for ME-IR after a four fold dilution in RIA buffer; pmol ME-IR / g adrenal gland before digestion (●——●) and after digestion (○——○);absorbance at 280 nm. (——)

total column volume (< 2 kDalton) demonstrated that there was little free enkephalin, approximately 50 pmol/g adrenal tissue representing only 2% of the level found in guinea pig striata. The ME-IR present in this region increased 250% following trypsin and carboxypeptidase B digestion.

4.4 Discussion

4.4.1 Sephacryl-300 chromatography of guinea pig striatal extracts

(i) Opioid peptide content before digestion

Following Sephacryl-300 chromatography of guinea pig striatal tissue, direct RIA using ME and LE antisera revealed a peak of immunoreactivity eluting almost exclusively in the total column volume. This single peak contained ME- and LE-IR in the ratio 4.8:1, which is comparable with the 4.5:1 ratio for these peptides found by Ikeda et al., (1982) in the same tissue. This value is in agreement with the 4:1:1:1 ratio for ME : LE : ME-Arg^{6}-Phe^{7} : ME-Arg^{6}-Gly^{7}-Leu^{8} contained within the sequence of adrenal proenkephalin and supports the suggestion that processing in the striatum produces these products in a stoichiometric ratio from a similiar precursor. Ikeda et al., (1982) found an approximate ratio of 4:1:1:1 for ME, LE, ME-Arg^{6}-Phe^{7} and ME-Arg^{6}-Gly^{7}-Leu^{8} immunoreactivities in the striata of guinea pig, rat and golden hamster and these peptides have been released in this ratio from rat striatal slices (Patey et al., 1983a). A minor component of immunoreactivity eluted before the total column volume. This material probably represents larger peptides, with ME at the COOH terminus, which cross-react with the ME antiserum used. For example, this ME antiserum has a 28% cross-reactivity (on a molar basis) with the 8.6

kDalton adrenal proenkephalin species (Liston and Rossier, 1984a).

(ii) Opioid peptide content after digestion

a. Bioassayable opioid activity

The opioid-containing peptides within guinea pig striatal extracts were resolved into four separate peaks by Sephacryl-300 chromatography, eluting with apparent M_r's of > 70000 (Peak I), 66000 (Peak II), 29000 (Peak III) and 16000-6000 (Peak IV), in addition to a single peak eluting in the total column volume. Digestion of Peak I, by trypsin and carboxypeptidase B, liberated peptide(s) which have naloxone-reversible activity on the MVD bioassay, but show little cross-reactivity with either ME or LE antisera. Bioassayable opioid activity was not detected before digestion, or after digestion with trypsin alone, indicating that these opioid peptide(s) were released from a large precursor molecule by the common mechanism of cleavage at basic residues. The inhibition of the MVD tissue twitch was rapid in onset and of short duration, a profile typical of metabolically labile opioids (Corbett et al., 1982). In constrast, Peaks II, III and IV all contained bioassayable opioid activity and both ME-IR and LE-IR after digestion.

b. Immunoreactivity

The ME-IR and LE-IR released after digestion showed a parallel distribution to that of bioassayable opioid activity, with ME-IR the major immunoreactive species at each molecular weight. Lower values were obtained for total bioassayable opioid activity, as compared with total immunoreactivity, for Peaks III, IV and the total column volume peak. As discussed earlier, this discrepancy could be due to the

presence of NH_2 extended peptides, or the formation of an oxidised form of ME, which are less potent on the MVD bioassay.

The ratio of ME-IR : LE-IR eluting in the total column volume changed from 4.8:1 to 2:1 following digestion. This indicates either a preferential loss of ME or a generation of LE. Trypsin and carboxypeptidase B digestion would not only liberate LE from proenkephalin derived peptides but also from prodynorphin derived peptides. Cone et al., (1983) have detected levels of α-neoendorphin-, β-neoendorphin-, DYN-(1-8)-, DYN-A- and DYN-B-IR in rat striatum which could generate in the region of 200 pmol LE-IR/g tissue after digestion with trypsin and carboxypeptidase B. All these species have M_r's < 2000 and similar peptides, if present in guinea pig striatum, would generate LE-IR in the total column volume after digestion, altering the observed ratio of ME : LE. Higher molecular weight prodynorphin intermediates may also be present in guinea pig striata, generating LE-IR after digestion (Seizinger et al., 1981; Cone et al., 1983). Therefore ME is a more accurate index for the presence of proenkephalin derived peptides, since this peptide is not contained within prodynorphin. It is also necessary to treat with caution ratios of opioid peptides estimated following the digestion of impure proteins.

These investigations confirm reports of high molecular weight putative opioid precursors in striatal tissue (Lewis et al., 1978; Beaumont et al., 1980; Kojima et al., 1982). In constrast, Ikeda et al., (1982 and 1983) have reported finding only the free penta- hepta- and octapeptides in the striata of guinea pig, rat, golden hamster, cow and human. Liston et al., (1983) have found no evidence for the existence of high molecular weight putative opioid precursors in bovine striata and others have detected only small enkephalin-containing

fragments, such as the dodecapeptide BAM 12P (Pittius et al., 1983). Some of these studies have relied on direct RIA using ME, LE, ME-Arg^{6}-Phe^{7}, ME-Arg-Gly^{6}-Leu^{7} and BAM-12P antisera to identify high molecular weight forms (Ikeda et al., 1982; Ikeda et al., 1983 ; Pittius et al., 1983; Giraud et al., 1984a). In this study it has been demonstrated that the ME and LE antisera used did not cross-react directly with opioid-containing peptides present in guinea pig striata. This provides one possible explanation for the disparity.

It was felt that variation between species, or post-mortem changes, or differences in extraction procedure could also provide explanations as to why reports concerning the presence of high molecular weight opioid-containing peptides in striatum are inconsistent. These proposals were investigated.

(i) Species variations

Rat and bovine striata have been shown to contain putative opioid peptides eluting over the same M_r range as those from guinea pig striata (> 70000-2000), when chromatographed under similar conditions. Rat and guinea pig striatal opioid-containing peptides could be separated into Peaks I, II, III and IV. However these species were present in considerably lower quantities in the cow, approximately 20-25% of the levels found in rat and guinea pig, and were not as well resolved.

(ii) Post-mortem variations

Bovine striatal tissue obtained fresh from the slaughter house was frozen within 30 min of death. As a comparison, guinea pig striata

were incubated at 25°C for 30 min before freezing. Although this could not be judged an equivalent tissue state, it was felt that any gross change in opioid-containing peptide levels occuring before extraction could be observed. There was no evidence of a diminution in the levels of the major Peak's III and IV species of putative opioid precursors, present in guinea pig striata, as a result of this treatment.

(iii) Extraction procedure

It was also demonstrated that the use of acetic acid as the extraction medium, in place of Tris-8M urea, did not dramatically reduce the levels of opioid-containing peptides in guinea pig striatal extracts, although decreases were observed in the minor Peak's I and II.

In view of these observations, variations in tissue preparation do not explain the difference between our results and other reports, that opioid-containing peptides do not accumulate to any measurable extent in striatal tissue. The low levels of opioid-containing peptides observed in bovine striatum, as compared with these tissues from the guinea pig and rat, suggests that species differences may be more important. Giraud et al., (1984b) have also reported that trypsin digestion of rat brain releases ME-Arg^6-IR from high and low molecular weight opioid-containing peptides, whereas a similar treatment of bovine striatum releases ME-Arg^6-IR only from low molecular weight species. Cupo et al., (1984) have also used ME-Arg^6 antiserum to detect putative opioid precursors in the rat striatum. In their study the level of ME-Arg^6-IR associated with the precursor fraction was six fold higher than the level of ME-IR reported here and 160% of the value for the total ME-Arg^6-IR content of rat striata found by Giraud et

al.,(1984b).

In conclusion, guinea pig, rat and bovine striata have been found to contain putative opioid precursors in the M_r range 2000- > 70000 and these species from rat and guinea pig have been resolved into four separate peaks. Levels of opioid-containing peptides were similar in rat and guinea pig but significantly lower in the cow. However, the percentage of ME-IR incorporated into these species was highest in the guinea pig. The major opioid-containing peptide species present in all striatal tissues exa mined was Peak III. This represented 8.94±1.5%* of all ME-IR after digest in the guinea pig striatum. Peak I, containing bioassayable opioid activity, but little or no immunoreactive component was also consistently present in all these extracts.

4.4.2 Adrenal extracts

The structures of many putative opioid precursors (M_r range 2000-18200) have been elucidated from bovine adrenal medulla (Udenfriend and Kilpatrick, 1983). The ME-IR profile obtained following digestion of a Sephacryl-300 separation of guinea pig adrenal extract was in agreement with the presence of similar putative precursors in this tissue, with the majority of opioid-containing peptides eluted over the M_r range 6000-25000. Although a minor component of ME-IR was observed eluting between 30-25 kDalton, in guinea pig adrenal, this was not present as the resolved Peak III species observed in striatum. In addition, putative opioid precursors with an apparent M_r > 30000 were not dectected after digestion of the adrenal chromatogram, either by RIA or MVD bioassay.

The value for ME-IR detected in the total column volume before

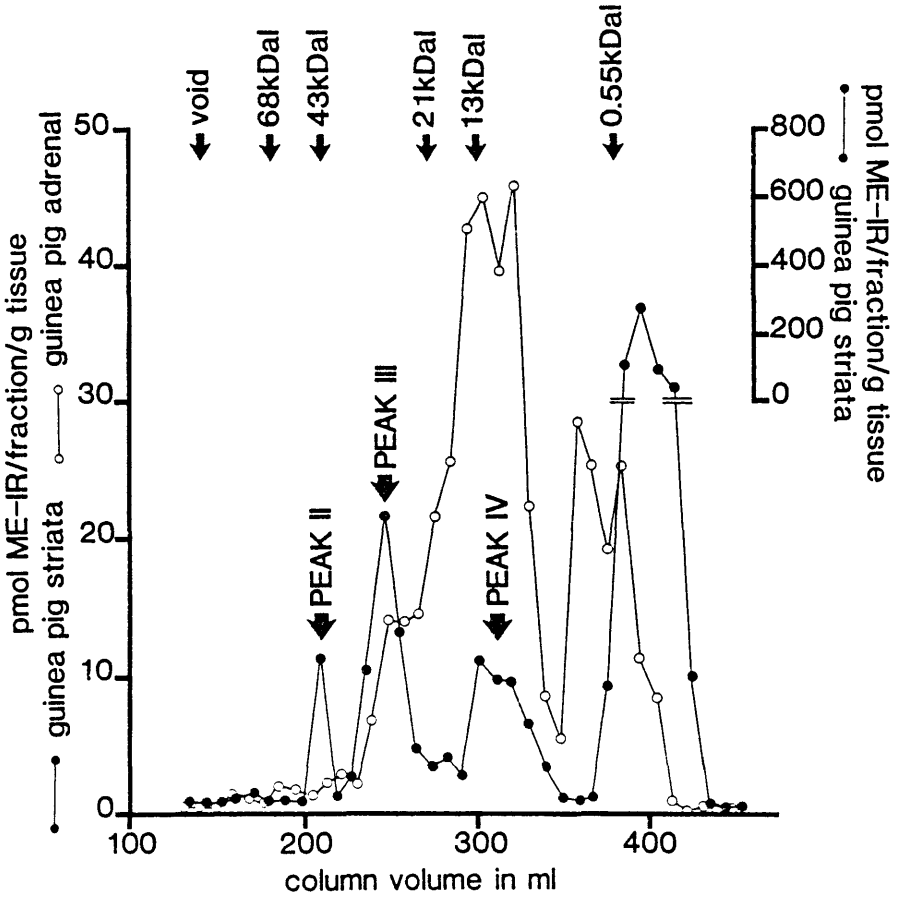
* mean ± S.E. where n = 11

digestion was comparable with the low levels of free enkephalin found in other adrenal tissues (Hexum et al., 1980; Kojima et al., 1982; Chaminade et al., 1984). The increase in immunoreactivity after digestion suggested the presence in guinea pig adrenal gland of ME-Arg^{6}-Phe^{7}, ME-Arg^{6}-Gly^{7}-Leu^{8} and other enkephalin congeners previously detected in bovine adrenal medulla (Stern et al., 1980; Giraud et al., 1984a).

In summary, the patterns of proenkephalin derived peptides from guinea pig adrenal and guinea pig striatal tissues showed considerable differences, Fig. 22. In guinea pig striatal tissue around 20% of ME-IR was contained within larger precursor forms in comparison with 90% in the guinea pig adrenal gland.

FIG.22

SEPHACRYL-300 CHROMATOGRAPHY OF GUINEA PIG STRIATAL AND ADRENAL GLAND EXTRACTS.



Guinea pig striata and adrenal glands were extracted in 50mM Tris-HCl pH 8.0 at 20°C containing 8M urea and chromatographed over Sephacryl-300 in the same buffer,as described in FIG. 17 .Aliquots were digested with trypsin followed by carboxypeptidase B and analysed for ME-IR; pmol ME-IR / g guinea pig striatal (●——●) and adrenal (○——○) tissues.

CHAPTER FIVE

CHARACTERISATION OF GUINEA PIG STRIATAL OPIOID-CONTAINING PEPTIDES

Chapter five

Characterisation of guinea pig striatal opioid containing peptides

5.1 Introduction

Opioid-containing peptides resolved by gel filtration of guinea pig striatal extracts were characterised on the basis of their molecular weight, opioid peptide content and/or pI.

The high molecular weight region of putative opioid precursors separated over Sephadex G-200 was subjected to ion-exchange chromatography, IEF and Sephacryl-300 chromatography. This region was found to contain a species which eluted in the position of Peak I and liberated bioassayable opioid activity after digestion.

Peak III, the major opioid-containing species separated by Sephacryl-300 chromatography, was partially purified by chromatofocusing and SDS-PAGE. The opioid peptide content of this peak was determined by specific RIA and reverse phase HPLC following digestion.

Peak IV, also separated by Sephacryl-300, was resolved by chromatofocusing in order to investigate the complexity of the opioid-containing peptides within this species.

