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A STUDY OF NEUROMEDIN U-LIKE PEPTIDES

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ABSTRACT

Neuromedin U8 and its N-terminally extended form neuromedin U25, were recently isolated from porcine spinal cord. Both peptides stimulate uterine contraction *in vitro* and exert pressor effects when administered systemically into rats. Using synthetic neuromedin U8, a specific radioimmunoassay was developed.

Neuromedin U-like immunoreactivity was found in the central nervous system, gastrointestinal tract, pituitary gland, thyroid gland and urogenital tract. Complementary chromatographic analysis characterized the predominant immunoreactive form present in porcine tissue as neuromedin U25. In all other species, the immunoreactivity failed to co-elute with either of the porcine standards suggesting a high degree of inter-species molecular heterogeneity.

Using preparatory techniques, neuromedin U was subsequently isolated from the rat gastrointestinal tract and found to be a C-terminally amidated, 23 residue peptide. Despite its shorter length, rat neuromedin U bears a high degree of sequence similarity with neuromedin U25 as does frog neuromedin U, a 25 residue peptide also purified from the gastrointestinal tract. Using synthetic forms of both peptides, their

comparative bioactivity was examined on preparations of rat uterus and blood pressure and the results compared to neuromedin U25 and neuromedin U8.

Experiments which altered the endocrine status of the rat revealed that chronic treatment with TRH and adrenalectomy increased, whilst gonadal steroids and dexamethasone decreased, the anterior pituitary content of neuromedin U immunoreactivity. The response induced by TRH treatment was investigated further and found to be dependent upon the circulating levels of thyroid hormone. In addition, the effect of adrenalectomy in male rats, was dependent upon the presence of intact gonads.

In vitro studies revealed that neuromedin U stimulates the secretion of both LH and TSH from the rat pituitary. Perifusion of pituitary pieces demonstrated that the secretion of immunoreactive neuromedin U can be induced by both TRH and membrane depolarization using potassium chloride. These results, together with the selective changes in pituitary content observed following endocrine manipulation, indicate that neuromedin U may be a novel local regulator of anterior pituitary hormone secretion.

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This thesis is dedicated to Samantha and Michael my children, Teodora my mother and to the memory of Mateusz my father.

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CHAPTER 1

GENERAL INTRODUCTION

Historical Background

By the latter half of the 19th century, surgical studies had established that the selective removal of a gland could produce a deficiency which manifested itself as a clinical syndrome. Such symptoms could then be treated with selective replacement of the excised tissue. In this way, Berthold demonstrated in 1849 that the effects of castration could be reversed by testicular grafts devoid of all nervous connections and Minkowski with von Mering in 1890 produced diabetes mellitus in dogs by total pancreatectomy (see Everett, 1978).

Shortly thereafter, Bayliss and Starling demonstrated the existence of a substance which was released upon acidification of the duodenal mucosa and was conveyed to the pancreas via the circulation where it stimulated the secretion of pancreatic juice (Bayliss and Starling, 1902). Their carefully controlled studies proved, beyond doubt, that chemical integration could occur without assistance from the central nervous system. They coined the term "hormone" in reference to this new substance

and thus began a new era in modern endocrinology.

In 1922 Banting and Best isolated insulin from pancreatic extracts, and, almost overnight, the outlook for the severely diabetic patient changed from that of rapid decline and death to that of an almost normal individual (Banting et al, 1923). Studies by von Euler and Gaddum revealed that extracts of horse intestine contained a substance which stimulated atropine-resistant contractions of rabbit ileum. They also found that the bioactive substance they called "preparation" (or substance) P for "powder" was also present in brain extracts (von Euler and Gaddum, 1931). In 1959 Viktor Mutt purified secretin, the substance described by Bayliss and Starling (Mutt V, 1959).

Technological developments in the 1950's such as those pioneered by Sanger and Edman allowed the primary structure of peptides and proteins to be determined, the first of which was insulin (Sanger F, 1959). In addition, development of improved chromatographic matrices such as the polymerized carbohydrate derivative Sephadex, facilitated a more efficient fractionation of tissue extracts. Such advances paved the way for Mutt and his group based at the Karolinska Institute in Stockholm to fully characterize many gastrointestinal hormones including cholecystokinin (CCK) (Mutt and Jorpes, 1968), secretin (Mutt et al, 1970), vasoactive

intestinal polypeptide (VIP) (Mutt and Said, 1974) and gastric inhibitory peptide (Brown and Dryburgh, 1971). Of these peptides, both CCK and VIP were subsequently found to be present also in the central nervous system. Around this time, other groups were independently purifying peptides from the brain including substance P, the material described by von Euler (Chang et al, 1971), somatostatin (Burgus et al, 1973) and neurotensin (Carraway and Leeman, 1975). All three were subsequently found to be present in the gastrointestinal tract.