5.2 Peak I

5.2.1 DE52 anion exchange chromatography of the high molecular weight region separated over Sephadex G-200

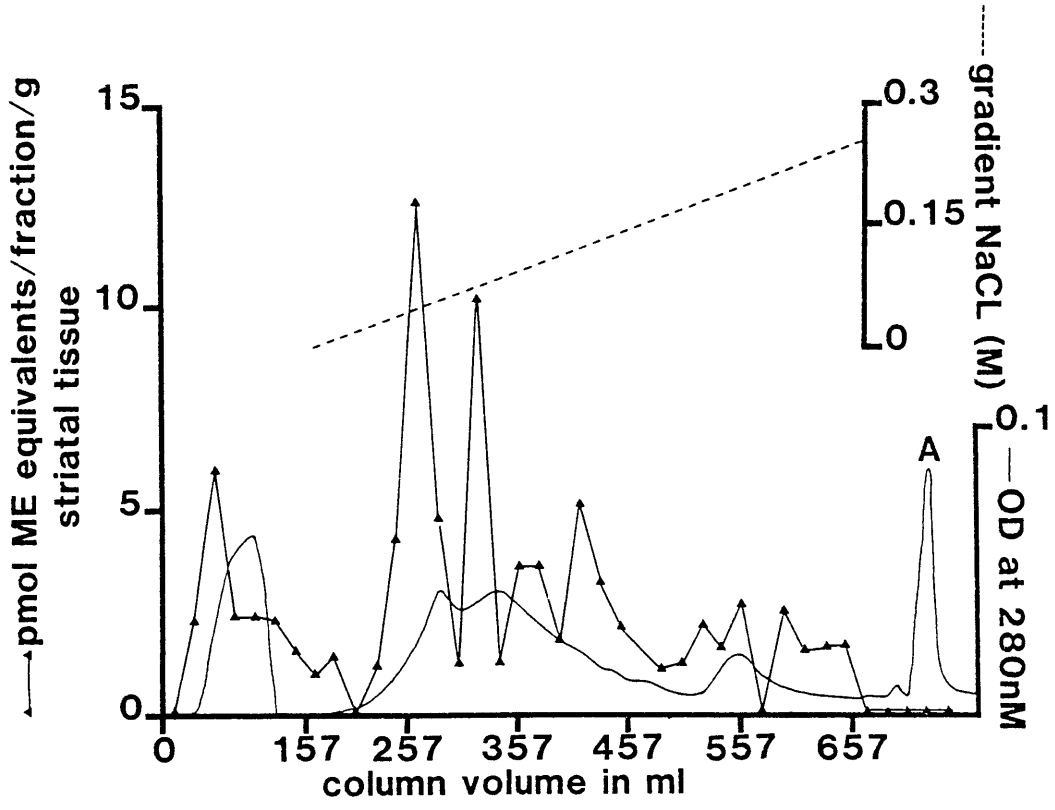
The high molecular weight region from a Sephadex G-200 separation of guinea pig striatal extract was subjected to DE52 anion exchange chromatography. The profile of bioassayable opioid activity liberated after sequential digestion by trypsin and carboxypeptidase B showed that 22% of the opioid-containing peptide(s) applied to the anion exchanger at pH 8.8 was not adsorbed on to the column. This indicated that at least one species of putative opioid precursor had a pI of > 8.8 under these conditions, Fig.23. Digestion of fractions corresponding to the adsorbed species, eluted under conditions of increasing ionic strength, revealed the presence of additional species of opioid-containing peptides which were not fully resolved.

5.2.2 Characterisation of non-adsorbed material separated by DE52 anion exchange chromatography by

a. Isoelectrifocusing

When the non-adsorbed material separated by DE52 anion exchange chromatography was subjected to IEF a single zone was detected which liberated bioassayable opioid activity after digestion with trypsin and carboxypeptidase B, Fig.24. This zone equilibrated at an isoelectric pH of 9.6.

FIG.23 . DE 52 ANION EXCHANGE CHROMATOGRAPHY OF THE HIGH MOLECULAR WEIGHT PEAK SEPARATED OVER SEPHADEX G-200.

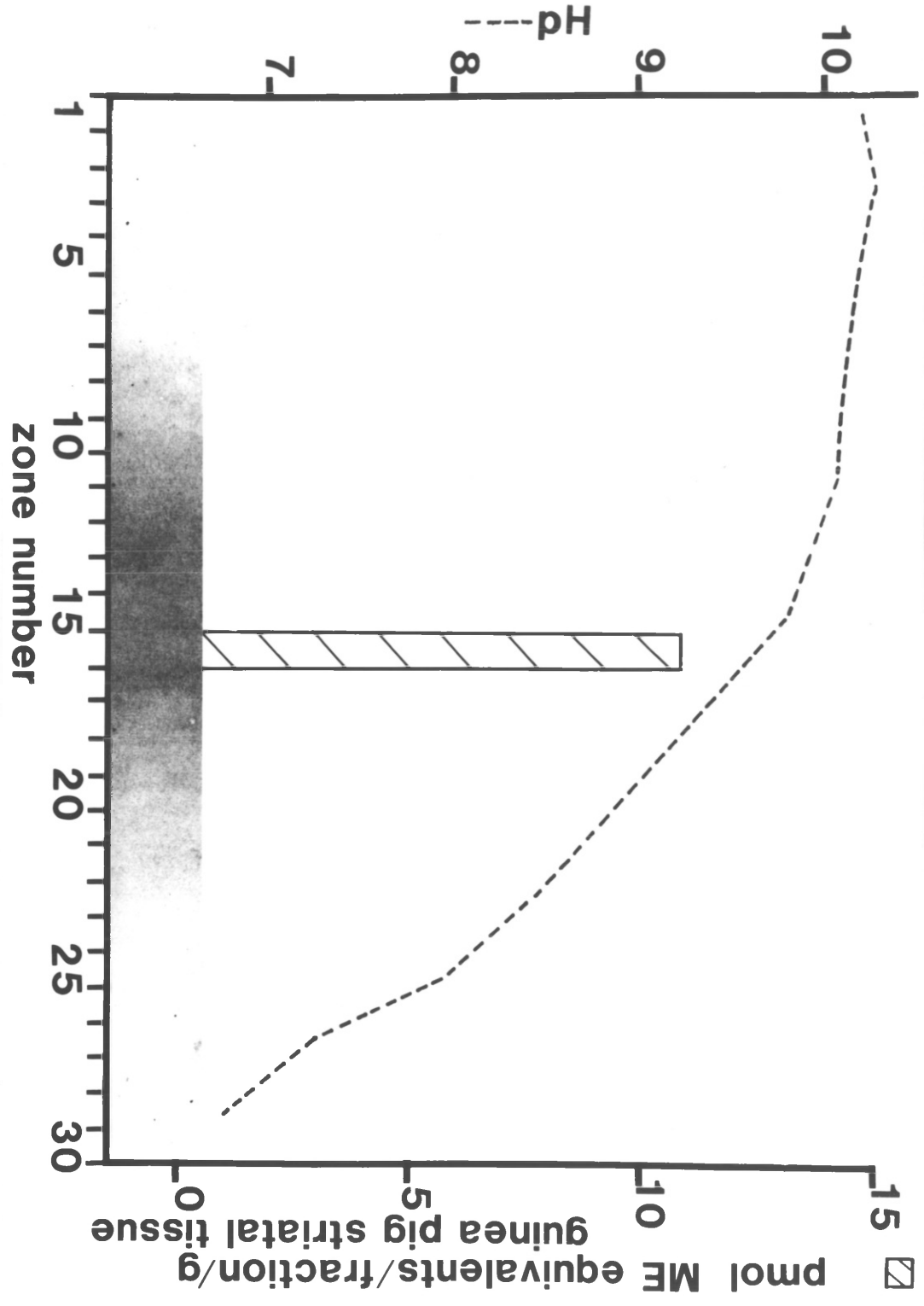


Guinea pig striatal extract was chromatographed over Sephadex G-200 as described in FIG.15 . Fractions corresponding to the high molecular weight region (60-90 kDal.) were pooled and protein precipitated with 15% saturated ammonium sulphate. The precipitated material was resuspended in 50mM Tris-HCl buffer pH 8.8 at 4°C containing 4M urea, applied to a DE 52 anion exchange column in the same buffer and adsorbed material eluted with a continuous salt gradient (0-0.25M NaCl). The column was then washed with 200mM HCl-8M urea. Eluate fractions were digested with trypsin followed by carboxypeptidase B and their opioid peptide content estimated using the MVD bioassay; pmol ME equivalents/ fraction/ g striatal tissue (▲——▲); NaCl gradient (- - -); absorbance at 280 nm (———). A = acid wash

FIG.24 . ISOELECRICFOCUSING OF A NON-ADSORBED SPECIES SEPARATED BY DE 52 ANION EXCHANGE CHROMATOGRAPHY OF HIGH MOLECULAR WEIGHT GUINEA PIG STRIATAL PROTEINS.

Fractions corresponding to non-adsorbed material separated by DE52 anion exchange chromatography of a high molecular weight fraction of guinea pig striatal proteins,were pooled and concentrated by ultrafiltration. The sample was then diluted to a final volume of 95ml containing 50mM Tris-HCl / 2M urea pH 8.0 at 20°C, incorporated in a 4% (w/v) Ultradex gel containing 1.6% Ampholine pH 9-11 and 0.4% Ampholine pH 7-9 and subjected to IEF at 8W for 16h at 10°C. After IEF material was eluted from the focused protein zones and residual Ampholine removed by mixed bed ion-exchange chromatography. Samples were then adjusted to 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$ and digested with trypsin followed by carboxypeptidase B prior to MVD bioassay; pmol ME equivalents / fraction / g striatal tissue(▨); pH gradient (------); protein bands visualised by Coomassie blue are shown below.

FIG. 24 . ISOELECTRICFOCUSING OF A NON-ADSORBED SPECIES SEPARATED BY DE 52 ANION EXCHANGE CHROMATOGRAPHY OF HIGH MOLECULAR WEIGHT GUINEA PIG STRIATAL PROTEINS.



b. Sephacryl-300 chromatography

The putative opioid precursor detected at pH 9.6 after IEF was rechromatographed over Sephacryl-300 by direct application of the IEF eluate (2 ml in 2M urea) to the column. Sephacryl-300 chromatography was performed as previously described in Fig.17. Monitoring u.v. absorbance at 280 nm revealed a small peak eluting after 120 ml of column volume (apparent M_r > 70000)and a large peak eluting just before and during the total column volume (data not shown). Digestion of fractions corresponding to the small u.v. peak (> 70 kDalton) liberated bioassayable opioid activity. The large u.v. peak probably represented the Ampholine which coeluted with protein from the IEF gel.

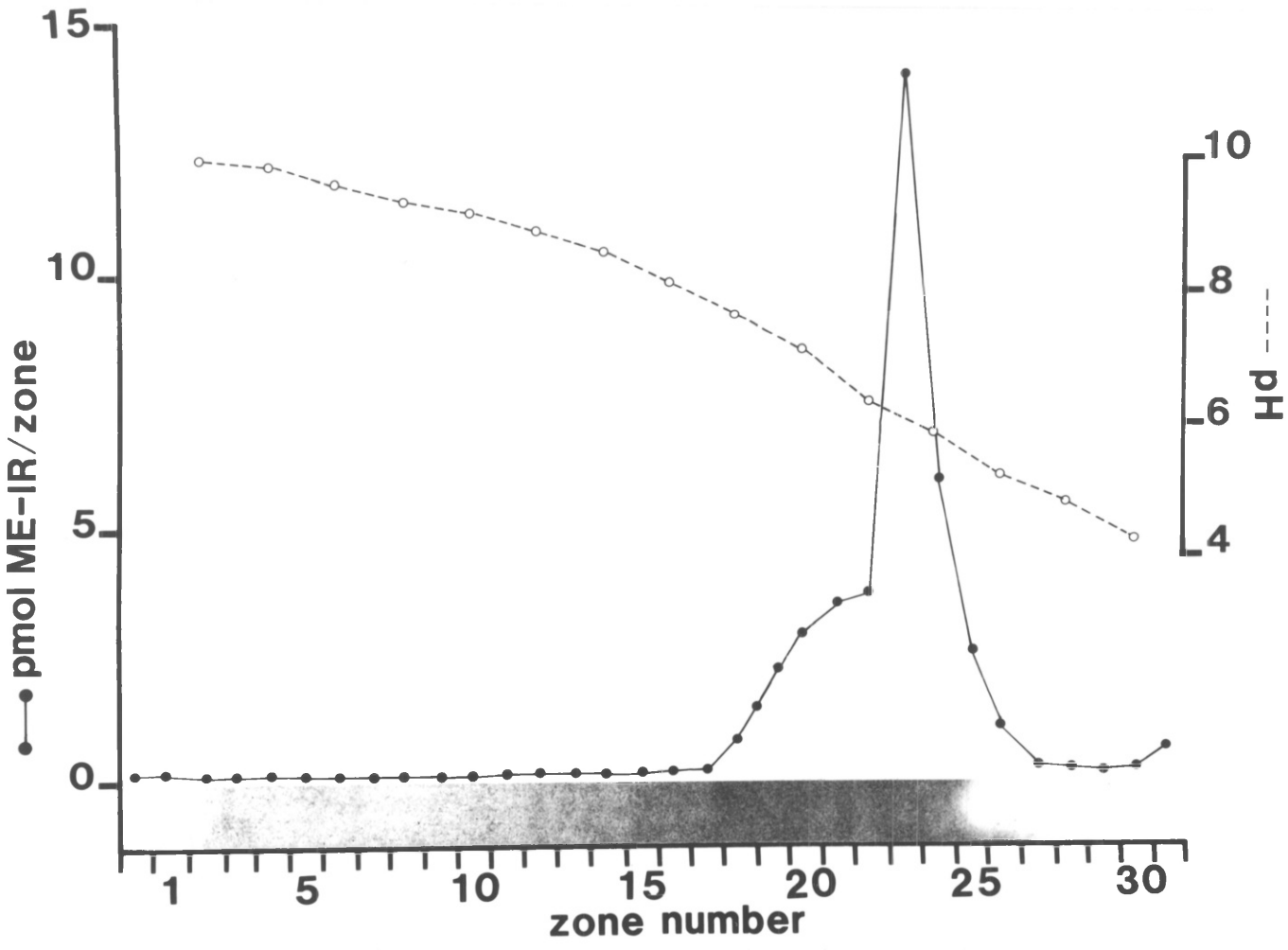
5.3 Peak III

5.3.1 Determination of pI for Peak III by Isoelectricfocusing and chromatofocusing

Flat-bed IEF over the pH range 3.5-10 was used to establish the pI values of opioid-containing peptides detected within Peak III in order to determine the correct pH range for preparative chromatofocusing. Peak III material was found to contain one major species, equilibrating at pH 6.0, which liberated ME-IR after enzymatic digestion, Fig. 25.