The number of peptides with a brain-gut distribution increased rapidly and the distinction between brain peptides and gut hormones has all but disappeared. Today it is recognised that regulatory peptides can function either as hormones, locally active (paracrine) substances, neurotransmitters/ neuromodulators or a combination of all three.

Discovery and Purification of Neuromedin U

In the early 1980's Naoto Minamino and his colleagues began a systematic search for novel mammalian peptides present in porcine spinal cord. They followed each purification using the ability of the peptide to contract smooth muscle *in vitro*. They termed each peptide they purified a "neuromedin", suffixed by a letter denoting sequence similarity with existing

peptide families (Minamino et al, 1985a). Consequently, neuromedins K and L are kassinin-like, neuromedins B and C are bombesin-like and neuromedin N is neurotensin-like (Figure 1: 1). A table of amino acid abbreviations used throughout this thesis is shown in Table 1: 1.

Neuromedin U, the latest neuromedin to be isolated, lacked sufficient sequence similarity to be classified in this manner and was suffixed U because it stimulated rat uterine contraction (Minamino et al, 1985b).

Unfortunately, the neuromedin nomenclature has been plagued with problems. It was noted that the sequence of neuromedin C was identical to that of gastrin releasing peptide (GRP_{18-27}) a peptide isolated one year earlier from canine gut and a human pulmonary carcinoid tumour (Reeve J R Jr et al, 1983). In addition, the amino acid sequence of neuromedin L is identical to that of neurokinin A and substance K whilst neuromedin K is identical to neurokinin B (Kimura S et al, 1983).

Current guidelines for peptide nomenclature recommend that the terms neuromedin C, K and L be dropped in favour of the more appropriate generic names.

Table 1:1 A list of amino acid abbreviations used throughout this thesis.

	SYMBOLS	
Amino Acid Residue	Three letter	Single letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

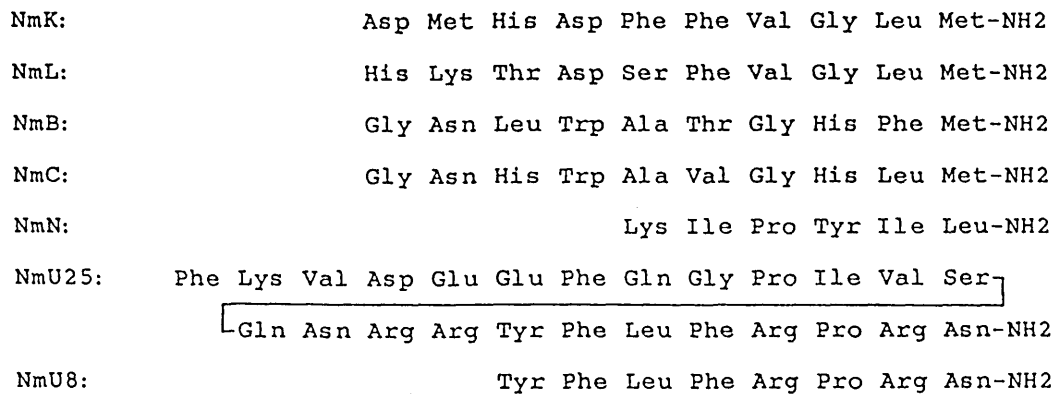


Figure 1.1 Primary structures of the neuromedins isolated from porcine spinal cord. For the sake of clarity, the "big", N-terminally extended forms of neuromedin B, NmB-30 and NmB-32 have been omitted.

Primary Structure of Neuromedin U

Neuromedin U was isolated as two, C-terminally amidated forms, an octapeptide (neuromedin U 8) and an N-terminally extended form (neuromedin U 25). Both peptides were obtained from side fractions generated during the purification of other neuromedins. Since the amino acid sequence of neuromedin U 8 is immediately preceded by two arginine residues, it was thought to be produced *in vivo* by a trypsin like cleavage of the larger form. Although neuromedin U cannot be grouped with any existing peptide family, it does bear limited sequence similarity to pancreatic polypeptide at its carboxyl terminus (Figure 1:2). In addition to the porcine forms, three other neuromedin Us have been characterised from rat (Conlon et al, 1988), frog (Domin et al (1), in press) and rabbit (Conlon et al 1989) (Figure 1:3).

Bioactivity

In addition to their potent effects on uterine smooth muscle, both forms of porcine, rat and frog neuromedin U display marked hypertensive effects in rats producing a rapid and sustained increase in arterial blood pressure (Minamino et al, 1985b: Domin et al (1) in press and Chapter 8). Interestingly, carboxyl deamidated neuromedin U 8 displays no appreciable biological

NmU8: Y F L F R P R N-NH2

NmU25: F K V D E E F Q G P I V S Q N R R Y F L F R P R N-NH2

Porcine PP: ...A T P E Q M A Q Y A A E L R R Y I N M L T R P R Y-NH2

Rat PP: ...A T H E Q R A Q Y E T Q L R R Y I N T L T R P R Y-NH2

Figure 1: 2 Comparison of the primary structure of neuromedin U and the carboxyl-terminal region of pancreatic polypeptide (PP). The sequence of neuromedin U8 (NmU8) is contained in neuromedin U25 (NmU25), as indicated by underlining. Sequence similarity with porcine and rat PP is also underlined.

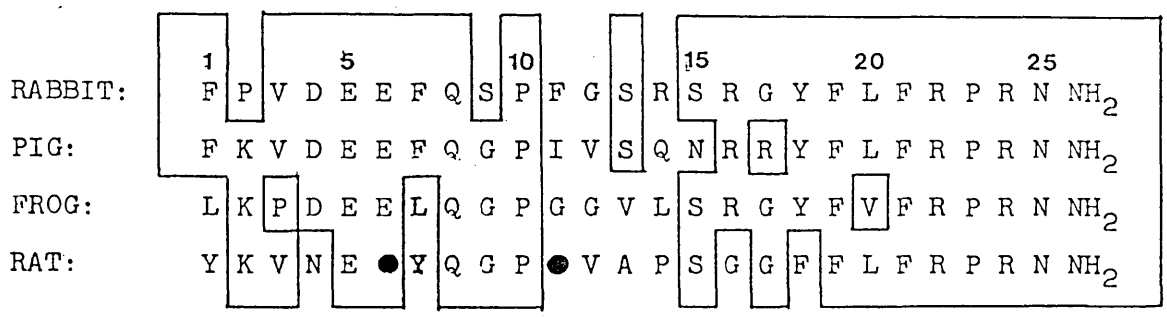


Figure 1: 3 Primary structures of porcine, rat, frog and rabbit neuromedin U.

activity in either bioassay system. More sophisticated cardiovascular studies revealed that in both dogs and rats, both porcine and rat neuromedin U selectively reduce splanchnic blood flow (Sumi et al, 1987, Gardiner et al, in press). Neuromedin U 8 has also been found to modify selectively intestinal ion transport through an interaction with non-cholinergic enteric neurons (Brown and Quito, 1988).

Discussion And Purpose Of This Study

At the time when work for this thesis began, nothing was known of neuromedin U other than it was present in porcine spinal cord and that it was biologically active (Minamino et al, 1985b). Today it is recognised that neuromedin U-like peptides are present in a variety of species with the concentrations of immunoreactivity being especially high in the gastrointestinal tract. The most promising findings in relation to ascribing a biological role for this peptide appear to be those demonstrating selective effects on splanchnic blood flow and trans luminal transport. Future studies in this area will be of great interest and may hold great potential in their use during surgery of the gastrointestinal tract.

This thesis begins by describing the development of a radioimmunoassay to quantify neuromedin U-like

immunoreactivity (Chapter 2). The optimal conditions required to extract the peptide from tissue extracts are investigated in chapter 3 in addition to a comparative assessment of the molecular forms of neuromedin U-like immunoreactivity present in mammalian CNS and gastrointestinal tract. Chapter 4 describes a more detailed study of the distribution and molecular forms of neuromedin U-like peptides in both mammalian and a non-mammalian species. As a consequence of the chromatographic differences observed between porcine neuromedin U and the immunoreactivity in tissue extracts from rat and frog, chapter 5 describes the purification and sequence analysis of this peptide from the gut of these two species. In chapter 6 the effect of various endocrine manipulations are examined on the content of neuromedin U in the pituitary gland. This theme is continued in chapter 7 where the effect of alterations in thyroid status are examined. The results of comparative bioactivity studies involving porcine, rat and frog neuromedin U are presented in chapter 8, in addition to studies investigating the effect of neuromedin U upon rat pituitary hormone secretion.