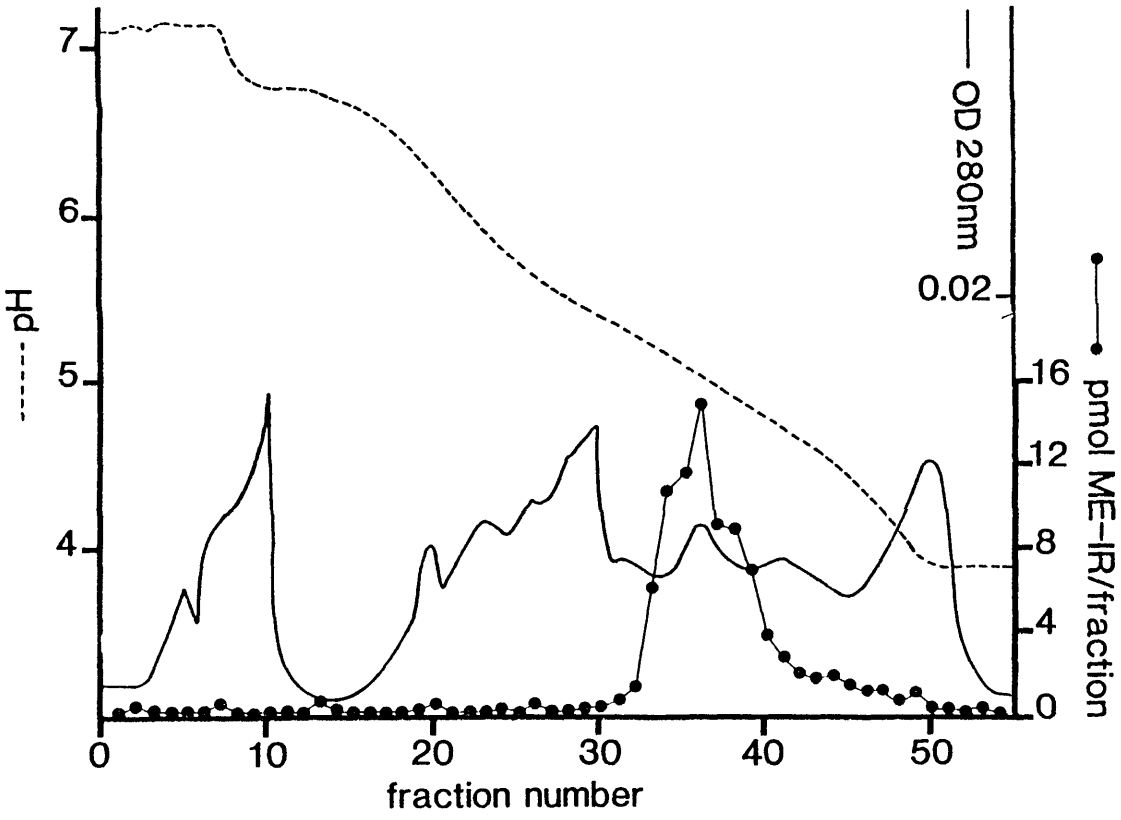
Chromatofocusing of Peak III, followed by digestion with trypsin and carboxypeptidase B, revealed one peak of ME-IR, eluting over an isoelectric pH range of 4.6-5.25, Fig. 26.. Digestion of the NaCl wash did not yield any additional ME-IR (data not shown). The recovery of

FIG. 25 . ISOELECTRICFOCUSING OF PEAK 111



Fractions corresponding to Peak III from a Sephacryl-300 separation of guinea pig striata were pooled and diluted to a final volume of 95 ml with 50mM Tris-HCl pH 8.0 at 20°C containing 6M urea. The sample was slurried in a 4% (w/v) Ultradex gel containing 2% (v/v) Ampholine pH 3.5-10 and subjected to IEF at 8W for 16 h at 10°C. Protein was eluted from the gel zones with 6M urea and the eluates adjusted to 50mM Tris-HCl/2mM $CaCl_2$ pH 7.9 at 37°C containing 2M urea. Eluates were then digested with trypsin and carboxypeptidase B prior to assay for ME-IR (●——●); pH gradient (o- - -o); protein bands visualised by Coomassie blue staining are shown below.

FIG. 26 . CHROMATOFOCUSING OF PEAK III



Pooled fractions corresponding to Peak III from a Sephacryl-300 separation of guinea pig striata were equilibrated by ultrafiltration in 25mM imidazole-HCl, pH 7.2 containing 6M urea and applied to a column (1 x 25cm) of PBE 94 equilibrated in the same buffer. The column was eluted with 12.5% (v/v) PB 74-HCl pH 3.8 containing 6M urea at a flow rate of 15ml / h at room temperature and then washed with 1M NaCl containing 6M urea. Aliquots were sequentially digested with trypsin and carboxypeptidase B and analysed for ME-IR; pmol ME-IR / fraction (●——●); pH gradient (------); absorbance at 280nm (———).

Peak III ME-IR after chromatofocusing under these conditions was 108%.

5.3.2 SDS-PAGE and electrophoretic transfer blot of Peak III

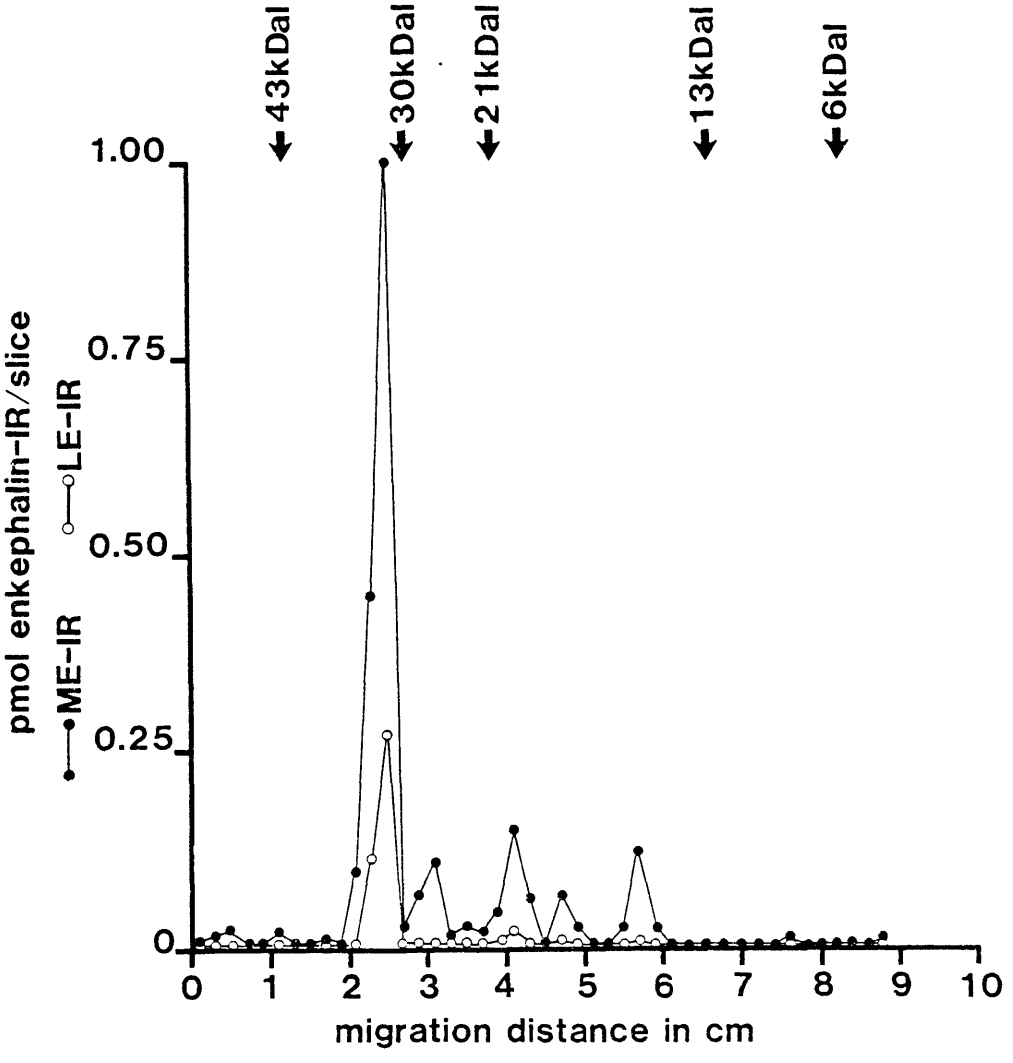
Peak III from a Sephacryl-300 separation of guinea pig striatal extract was subjected to SDS-PAGE followed by electrophoretic transfer of the resolved proteins on to nitrocellulose. The nitrocellulose was further processed by immunoblot, using an anti-ME primary serum and then treated enzymatically, prior to RIA for ME and LE as described in Fig. 27.

The SDS-polyacrylamide gel sample lane was loaded with 750 µg of protein containing 5.15 pmol of ME-IR. The recovery after trypsin and carboxypeptidase B treatment of the nitrocelluose was 2.3 pmol ME-IR, representing an overall yield of 45% for this protocol comprising SDS-PAGE, electrophoretic transfer of protein, immunoblot and digestion.

Enzymatic treatment of the individual nitrocellulose strips, followed by specific RIA for ME and LE, revealed a major peak of immunoreactivity containing ME-IR and LE-IR in the ratio 4:1. This peak migrated with an apparent M_r of 31000 on SDS-PAGE and represented 67% of all ME-IR recovered after digestion of the nitrocellulose. It was also possible to identify several minor species of ME-IR, with apparent M_r's of 15000-30000, Fig. 27.

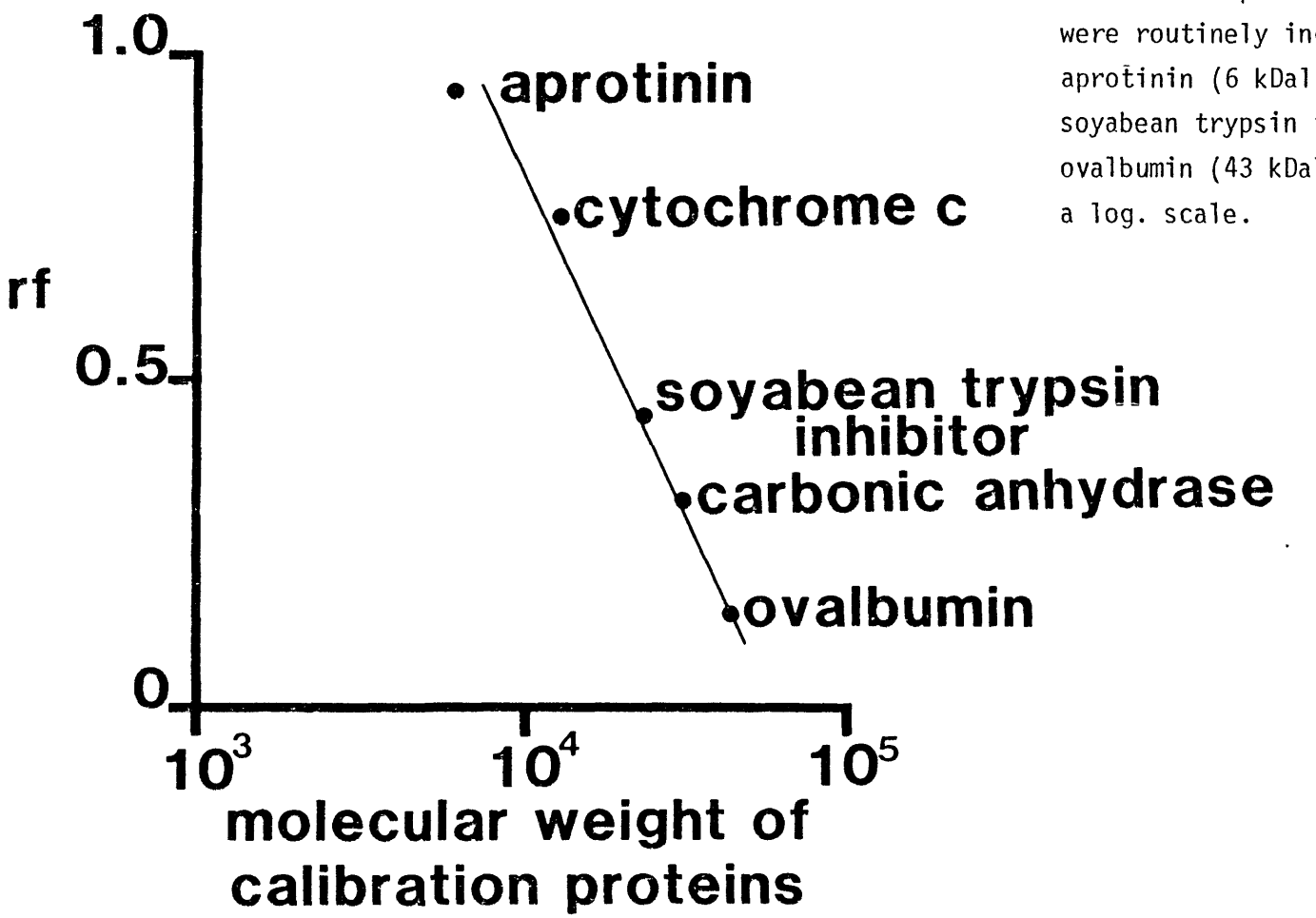
The immunoreactive material, liberated by trypsin and carboxypeptidase B treatment of the nitrocellulose corresponding to the 31 kDalton species, was serially diluted with 50mM Tris-HCl pH 7.9 at 37°C containing 2mM $CaCl_2$ and analysed for ME-IR. The dilution

FIG.27 . SDS-PAGE AND ELECTROPHORETIC TRANSFER BLOT OF PEAK III



Fractions corresponding to Peak III from a Sephacryl-300 separation of guinea pig striata were pooled and equilibrated by ultrafiltration in 5% acetic acid prior to lyophilisation. Lyophilised protein (750ug) was subjected to SDS-PAGE followed by electrophoretic transfer of the resolved proteins on to nitrocellulose. The lane corresponding to calibration proteins was removed and standards visualised by Amido Black staining. The sample lane was further treated by immunoblot using ME primary antiserum. Finally, the nitrocellulose was sliced into 2mm strips along the length of the sample lane and analysed for ME-IR (●——●) and LE-IR (○——○) following sequential digestion with trypsin and carboxypeptidase B. Calibration is shown in FIG.27B.

FIG.27B. SDS-PAGE CALIBRATION



Sample lanes containing the following calibration proteins (2.5µg / protein / lane) were routinely included during SDS-PAGE: aprotinin (6 kDal.), cytochrome c (13 kDal.), soyabean trypsin inhibitor (21 kDal.) and ovalbumin (43 kDal.). M_r is represented using a log. scale.

curve obtained was parallel to the synthetic ME RIA displacement curve constructed during the same assay, Fig. 28.