CHAPTER 2

RADIOIMMUNOASSAY OF NEUROMEDIN U

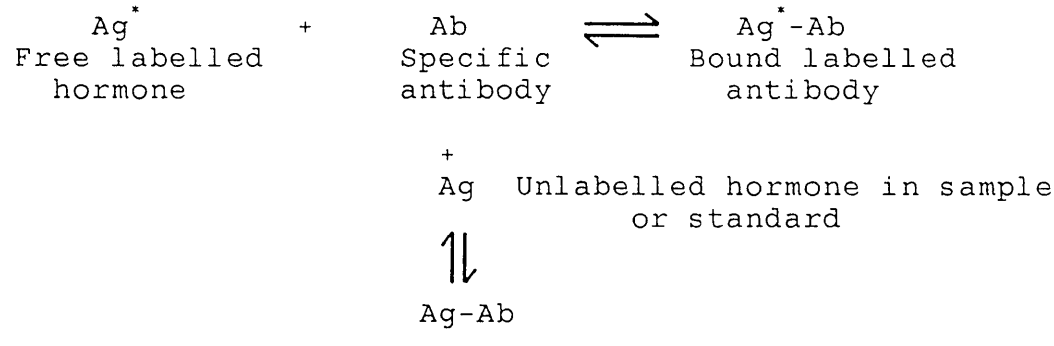
Introduction

In the mid 1950's, Solomon Berson and Rosalyn Yalow noted that established insulin dependent diabetics produced a protein which bound ^{131}I labelled insulin and consequently delayed its metabolism. Furthermore, once bound, the tracer could be displaced from the protein (antibody) by high concentrations of unlabelled hormone. The degree of displacement was quantitatively related to the total amount of insulin present (Berson et al, 1956). This work formed the basis of a sensitive analytical technique termed radioimmunoassay (RIA) which was initially applied to the measurement of plasma insulin in man (Yalow and Berson, 1960). The potential of this work was soon recognised by some and levels of circulating thyroxine were quantified in man shortly thereafter, using thyroxine-binding globulin (Ekins, 1960). Interestingly however, few other groups cited this method in the years which immediately followed, but by the late 1960s RIA was established as a major investigative tool. The advent of RIA brought several distinct advantages. Firstly, it presented an approach

immediately applicable to most antigens. Secondly, it fulfilled the need for hormone assays which was not being answered by existing bioassay methods. High assay sensitivity also became available, usually by several orders of magnitude, and, finally, it offered a robust system amenable to automation in an laboratory equipped for the measurement of radioactivity.

Principles Of Radioimmunoassay

In theoretical terms, RIA is based upon the binding of antigen (Ag) to a specific antibody (Ab).



At equilibrium, the formation of the antigen-antibody complex (Ag-Ab) is equal to the dissociation of this complex back to free antigen (Ag) and antibody (Ab). This reaction can be summarized thermodynamically by the law of mass action which states that at equilibrium, the concentration of the products on the two sides of the equation will be constant:

$$K_a = \frac{[Ag\ Ab]}{[Ag]\ [Ab]}$$

where K_a is the affinity or association constant. The affinity constant describes the avidity of the antiserum and for a highly avid antiserum is of the range 10^{10} -10^{12} l/mol.

Radioimmunoassays are based on the incubation of a constant amount of antibody and iodinated antigen together with the sample containing an unknown amount of antigen. On the assumption that both labelled and endogenous antigen behave identically, a competition occurs for antibody binding sites. Following an incubation period, free and antibody-bound antigen is separated and the radioactivity in one or both of the fractions determined. Each assay also contains known additions of exogenous antigen. This is referred to as the standard curve which also contains a volume of peptide-free medium equal to that of the sample added. The greater the amount of endogenous antigen in the sample or exogenous peptide added, the greater the competition for binding sites and the less labelled antigen will bind to the antibody.

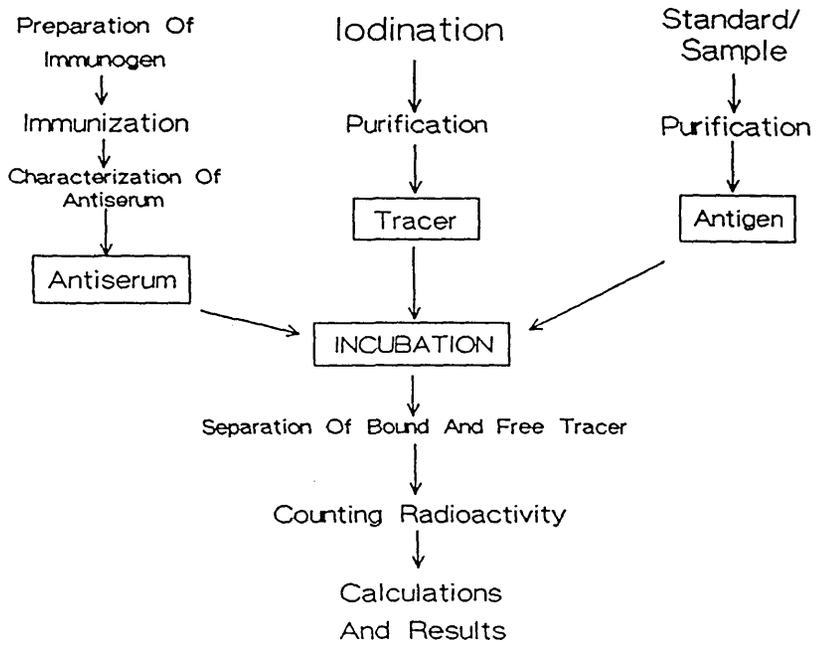


Figure 2: 1 A diagrammatic representation of the steps involved in the development and execution of a radioimmunoassay.

The concentration of antigen in a sample is estimated by comparing the amount of antibody bound tracer with that obtained using increasing concentrations of standard. The steps involved in developing a radioimmunoassay are shown schematically in Figure 2: 1

There are a number of requirements for the production of a successful RIA; (1) a radiolabelled tracer; (2) an antiserum having high specificity and affinity for the substance measured; (3) a method for separating free from bound tracer; (4) an antigen of sufficient purity to use as standard against which all estimations are made and (5) appropriate incubation conditions.

1. Production Of Radiolabelled Antigen

The preparation of a radiolabelled antigen involves the choice of an appropriate radioactive isotope, its incorporation into the antigen in such a way so as to maintain antigenicity and finally the purification of the radiolabelled product from unreacted reagents and reaction side products.

Choice Of Isotope

A large number of radioisotopes have been utilized for RIA although the two most commonly used are ^{3}H and ^{125}I. The choice of isotope influences the potential

sensitivity of an assay since a labelled antigen having a low specific activity will have a high peptide: radioactivity ratio. This is ultimately manifested in the form of poor sensitivity irrespective of antiserum avidity. For the quantitation of regulatory peptides, ^{125}I is often the isotope chosen as it offers high specific activity and requires no special sample preparation. On theoretical grounds, ^{131}I is an alternative although this radiolabel has a high degree of contamination with non-radioactive iodine and has a shorter half life.

Methods Of Iodination

There are several methods for radioiodinating peptides but they can be divided into two approaches. Firstly, direct methods such as the chloramine T technique (Greenwood et al, 1963) where the radioiodine is principally incorporated into tyrosine residues although incorporation into histidine, tryptophan or sulphydryl groups may also occur. Secondly, conjugation methods such as those involving Bolton and Hunter reagent (N-succinimidyl 3-(4-hydroxy 5-[^{125}I] iodophenyl)proprionate) where this radioiodinated ligand forms a covalent amide bond with free amino groups such as those present in lysine residues (Bolton and Hunter, 1973).

The Chloramine-T Method

This technique is by far the most commonly used for the radioiodination of peptides for RIA purposes. Chloramine T is the sodium salt of the N-monochloro derivative of p-toluene sulphonamide. When placed in aqueous solution it slowly degrades to form hypochlorous acid and is consequently a mild oxidising agent. In the presence of this reagent NaI is oxidised to form cationic iodine (I^+). At pH 7.5, tyrosine is only slightly ionized to the anion but this is thought to be sufficient for the reaction to proceed through these ionized groups. The iodine atoms then substitute ortho to the hydroxyl group in the phenolic ring of tyrosine. The reaction is terminated by the addition of sodium metabisulphite which reduces I^+ back to I^-.