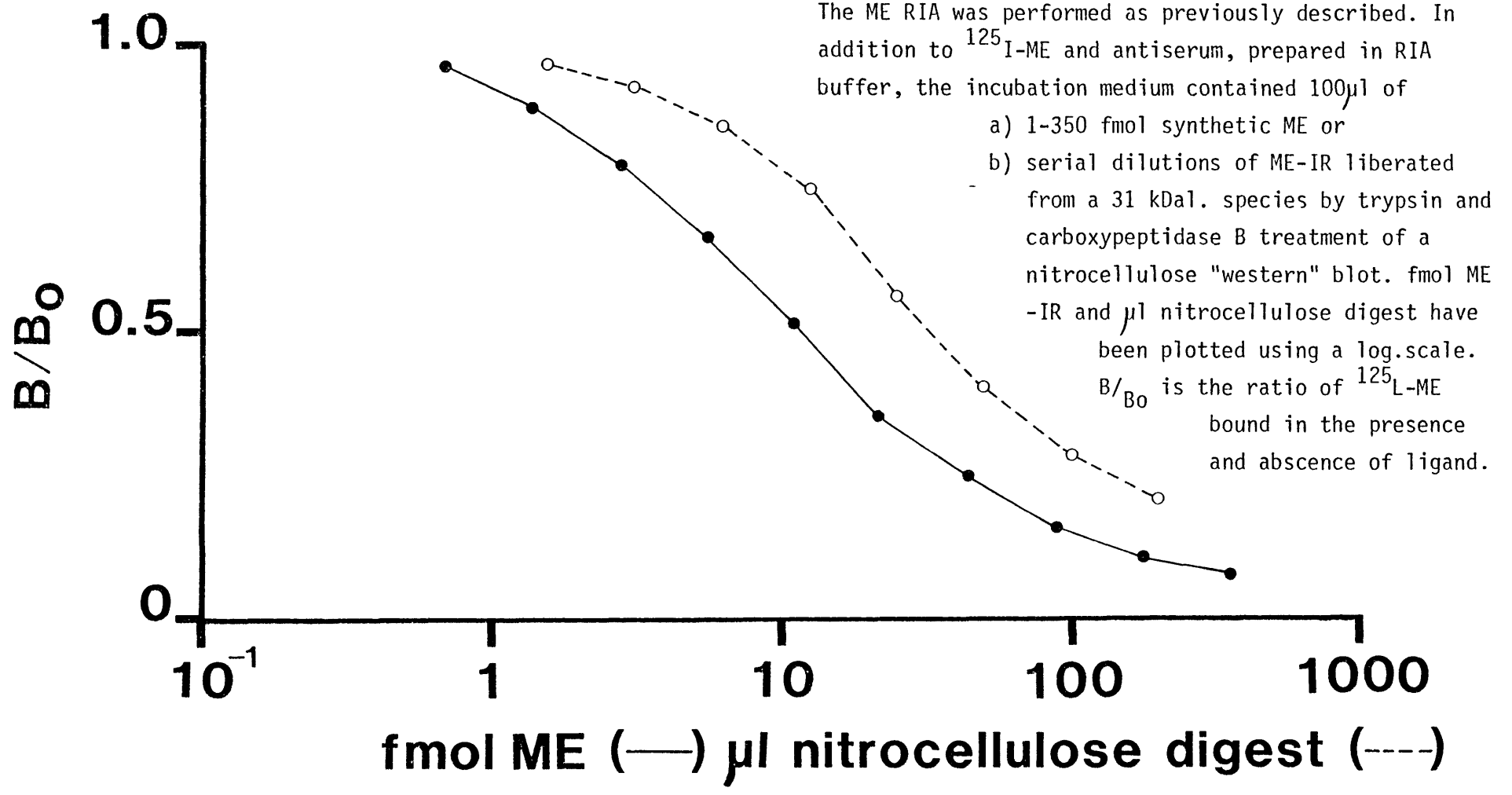
When developed, the immunoblot was stained in many bands along the sample lane. There was no evidence of a particularly heavily stained band corresponding to the position of the immunoreactive material detected after digestion.

5.3.3 SDS-PAGE and electrophoretic transfer blot of the pI 5.0 ME immunoreactive species

(i) SDS-PAGE and silver staining

The ME immunoreactive species which eluted at pI 5.0 during chromatofocusing of Peak III was further analysed by SDS-PAGE. Following electrophoresis the resolved proteins were visualised by silver staining. It was therefore necessary to determine a suitable protein concentration to load on to the gel. For this purpose a preliminary SDS-PAGE was conducted using fractions (46-48) obtained from the chromatofocusing of Peak III, Fig. 26. This region contained a similar concentration of protein to the region comprising the immunoreactive species but did not contain any immunoreactive material. Electrophoresis followed by silver staining was conducted as described in Fig. 29. Silver staining artifacts occuring across the width of the gel, including gel lanes containing only sample buffer and particularly at the stacking-resolving gel interface. This effect was most marked when the gel was stained to maximum sensitivity. As shown in Fig. 29, lane A was over developed for this gel, but lane B indicated good resolution of protein bands, with fainter staining. The protein concentrations in lanes C, D, E and F were too low to detect

FIG.28 . COMPARISON OF THE ME RIA DISPLACEMENT CURVE WITH THE DISPLACEMENT CURVE OBTAINED BY SERIAL DILUTIONS OF ME-IR LIBERATED FROM A 31 kDALTON SPECIES BY TRYPSIN AND CARBOXYPEPTIDASE B TREATMENT.

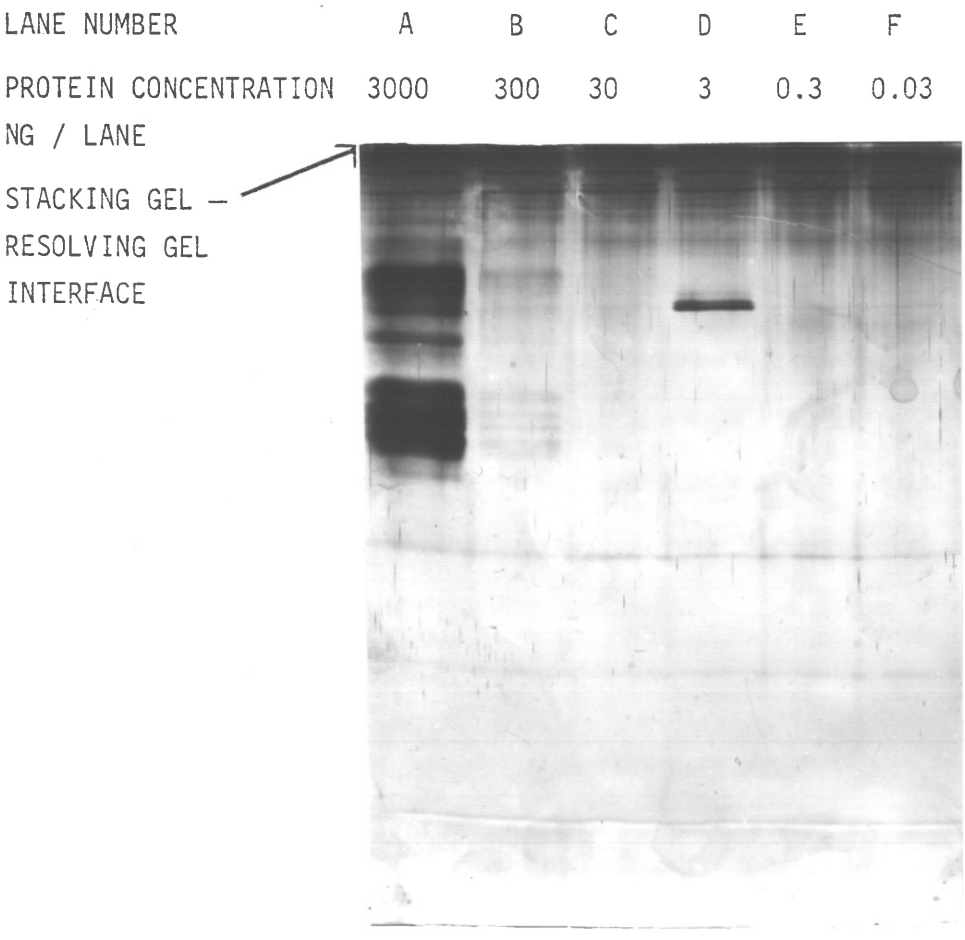


The ME RIA was performed as previously described. In addition to ^{125}I-ME and antiserum, prepared in RIA buffer, the incubation medium contained 100µl of

a) 1-350 fmol synthetic ME or

b) serial dilutions of ME-IR liberated from a 31 kDal. species by trypsin and carboxypeptidase B treatment of a nitrocellulose "western" blot. fmol ME -IR and µl nitrocellulose digest have been plotted using a log.scale. $B/_{Bo}$ is the ratio of ^{125}L-ME bound in the presence and abscence of ligand.

FIG. 29 . SILVER STAINING OF PROTEINS CORRESPONDING TO FRACTIONS 46-48 SEPARATED BY CHROMATOFOCUSING OF PEAK III



Fractions (46-48) eluted during the chromatofocusing of Peak III were pooled and equilibrated by ultrafiltration in 5% acetic acid prior to lyophilisation. Lyophilised protein was resuspended and serially diluted in ten fold dilutions of sample buffer to give a final concentration range of 3000 (A) - 0.003 (F) ng / 150µl capacity gel lane. Samples were subjected to SDS-PAGE and the resolved proteins were visualised by silver staining.

single bands in this experiment. Therefore a protein concentration of 3-0.3 µg/150 ul capacity gel lane gave good visualisation of the components of this mixture. This represents a protein concentration of 235-23.5 ng/mm^2 of gel lane surface area.

Although the protein species within fractions 46-48 were not the same as those in the immunoreactive peak both samples contained a similar M_r range of proteins, as determined by Sephacryl-300, at a similar concentration. Therefore, the estimation of 3-0.3 µg of protein/gel lane was adopted as a suitable concentration range to employ when visualising the proteins resolved by SDS-PAGE of the pI 5.0 immunoreactive peak.

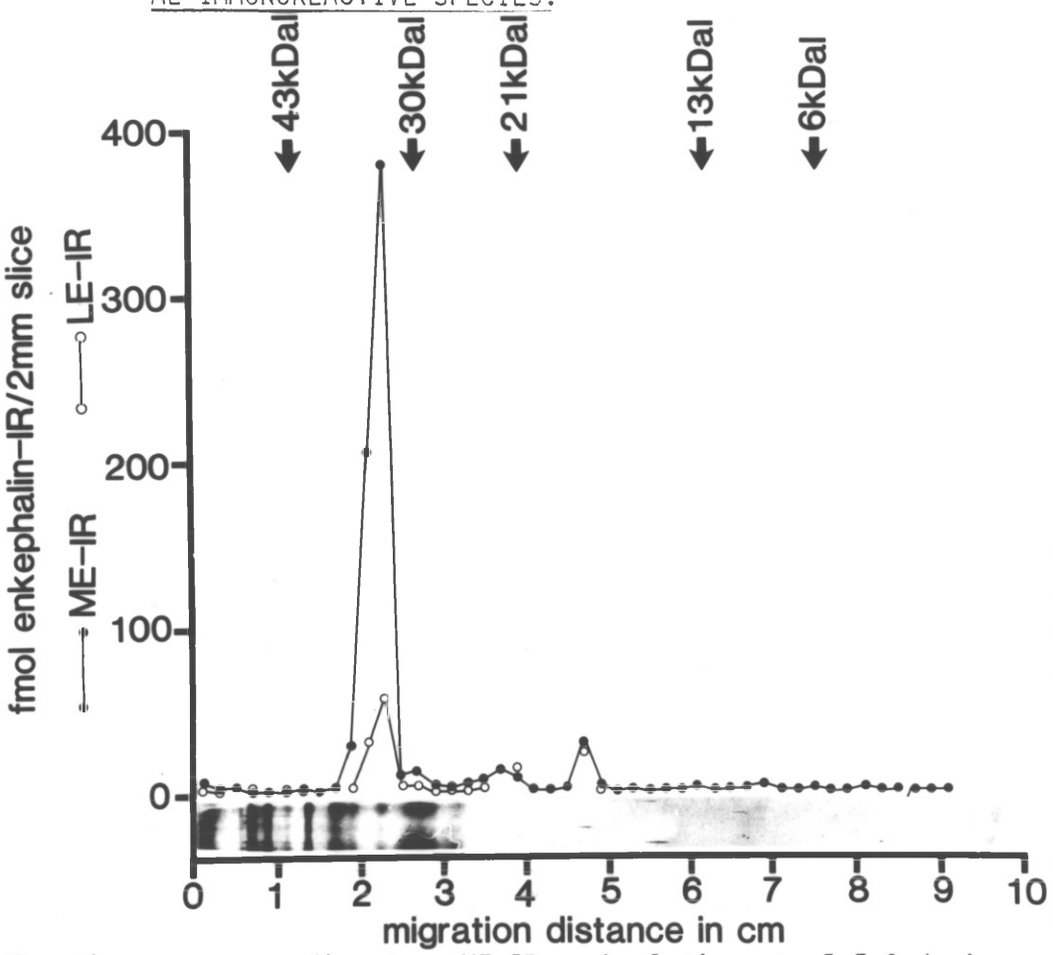
The pI 5.0 enkephalin immunoreactive species was subjected to SDS-PAGE and silver staining. The resolved proteins are shown in the gel lane at the bottom of Fig. 30.

(ii) Electrophoretic transfer blot

Electrophoretic transfer of the protein bands resolved by SDS-PAGE on to nitrocellulose was performed as described in Fig. 30. Treatment of the nitrocellulose paper with trypsin and carboxypeptidase B, followed by RIA for ME and LE, showed the presence of a single major band of immunoreactive material with an apparent M_r of 31000, Fig. 30. Both ME-IR and LE-IR were detected in this band with the ratio of ME to LE being 6.3:1. The ME : LE ratio of the sample before SDS-PAGE was 5.6:1 (this corresponded to 4.69 $\pm$ 0.28* fmol ME-IR and 0.83 $\pm$ 0.13* fmol LE-IR/µg of lyophilised protein).

* mean $\pm$ S.E. where n = 4

FIG.30 . SDS-PAGE AND ELECTROPHORETIC TRANSFER BLOT OF A pI 5.0 ME-IMMUNOREACTIVE SPECIES.



Fractions corresponding to a ME-IR peak eluting at pI 5.0 during chromatofocusing were pooled and equilibrated in 5% acetic acid,by ultrafiltration, prior to lyophilisation. Protein (153µg) was resuspended in sample buffer and an aliquot corresponding to 135 µg loaded on to the gel lane required for electrophoretic protein transfer. Samples required for silver staining were prepared by serial dilution of the remaining aliquot in sample buffer to give a final protein concentration of 2µg and 0.2µg/gel lane.Following SDS-PAGE protein bands were either electrophoretically transfered to nitrocellulose or visualised by silver staining (silver stain shown at base of figure). The nitrocellulose was further processed by immunoblot and digestion as described in FIG.27 . fmol ME-IR/2mm slice (●—●) and fmol LE-IR/2mm slice (○—○). M_r calibration is shown in FIG.27B . RIA data has been adjusted with respect to antisera cross-reactivity.