Purification Of Tracer

Once the iodination reaction has been halted, labelled peptide is separated from unreacted sodium ^{125}I, damaged and unreacted peptide. Early methodology employed gel permeation on Sephadex® gel columns (Pharmacia UK). However, the specific activity of the labelled peptide preparations tended to be rather low because of incomplete separation. Today, reversed phase high performance liquid chromatography (HPLC) is the method of choice. The mixture of reactants and products is

loaded onto a silica matrix coupled to C_{18} aliphatic chains and eluted with an aqueous solution. A gradient of an organic solvent, such as acetonitrile, is then developed which decreases the polarity of the mobile phase. At a certain solvent concentration, the tracer leaves the stationary phase and is collected as it leaves the column.

2. Production Of Antisera

Large polypeptides and proteins of molecular weights in excess of 3000 can promote an immunogenic response when injected into an animal to which the substance is foreign. Peptides with a lower molecular weight are themselves poorly immunogenic, and, generally, require coupling to a larger macromolecule, typically a protein, to induce immunogenicity (Odell et al, 1972). Antisera produced in this way will contain not only antibodies directed against the hapten (peptide) but also against the carrier. However, since the carrier is not present in the radiolabelled form, there is no interference in the assay using such an antiserum. Subsequent antibody specificity depends upon the orientation of the hapten in relation to the carrier protein. Several agents can couple peptides to proteins, of which the following three are the most commonly used: -

(a) Glutaraldehyde Coupling

Glutaraldehyde (glutaric dialdehyde) forms a bridge between primary amino groups such as those at the N-terminus or lysyl residues on both the protein and peptide. The scheme for the glutaraldehyde coupling reaction is shown in figure 2: 2

(b) Carbodiimide Coupling

Carbodiimides e. g 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide condense free carboxyl and primary amino groups, whether N- or C-terminal or side chain (ie. lysyl, aspartate or glutamate residues) to form peptide bonds. Since this agent can cause coupling at several positions it may give rise to a variety of antigenic responses. One possible reaction is shown in figure 2: 3

(c) Bis-Diazonium Salts

These usually bridge tyrosyl or histidyl residues. Overnight treatment of benzidine with nitrous acid (hydrochloric acid and sodium nitrite) at 4^0C results in diazotisation of the two amino groups. These groups then allow coupling at both ends of the molecule (Figure 2: 4). Although a large variety of carrier proteins can be used, bovine serum albumin (BSA) tends to be the most commonly employed.

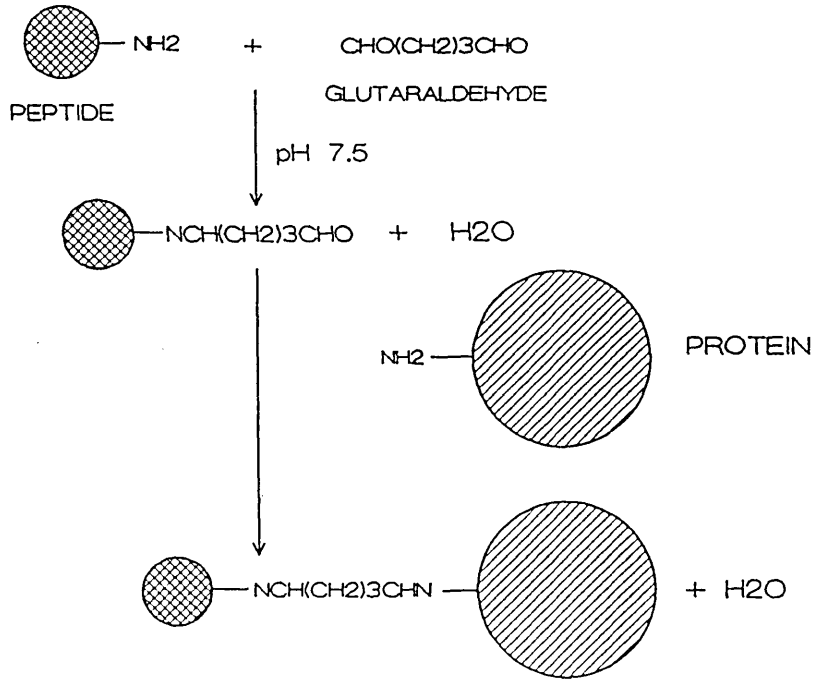


Figure 2: 2 A proposed reaction pathway for glutaraldehyde coupling.