5.3.4 Estimation of the ME : LE ratio in Peak III by reverse phase HPLC

Preliminary experiments showed that peptides liberated by sequential digestion of partially purified guinea pig striatal extracts were separated into two peaks of ME-IR by reverse phase HPLC. These peaks had the same retention times as synthetic ME and an oxidised form of ME. Therefore, to ensure only one form of ME was present in samples required for reverse phase HPLC, this peptide was routinely converted to an oxidised form by incubation with hydrogen peroxide. As previously stated, the ME antiserum used in these studies displayed 100% cross-reactivity, on a molar basis, with oxidised ME. Therefore, oxidation of ME prior to reverse phase HPLC did not affect ME values.

Peak III material, from a Sephacryl-300 separation of guinea pig striatal extract, was sequentially digested with trypsin followed by carboxypeptidase B and subjected to reverse phase HPLC as described in Fig. 31. ME-IR (4.17 ± 0.24 pmol)* and LE-IR (0.61 ± 0.05 pmol)* were detected eluting with the same retention times as synthetic oxidised ME and synthetic LE, respectively. ME-IR was not detected eluting in the position corresponding to non-oxidised synthetic ME. Neither ME-IR nor LE-IR were detected in any other fraction collected over the 25 min elution time. Therefore, the ME-IR and LE-IR liberated by a sequential trypsin and carboxypeptidase B digestion of Peak III had the same characteristics as synthetic ME and LE, when subjected to reverse phase HPLC under these conditions.

* mean ± S.E. where n=3

FIG.31 . ESTIMATION OF THE ME:LE RATIO WITHIN PEAK III BY REVERSE PHASE HPLC

SAMPLE PREPARATION

Fractions corresponding to Peak III, resolved by Sephacryl-300 chromatography of guinea pig striatal extract, were pooled and diluted to a final concentration of 2M urea with 50mM Tris-HCl, pH 7.9 at 37°C, containing 2mM $CaCl_2$. The sample was then digested with trypsin (50µg / ml; 16h at 37°C) followed by carboxypeptidase B (0.1µg/ml; 1h at 37°C). After each incubation the enzyme was heat inactivated (20 min at 100°C) prior to sample concentration over Porapak Q. The resulting peptide fraction was incubated in 1.5% H_2O_2 for 1h at 37°C and then evaporated to dryness under vacuum. The residue was finally resuspended in the HPLC running buffer- 20mM ammonium acetate pH 4.0 containing 20% (v/v) acetonitrile.

REVERSE PHASE HPLC

The sample (50µl in HPLC running buffer containing an estimated 4 pmol ME-IR) was injected on to an Ultrasphereoctyl column (Altex 0.45 x 25 cm). Peptides were eluted isocratically in the HPLC running buffer at a flow rate of 1 ml / min. Fractions (500µl) were collected and suitable aliquots lyophilised prior to resuspension in RIA buffer and assay for ME-IR and LE-IR. Samples were radioimmunoassayed at several dilutions over the range 1-100 fmol enkephalin.

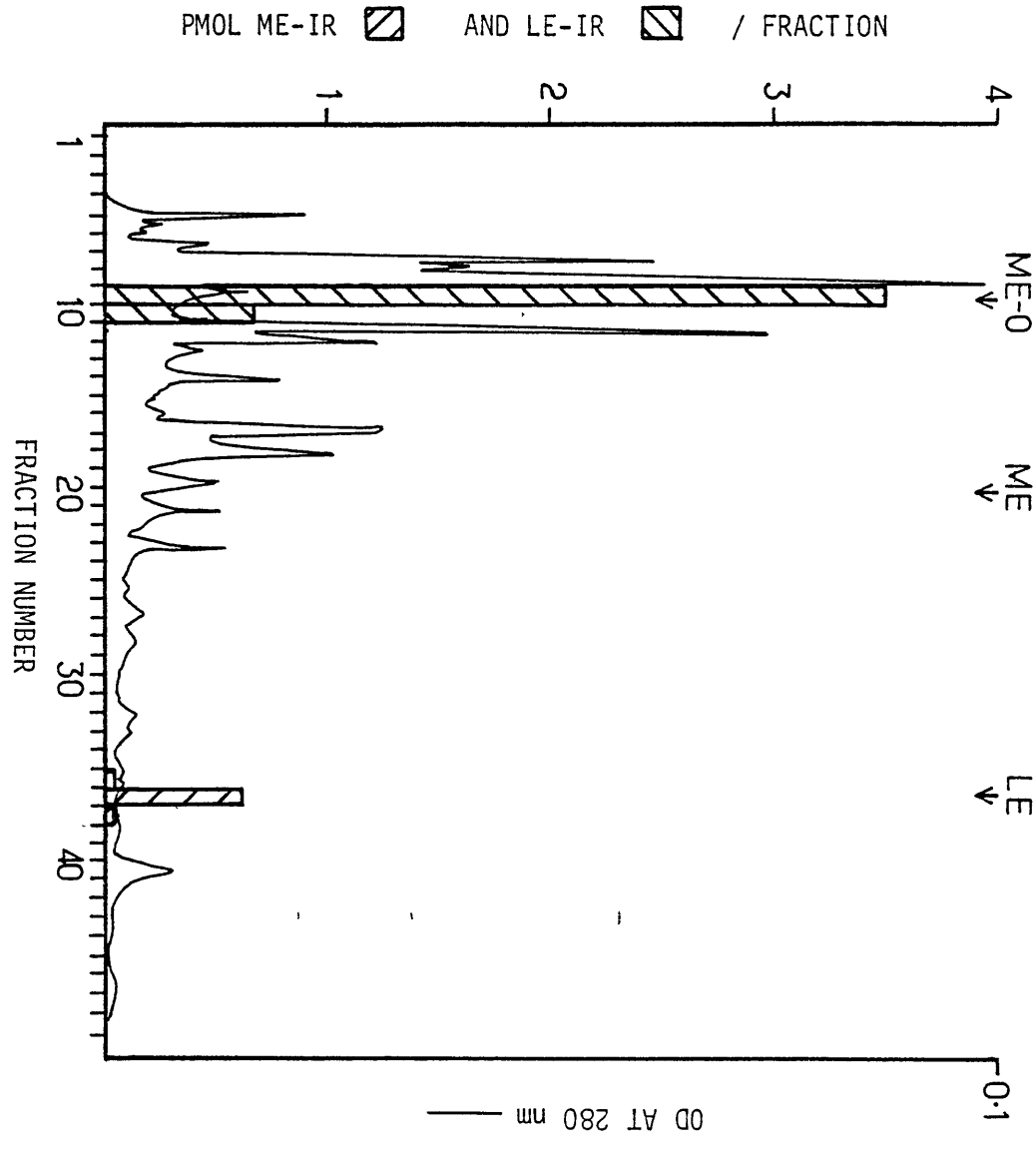
The reverse phase HPLC retention times (min) for the following synthetic peptides were:

ME (oxidised)	5.30
ME	10.40
LE	18.65

H_2O_2 represents hydrogen peroxide.

FIG. 31 .

ESTIMATION OF THE ME: LE RATIO WITHIN PEAK III BY REVERSE PHASE HPLC



5.3.5 ME-Arg^6-Phe^7 assay

The cDNA sequencing of adrenal proenkephalin has shown that ME-Arg^6-Phe^7 is present at the COOH-terminus of this molecule. The endopeptidase S. aureus protease cleaves at the carboxyl side of glutamate and aspartate residues (Houmard and Drapeau, 1972) and would therefore be expected to release the nonapeptide Lys-Arg-ME-Arg^6-Phe^7 from adrenal proenkephalin (Fig. 32). This nonapeptide could then be detected by a COOH-terminally directed ME-Arg^6-Phe^7 antiserum.

Peak III from a Sephacryl-300 separation of guinea pig striatal extract was characterised for ME-, LE- and ME-Arg^6-Phe^7-IR as described in Table 11. ME-Arg^6-Phe^7-IR was detected both before and after digestion with S. aureus protease. The value obtained from untreated samples was 0.95 pmol/mg of protein whereas in the corresponding digested samples the figure was 1.4 pmol/mg of protein. The ratios of opioid peptides derived from Peak III, as estimated by immunoreactivity after digestion, were :

ME : ME-Arg^6-Phe^7, 7.3:1

LE : ME-Arg^6-Phe^7, 1.4:1

ME : LE, 5.1:1

Enzymatic digestion of Peak III material liberated ME, LE- and ME-Arg^6-Phe^7-IR in the ratio 5.1:1:0.7.

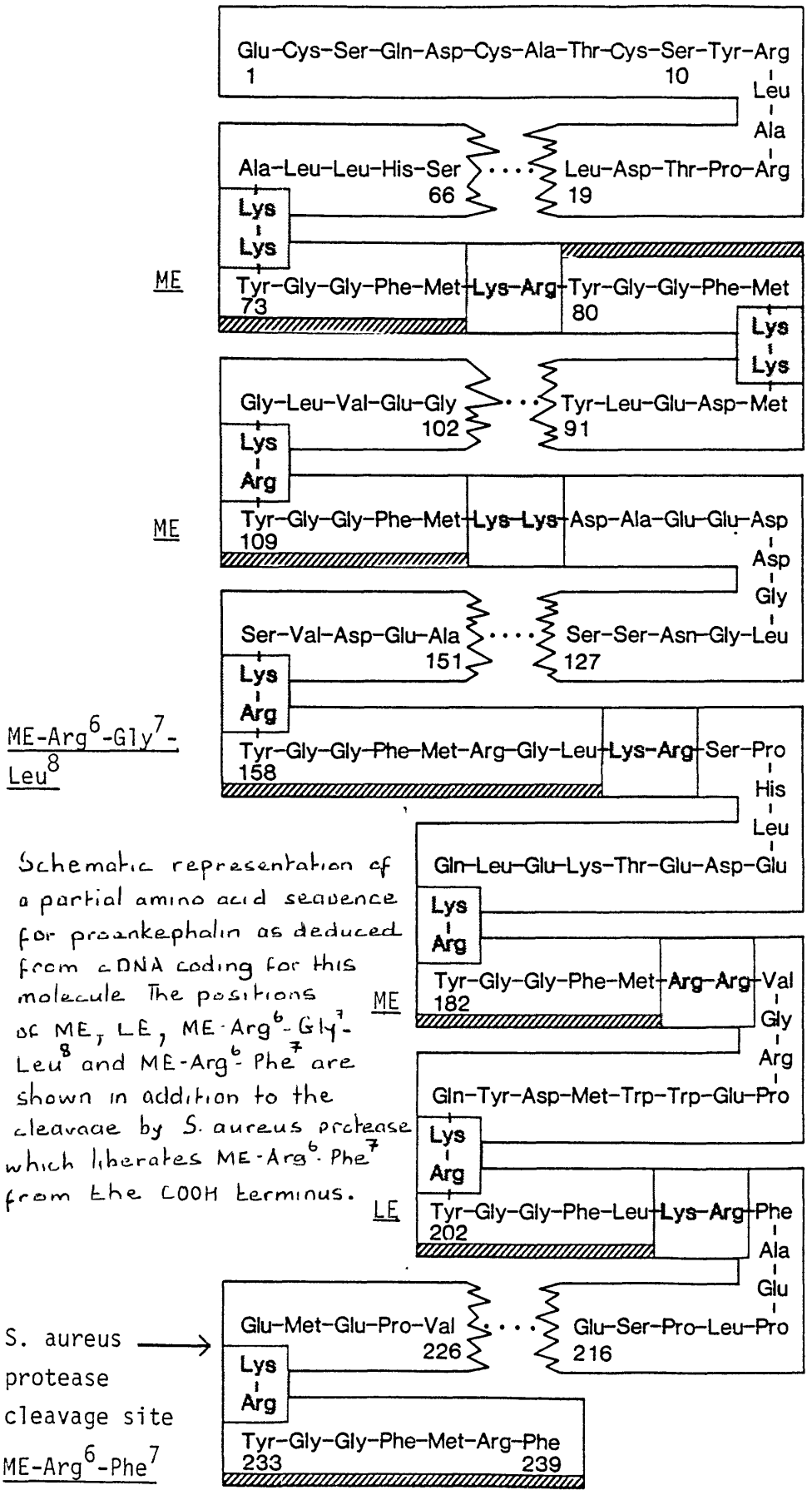
5.4 Peak IV

5.4.1 Determination of pI for Peak IV by Isoelectricfocusing and chromatofocusing

Peak IV was subjected to flat-bed IEF over the pH range 3.5-10.

Fig. 32.

SEQUENCE OF PRO-ENKEPHALIN modified from Rossier, 1982



Schematic representation of a partial amino acid sequence for proenkephalin as deduced from cDNA coding for this molecule. The positions of ME, LE, ME·Arg^6-Gly^7-Leu^8 and ME-Arg^6-Phe^7 are shown in addition to the cleavage by S. aureus protease which liberates ME-Arg^6-Phe^7 from the COOH terminus.

TABLE 11

ANALYSIS OF OPIOID PEPTIDE CONTENT OF PEAK III

ENZYME TREATMENT	RIA	P MOL IR / MG PROTEIN
Trypsin and carboxypeptidase B	ME	10.2
Trypsin and carboxypeptidase B	LE	2.0
S. aureus protease	ME-ARG^{6}-PHE^{7}	1.4
No enzymatic treatment	ME-ARG^{6}-PHE^{7}	0.95

Fractions corresponding to Peak 111 resolved by Sephacryl-300 chromatography of guinea pig striatal extract were pooled and equilibrated in 5% acetic acid, by ultrafiltration, prior to lyophilisation. Lyophilised protein was resuspended in 50mM Tris-HCl / 2mM $CaCl_2$ pH 7.9 at 37°C by agitation (1h at 37°C)and then centrifuged (30 min at 10000g_{av} at 4°C). Aliquots of the supernatant were digested either by trypsin (100µg / ml for 16h at 37°C) followed by carboxypeptidase B (0.1µg / ml for 1h at 37°C), or by S. aureus protease (40µg / ml for 16h at 37°C) and radioimmunoassayed as indicated in the TABLE.

Enzymatic digestion revealed two main peaks of ME-IR equilibrating at pH 6.0 and pH 4.5-5.5, Fig. 33.

Chromatofocusing of Peak IV yielded several peaks of ME-IR following trypsin and carboxypeptidase B treatment. The ME-IR species eluting within the range of the pH gradient had isolectric pH values below pH 4.7 with a major peak at pH 4.3. Digestion of the NaCl wash also liberated a large peak of ME-IR, Fig. 34. The recovery of Peak IV ME-IR after chromatofocusing under these conditions was 80%.