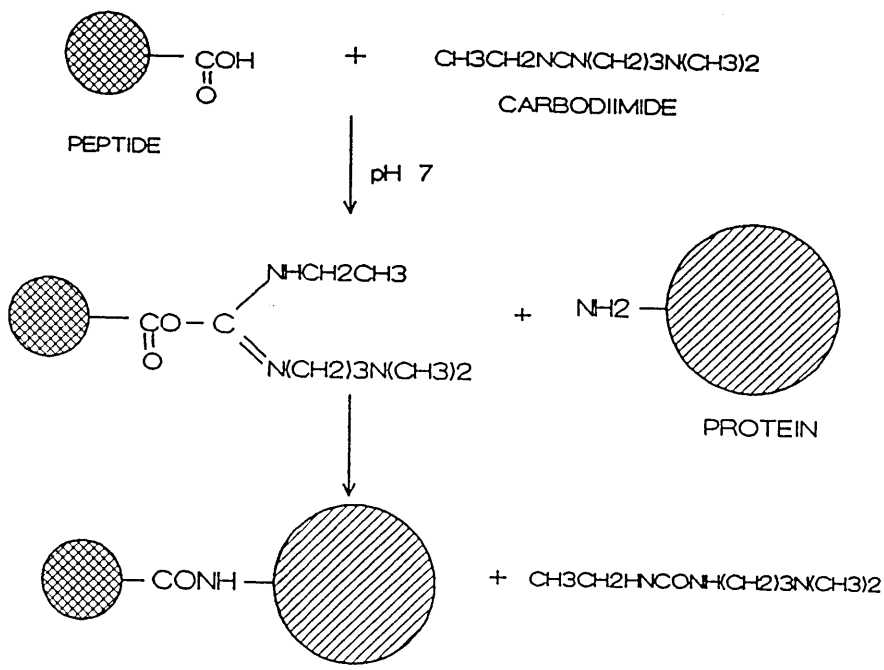


Figure 2: 3 A proposed reaction pathway for carbodiimide coupling

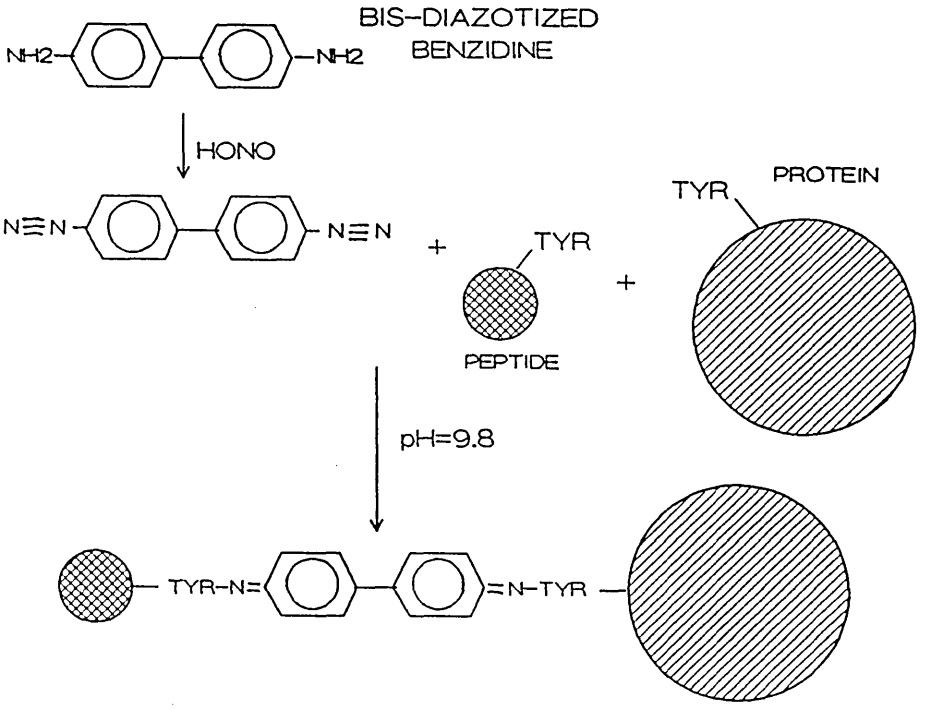


Figure 2: 4 A possible reaction pathway for coupling by bis-diazotized benzidine.

Immunization Procedure

Conjugate is generally administered in the form of an emulsion made with Freund's adjuvant (a mixture of n-hexadecane and the detergent Arlacel A in a 4:1 ratio). This procedure allows the slow release of the coupled hapten into the circulation thereby prolonging the antigenic response. Freund's adjuvant alone (incomplete) will cause an inflammatory response but this is enhanced, when made complete by the addition of heat killed mycobacterium. Such mixtures are then injected subcutaneously, intramuscularly or intradermally into a recipient animal. Rabbits are often chosen since they are usually passive and can be easily bled from the ear veins. The first dose given tends to be the largest and is followed by regular boosts spaced approximately eight weeks apart.