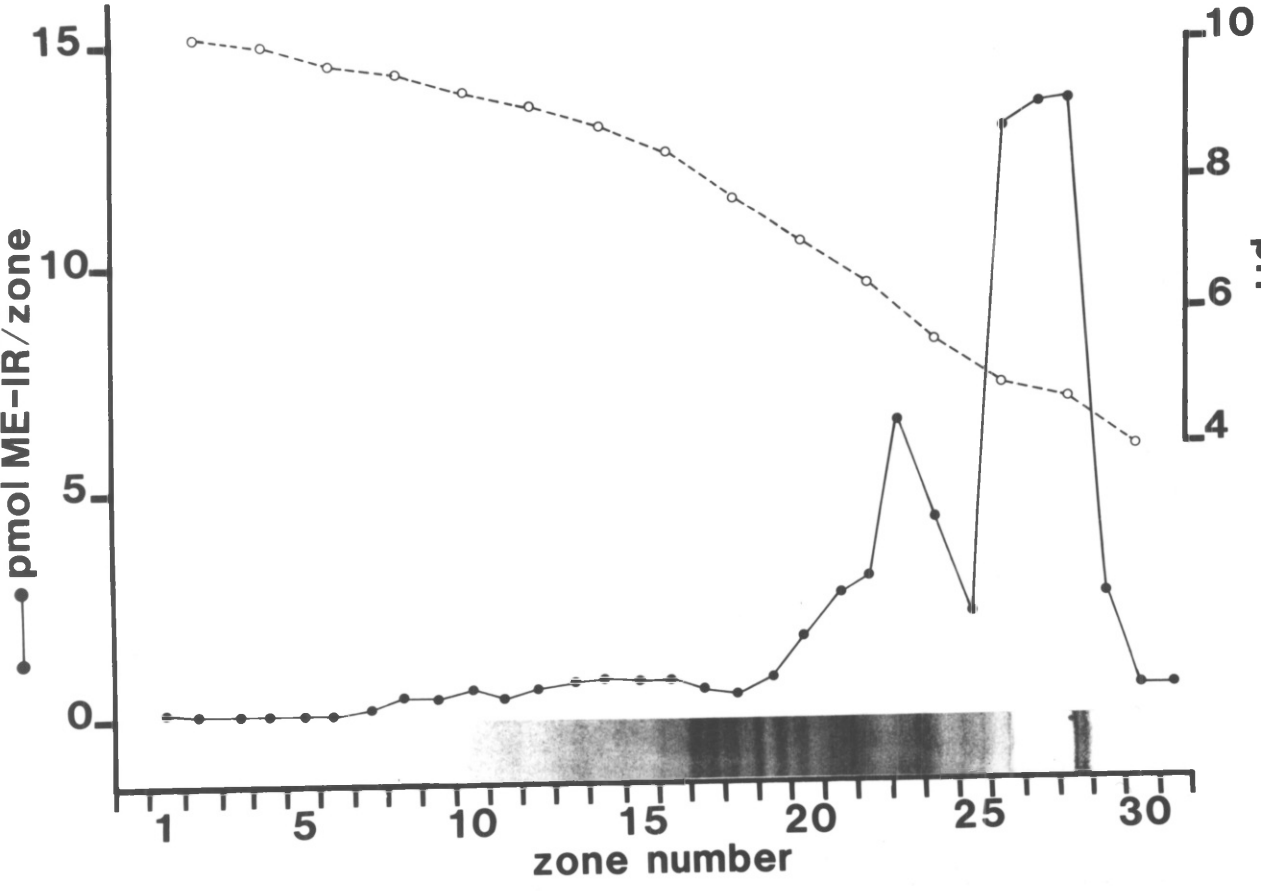
5.5 Discussion

5.5.1 Peak I (apparent M_r > 70000)

Initial studies exploited the parameter of pI to separate putative opioid precursors present in the high molecular weight region separated over Sephadex G-200. Anion exchange chromatography indicated the presence of several species of opioid-containing peptides in this region (this was subsequently confirmed by Sephacryl-300 chromatography). However, this technique did not fully resolve these species, with the exception of material with an apparent pI of > 8.8 which was not adsorbed on to the column. This non-adsorbed material focused in a single zone at pI 9.6 when subjected to IEF and probably represents a single species. When rechromatographed over Sephacryl-300 this species eluted with an apparent M_r > 70000, in the position observed for the Peak I material present in crude tissue extracts. These results indicate that the Peak I material consists of a single opioid-containing species which has a basic pI of 9.6, a M_r > 70000 and releases biossayable opioid activity, but not ME- or LE-IR, after digestion. This material was consistently present in the striata of

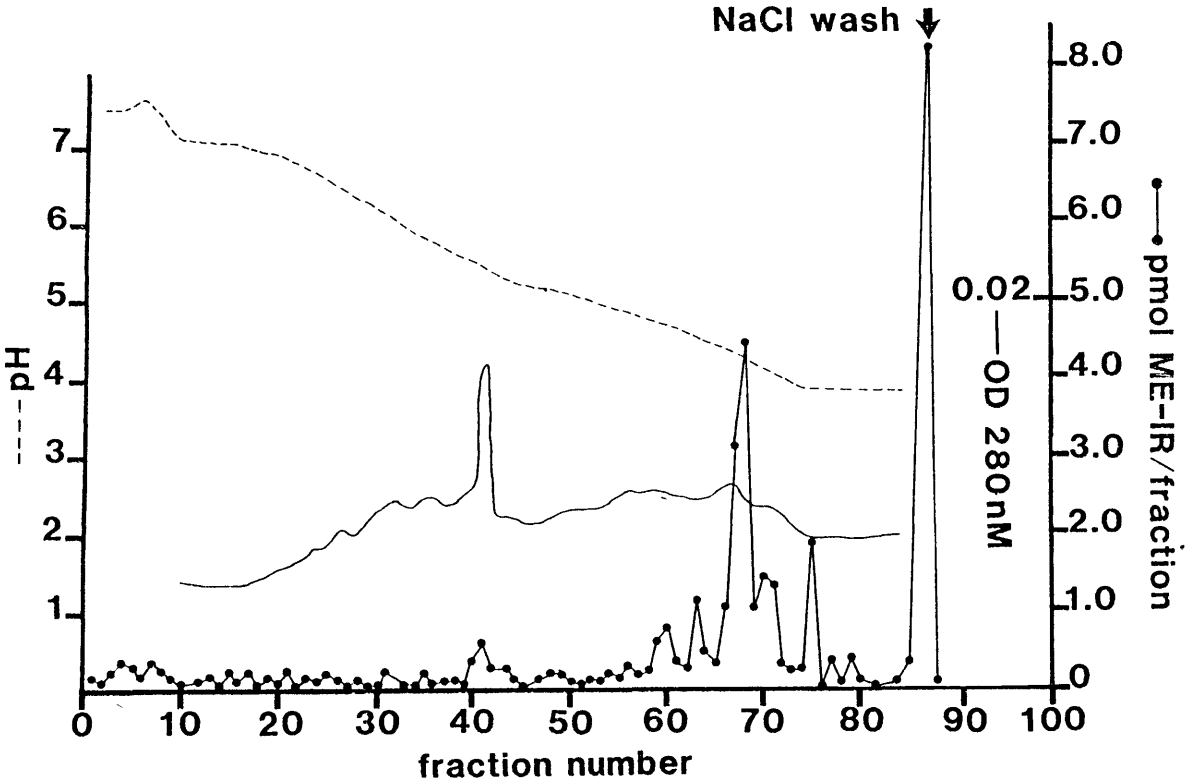
FIG.33 .

ISOELECTRICFOCUSING OF PEAK IV



Fractions corresponding to Peak IV from a Sephacryl-300 separation of guinea pig striata were pooled and subjected to IEF as described in FIG.25 . pmol ME-IR/ zone (●——●); pH gradient (○---○);protein bands visualised by Coomassie blue are shown below

FIG.34 . CHROMATOFOCUSING OF PEAK IV



Pooled fractions corresponding to Peak IV from a Sephacryl-300 separation of guinea pig striata were subjected to chromatofocusing as described in FIG. 26 . pmol ME-IR / fraction (●——●); pH gradient (------); absorbance at 280nm (———).

all species examined, but was not detected in guinea pig adrenal preparations. The presence of a putative opioid precursor with an apparent M_r of 90000 on SDS-PAGE has been reported by Beaumont et al., (1980). This protein was present in considerably higher levels than those observed for Peak I in this study and contained ME- and LE-IR. However, the specificity of the antisera used by Beaumont et al., (1980) was not well characterised.

The sequences of all known opioid peptides are contained within the structures of the three opioid precursors, proenkephalin, prodynorphin and POMC. Moreover the mRNA coding for these precursors could not code for a protein with the apparent M_r of Peak I. It is possible, therefore, that Peak I represents an as yet unknown opioid precursor which contains a novel opioid peptide(s). A less dramatic explanation would be simply that Peak I is an artifact of extraction and that digestion failed to liberate the free COOH-terminus necessary for antisera recognition.

Purification of Peak I presented a formidable task since this material represents a small percentage of the high molecular weight proteins present in striatal extracts. The problem was exacerbated by the absence of a sensitive assay since the use of the MVD bioassay, with a detection limit of $\approx$ 0.5 pmol ME equivalents, was demanding in terms of product. It would probably be more profitable to characterise the opioid peptide(s) released from this species by digestion in terms of their receptor binding specificity, M_r and HPLC characteristics.

5.5.2 Peak II (apparent M_r 66000)

Peak II was not further characterised following Sephacryl-300

chromatography. As in the case of Peak I this species, with an apparent M_r of 66000, could not be produced by translation of known opioid precursor mRNA's. Following digestion, Peak II liberated both ME- and LE-IR as well as bioassayable opioid activity. This observation, combined with the apparent M_r data, suggests that Peak II opioid-containing material may be formed by dimerization of Peak III, or agregation of unknown proteins with other opioid-containing peptides, present in the extract, which persists under dissociating conditions. This is supported by the observation that Peak II, in contrast to the other opioid-containing peptides, was not present in striatal tissue preincubated for 30 min at 25°C before extraction, suggesting non-specific degradation of this species.

5.5.3 Peak III (apparent M_r 29000)

Peak III appears to be the major enkephalin-containing species present in guinea pig striata. The protein, from guinea pig extracts, was partially purified by chromatofocusing (pI 5.0) and was found to have an apparent M_r of 31000 on SDS-PAGE.

Digestion of Peak III with trypsin and carboxypeptidase B, followed by RIA, showed the presence of both ME-IR and LE-IR. These immunoreactivities were found to have the same characteristics as the corresponding synthetic peptides when subjected to reverse phase HPLC. The dilution curve for ME-IR, liberated from the 31 kDalton species by digestion of the western blot, was parallel to the synthetic ME RIA displacement curve indicating the immunoreactivity corresponded to authentic ME. It was concluded that the Peak III opioid-containing species contained both ME and LE sequences. ME : LE ratios were estimated at several different stages of purification and the values

obtained following Sephacryl-300 chromatography and reverse phase HPLC (6.7:1), chromatofocusing (5.6:1) and electrophoretic transfer blotting (6.5:1) were found to be close to the ratio of these peptides found within adrenal proenkephalin (6:1). Therefore, combined with the molecular weight data, obtained from SDS-PAGE, these ratios suggested the presence of intact proenkephalin in striatal tissue.

As previously described, the heptapeptide ME-Arg^6-Phe^7 constitutes the COOH-terminal seven residues of proenkephalin. Further evidence for the presence of proenkephalin in striatal tissue was obtained when ME-Arg^6-Phe^7-IR was detected by a direct assay of extracts from Peak III, using a COOH-terminally directed antibody to ME-Arg^6-Phe^7. This immunoreactivity was increased after digestion with S. aureus protease, which, as previously discussed, would be expected to release Lys-Arg-ME-Arg^6-Phe^7 from the COOH-terminus of adrenal proenkephalin. After enzymatic digestions the peptide ratios obtained for ME : ME-Arg^6-Phe^7 (7.4:1) and LE : ME-Arg^6-Phe^7 (1.4:1) were again close to those expected from a similar treatment of adrenal proenkephalin (6:1 and 1:1 respectively). These data indicated that the ME-Arg^6-Phe^7-IR detected was located at the COOH-terminus of the Peak III species. The presence of this peptide could not be confirmed by HPLC since the synthetic peptide Lys-Arg-ME-Arg^6-Phe^7 was not available as a standard.

In conclusion, an enkephalin-containing species has been partially purified, from guinea pig striatum, which has an apparent M_r of 31000 on SDS-PAGE and contains ME-, LE- and ME-Arg^6-Phe^7-IR in a 5.1:1:0.7 ratio. Digestion of Peak III liberated the opioid peptides ME and LE in a 6.7:1 ratio, as determined by reverse phase HPLC. In addition, ME-Arg^6-Phe^7-IR has been liberated from the COOH-terminus of

this molecule. These data suggest that Peak III corresponds to intact proenkephalin. This postulation can only be confirmed by the purification and sequencing of the opioid-containing Peak III species.

It was noted that the apparent M_r of 31000 for the Peak III species was higher than the theoretical value of 27.3 kDalton expected for adrenal proenkephalin. However, high apparent M_r's on SDS-PAGE seem to be a common feature for all proenkephalin intermediates containing the 5.3 kDalton species purified from bovine adrenal medulla (Jones et al., 1982b; Kilpatrick et al., 1982a). This 5.3 kDalton peptide contains a glycosylation site. Glycoproteins are known to migrate with a high M_r on SDS-PAGE and, although no evidence has been found for glycosylation of adrenal opioid-containing peptides (Kilpatrick et al., 1983), this cannot be ruled out in the CNS. It is also possible that guinea pig proenkephalin, whilst showing considerable sequence homology with adrenal proenkephalin, may contain non-conserved sequences resulting in a higher M_r.

Attempts to visualise this 31 kDalton species using immunoblot were unsuccessful. This technique has been elegantly applied to the characterisation of proenkephalin intermediates in the bovine adrenal medulla (Patey et al., 1984). In this study, the concentration of the 31 kDalton opioid-containing peptide was probably below the sensitivity of the ME antiserum used, since a concentration of 10-100 pmol of ME-IR/band was necessary to visualise the adrenal species. A high background of non-specific staining was also observed which could have obscured specific labelling.

5.5.4 Peak IV (apparent M_r 16000-6000)

Peak IV was resolved into at least three separate ME-containing peaks on chromatofocusing (pI's < 4.7) and probably represents intermediates in the processing of proenkephalin. The presence of such intermediates is supported by the work of Lindberg and Yang (1984) who have identified a 8 kDalton form of ME-Arg^6-Gly^7-Leu^8-IR in several regions of rat brain including striatum, medulla pons and hypothalamus. Similiar intermediates have also been detected in large amounts in the bovine hypothalamic magnocellular neuronal system (Liston et al., 1984d).

CHAPTER SIX

DISCUSSION

Chapter six

Discussion

The primary amino acid sequence of proenkephalin was first deduced from the cloned cDNA which codes for this protein in the adrenal gland (Comb et al., 1982b; Noda et al., 1982). There has been some controversy over whether biosynthesis of opioid peptides in the CNS proceeds via the processing of a similar precursor (Beaumont et al., 1980). Evidence based on cell-free translation of striatal mRNA (Sabol et al., 1983) and the immunological similarities between adrenal and brain proenkephalin derived peptides (Watson et al., 1982; Liston et al., 1983; Lindberg et al., 1984) suggested that certain sequences within proenkephalin are highly conserved between adrenal and neural tissues. This hypothesis has recently been confirmed by the publication of the rat brain preproenkephalin cDNA sequence (Yoshikawa et al., 1984; Howells et al., 1984).

However, although many adrenal proenkephalin intermediates have been purified and sequenced (Udenfriend and Kilpatrick, 1983), similar opioid-containing peptides have not been isolated from the CNS. There have been conflicting reports as to whether putative opioid precursors accumulate in neural tissue (Beaumont et al., 1980; Kojima et al., 1982; Ikeda et al., 1982; Liston et al., 1983) and this has led to the belief that the processing of proenkephalin in the brain proceeds rapidly to the octa-, hepta- and pentapeptides.

This study on opioid peptide precursors in the striatum has attempted to provide information on several interrelated questions :

1. How homologous are adrenal and striatal proenkephalin ?

2. Do high molecular weight opioid-containing peptides accumulate in the striatum ?
3. Is the processing of proenkephalin different in adrenal and striatal tissues ?

The results from this investigation have been analysed in previous chapters and the remainder of this discussion will concentrate on the significance of the data when considering these questions.

6.1 Conservation of proenkephalin

Information concerning the structure of proenkephalin is now available at the nucleotide level for species as evolutionarily divergent as the toad (Xenopus laevis), rat, cow and human.

The primary structure of proenkephalin was first deduced from the sequence of cDNA cloned from bovine adrenal medulla (Noda et al., 1982). As previously described, bovine adrenal proenkephalin contains four copies of ME and one copy each of LE, ME-Arg^{6}-Gly^{7}-Leu^{8} and ME-Arg^{6}-Phe^{7}. The octa- and pentapeptide sequences are flanked by basic paired amino acids whilst the heptapeptide is located COOH-terminally, preceded by a similar pair of basic residues.

The primary structure of human adrenal proenkephalin is remarkably similar to bovine, differing only with respect to thirty-five amino acid residues (Comb et al., 1982b). Recently Yoshikawa et al.,(1984) and Howells et al.,(1984) have, for the first time, cloned the cDNA coding for neuronal proenkephalin. The deduced primary amino acid sequence of rat brain preproenkephalin is fundamentally the same as human and bovine. The opioid peptide

sequences, paired basic amino acids, NH_2-terminally located cysteine residues and the glycosylation site are all highly conserved.

Toad proenkephalin is again similar in organisation, containing seven enkephalin sequences separated by spacers comparable in length to those in other proenkephalin species. The opioid sequences are again highly conserved, in contrast to the spacer sequences, suggesting that only the opioid peptides may have a functional significance. It is of interest that, in the toad, LE is replaced by ME and the octapeptide is present as ME-Arg^6-Gly^7-Tyr^8 (Herbert et al., 1983; Martens et al.,1984).

Previously, only the structure of adrenal proenkephalin had been confirmed at the protein level, by the isolation and sequencing of biosynthetic intermediates. In this study, a 31 kDalton species has been partially purified from guinea pig striata which contains ME-, LE- and ME-Arg^6-Phe^7-IR in a 5.1:1:0.7 ratio, with the latter peptide present at the COOH-terminus. This is the first time a proenkephalin species containing all the enkephalin sequences has been detected in any tissue and it has been postulated that this molecule represents intact proenkephalin. The presence of this species in guinea pig striatum demonstrates that the proenkephalin gene is translated as well as transcribed in this tissue and supports the hypothesis that this molecule acts as a precursor for enkephalin in both neuronal and non-neuronal tissues.

In conclusion, whether from a mammalian, amphibian, neuronal or non-neuronal source the opioid peptide sequences within proenkephalin appear highly conserved.

6.2 Differential processing of opioid-peptide precursors

The three opioid peptide precursors POMC, proenkephalin and prodynorphin can give rise to a variety of opioid peptide products, Fig. 35. At least eighteen opioid peptides have been isolated and purified from mammalian brain, adrenal and pituitary tissues and all these sequences are contained within the three separate precursors (Nakanishi et al., 1979; Noda et al., 1982; Kakidani et al., 1982). Studies using a combination of chemical characterisation and highly specific antisera have unequivocally shown that the levels of peptide products from a particular opioid precursor vary between tissues in a manner suggestive of differential processing (Zakarian and Smyth, 1982; Cone et al., 1983; Liston et al., 1984d). Differential processing gives rise to products with differing receptor selectivities and pharmacological profiles and may, therefore, play a major role in the physiological function of the neuronal and non-neuronal endogenous opioid systems. The differences in processing could occur at the gene, transcription or protein level.

6.2.1 Processing at nucleotide level

So far, the various reports have described the structure of a single functional gene for POMC in mouse, rat, cow and human. Little information is available at the gene level for proenkephalin and, particularly prodynorphin, but preliminary experiments have indicated that only one gene for proenkephalin is present in human and rat (Herbert et al, 1983; Rosen et al., 1984).

It has been demonstrated that the separate precursors for calcitonin and calcitonin gene related peptide are derived in a tissue

specific manner by alternate splicing at the mRNA level (Amara et al., 1982). This type of post-transcriptional processing is also responsible for the production of the two precursors for substance P and related peptides, α- and β-preprotachykinin (Nawa et al., 1984). A similar phenomenon has been observed for POMC in the neurointermediate lobe of rat pituitary, where two species of mRNA occur as a result of splicing (Herbert et al., 1983). Therefore post-transcriptional modifications cannot be ruled out as a processing mechanism for opioid-peptide precursors in certain tissues. However, the detection of the mRNA coding for preproenkephalin in rat striatum (Yoshikawa et al., 1984), combined with evidence from this study, that the protein itself is present in guinea pig striatum, suggests that processing in this tissue occurs post-translationally.

6.2.2 Processing at post-translation level

Post-translational events, in particular proteolysis, are thought to play a profound role in determining the final opioid precursor products. The opioid peptides are most commonly liberated from their precursors by cleavage at paired basic residues, although exceptions to this general rule occur within all three opioid precursors. DYN-(1-8) and DYN-B require cleavage at a single basic amino acid to release their COOH-termini from prodynorphin (Kakidani et al., 1982; Fischli et al., 1982b), the proenkephalin fragments BAM 12P and BAM 22P are released at their COOH-termini by cleavage at glutamic acid-tryptophan and glycine-glycine residues (Mizuno et al., 1980a and 1980b) whilst γ - and α -endorphin are the result of leucine-phenylalanine and threonine-leucine cleavages (Graf et al., 1976; Guillemin et al., 1977). The most interesting of these "uncommon" cleavages occurs within prodynorphin as DYN-(1-8) and DYN-B

have been found as the major products from prodynorphin in the brain, occuring with a differential distribution (Cone et al., 1983).

The existence of a pair of basic residues does not guarantee that processing will occur at that site, as many opioid precursor derived peptides have been isolated which contain intact double basic amino acids, for example β-END-(1-31) from POMC (Li and Chung, 1976a), peptide E and peptide F from proenkephalin (Kilpatrick et al., 1981a; Kimura et al., 1980) and α-neoendorphin, DYN-A and DYN-B from prodynorphin (Kangawa et al., 1981; Goldstein et al., 1981; Fischli et al., 1982a).

It is of great interest that the extent to which all the opioid peptide precursors are processed is tissue specific and, in general, the smaller products predominate in the brain. The relative ratio of the peptide products varies between brain regions and between neuronal and non-neuronal tissues. Clearly the basic paired residues are not uniformly processed, with certain sites being less susceptible to cleavage. In the following section the information on the processing of POMC and prodynorphin will be outlined briefly, followed by a more detailed consideration of proenkephalin processing.

6.2.3 Proopiomelanocortin

The neurointermediate and anterior lobes of the pituitary process POMC differently, with the production of smaller and more modified products in the neurointermediate lobe. This generalisation holds true for both non-opioid and opioid regions of the precursor but only the latter will be considered here. In the anterior lobe the majority of β-END-(1-31) exists in the form of β-LPH with the authentic

β-END-(1-31) in an unmodified form. In contrast, the neurointermediate lobe contains little β-LPH and β-END-(1-31) is present mainly in the shortened forms β-END-(1-27) and β-END-(1-26). Moreover, all three forms of B-END occur principally as their non-opioid α-N-acetylated derivatives (Smyth and Zakarian, 1982; Akil et al., 1981).

The situation in the brain resembles the neurointermediate lobe, in that processing is generally more complete. The three forms of β-END vary in concentration between brain regions, with β-END-(1-31) the principle product in hypothalamus, midbrain and amygdala and the shorter peptides more prevalent in the hippocampus and brain stem. The α-N-acetylated forms are less common in the brain, but can be detected at high concentrations in brain stem regions (Zakarian and Smyth, 1982).

6.2.4 Prodynorphin

If the processing of prodynorphin went to completion at every basic pair of residues, the only opioid product from this precursor would be LE. However, it is still not known whether LE is ever generated from prodynorphin, as this peptide is always found in association with proenkephalin-derived peptides (Watson et al., 1982). Cleavage of prodynorphin is known to give rise to two series of peptides, the dynorphins and α - and β-neoendorphin. In the anterior lobe of the pituitary DYN-B and DYN-A (DYN-1-17) seem to be contained within a 6 kDalton species and there is little α - and β-neoendorphin. Processing is again more complete in the neurointermediate lobe. In this region α - and β-neoendorphin are present in similiar concentrations, in constrast to most brain areas, particularly the striatum, where α-neoendorphin is the predominant form. Similarly the

processing of DYN-A into DYN-(1-8) is more pronounced in the brain than in either neurointermediate lobe or spinal cord. Moreover, a comparison of the molar ratio of these peptides in different brain regions demonstrates that the degree of processing is not uniform, with higher concentrations of DYN-(1-8) in striatum and midbrain. Finally, in the neurointermediate lobe and most brain areas the predominant species associated with DYN-B-IR is authentic DYN-B, although higher molecular weight forms have also been detected (Weber et al., 1982 ; Cone et al., 1983 ; Seizinger et al., 1984).

6.2.5 Proenkephalin

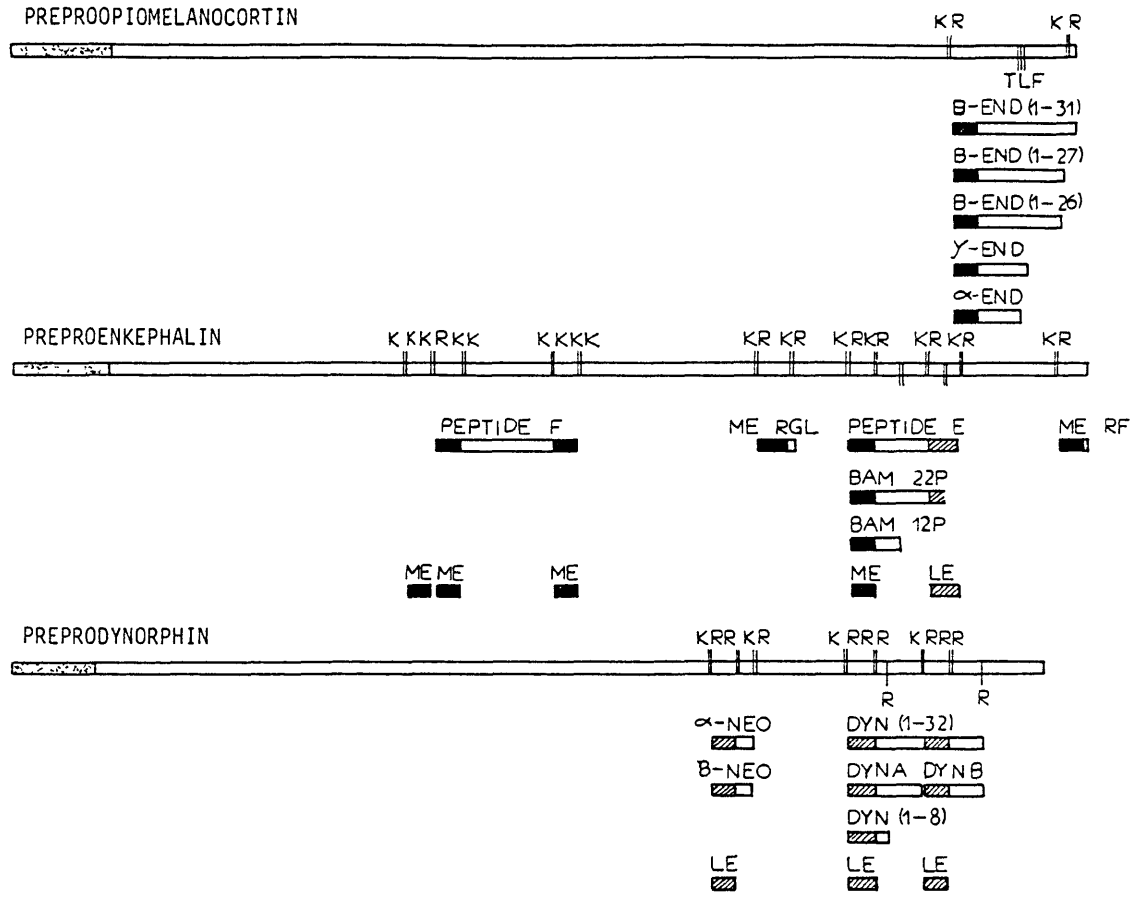
Although ME and LE were the first endogenous peptides to be discovered, little is known about the sequence of events which liberates them from proenkephalin. In this investigation, a comparison of the proenkephalin derived peptides present in guinea pig adrenal gland and striatum has demonstrated that the extent of precursor processing is very different in these tissues. The majority of ME in the adrenal gland is incorporated into high molecular weight species, whereas in the striatum ME-containing peptides represent around 20% of the peptide store. Similar results have been obtained when comparing bovine adrenal medulla with bovine striatum although, in this species, much lower levels of ME-containing peptides were generally detected in the latter tissue (Liston et al., 1983; Giraud et al., 1984a; Giraud et al., 1984b). Thus, the processing of proenkephalin appears more complete in the striatum.

However, if, as appears the case, all ME-containing peptides in both adrenal gland and brain are derived from proenkephalin, is the pattern of cleavages the same in both tissues ?

As previously described, the putative intermediates in the processing of adrenal medullary proenkephalin have been well characterized. All the high molecular weight enkephalin-containing peptides isolated from this tissue contain synenkephalin (proenkephalin-(1-70) at the NH_2-terminus. Therefore, a processing pathway has been proposed which involves several successive proteolytic cleavages beginning at the COOH-terminus of proenkephalin (Kojima et al., 1982). The first cleavage could occur at the COOH-terminus of the LE sequence within proenkephalin, Fig 35. This is supported by the observation that the largest synenkephalin-containing peptide detected in bovine adrenal medulla terminates with the LE sequence (Patey et al., 1984). This suggests that the initial cleavage step may involve the removal of peptide B, containing ME-Arg^6-Phe^7 and that this could occur rapidly in the Golgi before packaging into the secretory granule. Another possibility is that the first cleavage occurs at the COOH-terminus of ME-Arg^6-Gly^7-Leu^8. Baird et al. (1984) have detected a 8.5 kDalton peptide in bovine adrenal medulla which contains both BAM 22P- and ME-Arg^6-Phe^7-IR, which may be liberated from the COOH-terminus of proenkephalin as a result of this type of cleavage.

Less is known concerning the nature of the proenkephalin derived peptides in the brain. The most detailed study of proenkephalin processing in neural tissue has used the bovine hypothalamic magnocellular neuronal system as a model (Liston et al., 1984d). This system consists of cell bodies located in the supraoptic nucleus with axons which travel through the pituitary stalk and terminate in the neurohypophysis. These regions were examined for ME- and synenkephalin-containing peptides. In the supraoptic nucleus, although a large proportion of ME-IR was contained within high molecular weight

FIG. 35 . STRUCTURE OF THE OPIOID PEPTIDE PRECURSORS AND THEIR OPIOID PEPTIDE PRODUCTS.



Abbreviations: = putative signal peptide; = ME = methionine^5-enkephalin; = LE = leucine^5-enkephalin; END = endorphin; NEO = neoendorphin; DYN = dynorphin; K = lysine; R = arginine; G = glycine; T = threonine; E = glutamic acid; W = tryptophan; L = leucine; F = phenylalanine. Upward lines represent cleavage sites at paired basic residues and downward lines represent "unusual" cleavage sites. Only the potential opioid peptide products are shown.

Modified from Weber et al., 1983b.

material, this was not associated with synenkephalin-IR. In the pituitary stalk ME-containing peptides were present at lower concentrations and in the nerve terminals of the neurohypophysis processing was almost complete. The authors have suggested that, in this tissue, proteolytic cleavage may remove the amino fragment at an early stage and that subsequent cleavages may produce intermediates ending in ME-Arg^{6}-Gly^{7}-Leu^{8} and ME-Arg^{6}-Phe^{7}, which do not contain synenkephalin. In support of this hypothesis, extracts of the supraoptic nucleus were found to contain high molecular weight ME-Arg^{6}-Gly^{7}-Leu^{8} and ME-Arg^{6}-Phe^{7} immunoreactive species which did not cross-react with synenkephalin antiserum.

It is possible that processing in the striatum proceeds via a similar pathway to the one outlined for the hypothalamic system. Indeed, this study, demonstrating the presence of detectable levels of a ME-Arg^{6}-Phe^{7}-IR containing proenkephalin species in guinea pig striatum, supports the idea that the rapid removal of the COOH-terminus, which seems to occur in the adrenal, is not the first cleavage step in the brain.

Although proteolysis is central to the processing of opioid precursors, other post-translational modifications are known to occur during biosynthesis. As mentioned earlier, β-END related peptides can exist in an acetylated form which is not active at the opioid receptor. There is no evidence for acetylation of proenkephalin derived peptides. However, Unsworth et al., (1982) have shown that a percentage of LE in the striatum is rendered inactive by O-sulphation of the tyrosine residue.

An amidated octapeptide has been purified from both bovine

caudate nucleus and human phaeochromocytoma and has been called metorphamide and adrenorphin, respectively (Weber et al., 1983a; Matsuo et al., 1983). Metorphamide (adrenorphin) is derived by cleavage of proenkephalin at a single arginine residue, followed by transformation of the COOH-terminal glycine into an amide group. Metorphamide also shows a differential distribution indicative of tissue specific processing of proenkephalin, occuring in many areas of rodent brain, with the highest levels in striatum and hypothalamus. However, although a high concentration of this peptide is found in human adrenal medulla, little or no metorphamide is found in this tissue from cow or guinea pig (Sonders et al., 1984).

Recently, a novel COOH-terminally amidated opioid peptide, amidorphin, has been isolated from bovine adrenal medulla, which corresponds to the first 26 amino acid residues of peptide F. Amidorphin immunoreactive peptides were also detected in regions of bovine brain and pituitary (Seizinger et al., 1984).

6.3 Significance of differential processing

Many groups have now demonstrated that proenkephalin can give rise to a variety of peptides and that the relative concentration of these products can vary between tissues (adrenal gland v brain; Giraud et al., 1984a; Liston et al., 1984d; Metters et al., 1985), between regions within a tissue (bovine striatum and bovine hypothalamus; Liston et al., 1984a) and between the same tissue in different species (bovine striatum v rodent striatum; Giraud et al., 1984b; Metters et al., 1985). It is important to consider the potential role of every intermediate in the processing of a precursor and not to consider the pathway in terms of the biosynthesis of a particular product.

In the adrenal gland, the relatively high level of opioid-containing peptides could represent a large reserve of biologically active peptides. These species could be processed to produce these peptides in response to prolonged physiological stimuli. The depleted stores would be replenished over a longer time period by biosynthesis of proenkephalin via the protein synthesising machinery of the cell. The use of larger peptides for storage may provide protection against degradation and non-specific release of smaller moieties.

The situation is different in the brain. Many brain regions do not contain putative opioid precursors which could act as a tissue store of this type. Even in the guinea pig striatum, where high molecular weight opioid-containing peptides have been detected, the levels are too low to replenish a sustained release of peptides over a long period.

Another explanation for the differences in levels of the proenkephalin derived peptides present in adrenal gland and brain could be the variation in turnover between these two tissues. If the smaller peptides present in neuronal tissue are rapidly released and/or degraded, even when the neuronal firing is at basal level, the biosynthesis and processing of proenkephalin may be more rapid and more complete. It is possible that in the brain there are additional mechanisms, for example uptake, which can regulate neuronal proenkephalin derived peptide levels (Van Loon et al., 1982). In contrast, the frequency with which peptides are released from the adrenal gland may be reflected in generally slower processing of proenkephalin. The physiological stimuli which activate the proenkephalin peptidergic system in the CNS may have a feed back

effect on transcription and translation which differs the situation in the adrenal gland. This may result in varying rates of proenkephalin biosynthesis in these two tissues.

Similarly, differences in the proteolytic activities responsible for the processing of proenkephalin could also be responsible for the tissue specific patterns of products. Proenkephalin derived intermediates may have a role in the regulation of processing, for example via changes in their conformation or concentration.

A third possibility, when considering the differences in levels of opioid-containing peptides in the adrenal gland and brain, is that these species are not present simply as precursors but represent products with a specific functional role(s). The question as to which of the proenkephalin derived peptides have a physiological role remains unanswered.

Kilpatrick et al., (1980) have demonstrated that enkephalin and enkephalin-containing peptides are released by stimulation of the perfused beef adrenal gland. ME and ME congeners have also been shown to circulate in the plasma (Clement-Jones et al., 1980; Baird et al., 1984; Boarder and McArdle, 1984). The larger enkephalin-containing peptides may act as precursors for smaller peptides, which are metabolically labile and have a short half-life in the plasma, thus protecting them from degradation, or may act directly at target cells themselves. However Chaminade et al., (1984) have shown that the proportion of enkephalin-containing peptides released from the perfused cat adrenal in situ depends on the type of stimulation used to evoke release. Splanchnic nerve stimulation, which may be judged more physiological, releases 50% of all ME-IR in the form of ME,

ME-Arg^{6}-Phe^{7} and ME-Arg^{6}-Gly^{7}-Leu, whereas, following stimulation with acetylcholine or potassium chloride, a higher percentage of the ME-IR released was contained within higher molecular weight forms. The authors suggest that under more "normal" physiological conditions the material released by the adrenal medulla may be in the form of the hepta-, octa- and pentapeptides produced by the complete processing of proenkephalin.

It is possible that the enkephalins and related peptides circulating in the periphery have been released from tissues other than the adrenal gland, for example the neurohypophysis. It is of interest that the 8.5 kDalton species detected by Baird et al., (1984) in the plasma, is the type of COOH-terminal proenkephalin intermediate proposed by Liston et al., (1984d) in their study of processing in the hypothalamic magnocellular neuronal system.

Little is known about the non-opioid regions of proenkephalin and a possible functional role for some of these sequences cannot be ignored. Alessi et al., (1982) have shown that peptide F is processed into a non-opioid fragment in bovine, rat and guinea pig adrenal medulla which responds to environmental stimuli in a manner suggestive of a physiological role. Does the N-acetylation of β-END simply provide an inactivation mechanism for its opioid action or does this form have a non-opioid receptor mediated function ?

As discussed earlier, various reports have suggested that neural proenkephalin is processed rapidly to form ME, LE, ME-Arg^{6}-Phe^{7} and ME-Arg^{6}-Gly^{7}-Leu^{8} (Liston et al., 1983 ; Ikeda et al., 1982). Release of these products, from rat striatal slices, in the stoichiometric ratio expected from the complete processing of proenkephalin, has

supported the idea that these are the physiologically relevant peptides (Patey et al., 1983a). However, this study has shown that there are opioid-containing peptides present in the striatum which could have the potential to act as precursors for biologically active peptides other than the penta-, hepta- and octapeptides and that these may have a physiological role. The opioid peptides BAM 22P, BAM 12P, peptide F and metorphamide have all been detected by immunological techniques in nervous tissue and may provoke a receptor mediated response(s) (Mizuno et al., 1980a and 1980b; Höllt et al., 1982a; Weber et al., 1983a).

A particular peptide cannot be automatically judged more important than any other peptide simply because it is present in relatively high concentrations. Therefore, although ME is a major proenkephalin product in the brain and probably has a functional role, it may also be present as a metabolite of another opioid peptide. For example, although it may be difficult to accept the idea of ME as a metabolite, peptide F is a more potent opioid agonist, with a different receptor selectivity and conversion to ME may be a stage in the termination of the biological action of this peptide. It is also possible that more than one of the range of peptides released from within a single proenkephalinergic neurone may have an input into a responsive neurone, depending on the receptors expressed by that neurone. In this case the precursor may be processed to several important products.

The situation is further complicated by the presence of POMC and prodynorphin derived peptides, as well as those from proenkephalin, in neural tissue. A single neurone may receive an input from opioid peptides derived from more than one opioid precursor, although most

reports have indicated an independent distribution for these peptides (Rossier et al., 1977; Bloom et al., 1978; Watson et al., 1982; McGinty et al., 1983). It is difficult to interpret data based solely on concentrations of peptides found in distribution studies, or release experiments. It was felt that the key to understanding the role of the multiple opioid peptides may be in unravelling the relationship between these ligands and the different opioid receptor subtypes.

The brain has been shown to contain at least three opioid receptor subtypes (μ, δ and k) (Martin et al., 1976; Lord et al., 1977). Based on the observation that the enkephalins are δ-receptor selective, whereas the dynorphins can all interact with the k-receptor, it has been proposed that each of the opioid precursors is processed to produce ligands which act preferentially with one receptor subtype. Therefore, proenkephalin would produced δ ligands, prodynorphin k ligands and POMC μ ligands. However, the receptor selectivity of the opioid peptides is more complicated than this model would suggest, Table 12. β-END is equipotent at μ and δ receptors, the larger peptides from proenkephalin may not be δ selective, with metorphamide acting preferentially at the μ receptor while prodynorphin derived peptides have enhanced k selectively only when a basic residue is present at position 10. In addition, the receptor subtypes have not been found to have a delineated distribution commiserate with this hypothesis, although little information is available on the receptor subtypes situated on individual neurones (Egan and North, 1981; Zieglgansberger et al., 1982).

Perhaps a more realistic proposition is that the opioid precursors have the potential to produce a variety of opioid ligands

TABLE 12 OPIOID PRECURSORS AND THEIR RECEPTOR SELECTIVITIES

PRECURSOR	OPIOID PEPTIDE	RECEPTOR SELECTIVITY	REFERENCE
POMC	β-END	$\mu = \delta$	Lord, et al.,1977. McKnight, et al.,1982.
PROENKEPHALIN	LE ME	$\delta \gg \mu$	Lord,et al.,1977 McKnight, et al.,1983
	ME-Arg^{6}-Phe^{7} ME-Arg^{6}-Gly^{7}-Leu^{8}	$\delta \geq \mu \gg k$	Magnan, et al.,1982
	PEPTIDE E PEPTIDE F BAM 22P	$\mu \geq \delta$	Hollt, et al.,1982b
	metorphamide	$\mu > k \gg \delta$	Weber, et al.,1983a
PRODYNORPHIN	DYN A	$k \gg \mu \gg \delta$	Corbett,et al.,1982
	DYN B	$k \gg \mu > \delta$	
	DYN (1-8)	$k > \mu = \delta$	

modified from Zukin and Zukin,1984

and the nature of the final ligand-receptor interaction(s) is determined by the extent of processing and the relative concentration of the receptor subtypes present. This scheme would capitalise on the differing potencies and half-lives of the various opioid peptides. Thus, differential processing could provide an important mechanism for the regulation of the endogenous opioid systems.

It has also been shown that opioid peptides can coexist with other biosynthetically separable neuroactive substances, for example proenkephalin derived peptides coexist with catecholamines in the adrenal medulla (Roisin et al., 1983) and oxytocin in the hypothalamic magnocellular neurones (Vanderhaeghen et al., 1983) whereas prodynorphin derived peptides are associated with vasopressin within separate neurones in the latter tissue (Watson et al., 1982). Activation of the peptidergic system can therefore release many biologically active substances which may have opposing effects or may modulate the release of a co-stored ligand. For example, k ligands have been shown to be diuretic and hypotensive (Leander, 1982) whereas vasopressin has the opposite action.

6.4 Concluding remarks

This discussion has merely touched on the complexity of the interactions which may occur within the frame of endogenous opioid function. Many questions remain unanswered. How is the biosynthesis and processing of the precursors regulated at gene, mRNA and protein level? Can this regulation be controlled by other endogenous biologically active peptides ? What is the nature of the enzymes involved in precursor processing and are these species also tissue specific ? It is necessary to characterise the opioid receptor

subtypes. Do these receptors represent distinct proteins, interconvertible forms of the same protein or post-translationally modified forms of the same protein? How are these receptor subtypes related to the endogenous opioid ligands and what are the mechanisms for activation and termination of biological action at the synapse ?

Although opioid peptides have been implicated in the physiology of pain, stress, cardiovascular control, various psychiatric disorders and the immune response, as well as many other functions, very little precise information is available concerning their precise role. To understand the physiological and behavioural effects of the endogenous opioid systems remains the greatest challenge.

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