Antiserum Characteristics

Having obtained an aliquot of antiserum, it is essential to assess its properties. An antiserum dilution or titre is a reflection of both antibody concentration and its avidity for the antigen. It is generally defined as the final antiserum dilution required to bind 50% of the added tracer. Avidity is a measure of the strength with which antigen binds to a single antibody binding site and is usually in the range 10^{10}-10^{12} l/mol. Antisera

can however exhibit great heterogeneity with respect to their binding avidity due their polyclonal nature. Purification of specific antibodies within an antiserum may sometimes be possible eg. by affinity chromatography, although this is rarely undertaken. The use of monoclonal antisera is another possibility and although they offer the potential of world-wide assay standardization, they have not, as yet, been widely used for peptide RIA.

3. Separation Methods

Several methods can be employed to separate the free peptide from that bound to antibody. The two commonest are those using a second antibody or charcoal. The second antibody method utilizes the immunological difference between the antibody-labelled peptide complex and the free peptide. The second antibody binds the otherwise soluble complex and causes precipitation. Separation using the charcoal method on the other hand, exploits differences in size by acting as a non specific adsorbent. Usually the charcoal is coated with dextran to define the pore size and exclude large polypeptides and proteins (Gottlieb et al, 1965). Although the second antibody method is undoubtedly less dirty, separation using charcoal is simple, rapid and cheap.

4. Antigen

In the early years of RIA, most investigators were dependent for their supply of antigen from the group who performed the original isolation. Today, most peptides are synthesized chemically, purified at the site of production and sold commercially. This ease of availability can however lead to problems, since a successful assay is dependent upon the antigen behaving identically to the endogenous antigen. All lyophilized peptides contain residual moisture and counter ions such as acetate and formate because of their inherent charge. The peptide content of many preparations sold commercially can therefore vary from 50->90%. Several recent articles have highlighted the problems associated with using commercially produced peptides (Brown et al, 1986). These include impure preparations, wrong sequences and short measures. Peptides containing cysteine, methionine or tryptophan are also particularly sensitive to oxidation. Handling of peptides is also problematical since losses can occur due to surface adsorption. This problem is often underrated but it can be as much as 90% or more.

The study of a particular peptide can present problems regarding the appropriate antigen to use for assay purposes. This becomes important when inter- and intra-species molecular heterogeneity is evident. Intra-

species molecular heterogeneity can be further complicated when peptides differ not only from organ to organ (eg. enteroglucagon and pancreatic glucagon) but also within a given tissue (eg. cholecystokinin)

5. Incubation Conditions

Having established the optimal reagents to use, consideration must also be given to the incubation conditions. The two principle variables being incubation temperature and pH.

Incubation Temperature

Assay incubations are usually carried out to equilibrium, although this is not a prerequisite for a successful assay. The time to reach equilibrium conditions is dependent upon the incubation temperature. As the temperature is elevated, the time decreases at the expense of lowering the equilibrium constant and a corresponding loss of sensitivity. The choice of assay incubation temperature is therefore a balance between the sensitivity required and convenience.

pH

Antigen-antibody interactions are generally affected by fluctuations in pH outside the range 7.4-8.6. The

incubation medium must therefore be buffered to overcome this problem. Since ionic strength also affects antibody binding, low ionic strength buffers are often chosen.

Material And Methods

Iodination Of Neuromedin U8

Synthetic neuromedin U8 (CRB Ltd, Cambridge, UK) (20 nmol) was dissolved in 40 µl of 400 mmol/l phosphate buffer (pH=7.4) together with 1 mCi of carrier free $Na^{125}I$ and 40 nmol chloramine T dissolved in 40 mmol/l phosphate buffer. The reaction was left to proceed for 10-15 seconds, at the end of which time the reaction was terminated by the addition of 600 nmol sodium metabisulphite.

The iodination products were then purified by reversed phase HPLC. The contents of the reaction vial were diluted with 1ml 10% (v/v) aqueous acetonitrile containing 0.05% trifluoroacetic acid (TFA) and loaded onto a 5 µm octadecyl silysilica (C_{18}) column eluted with the same buffer. A linear gradient of acetonitrile/ water/TFA (25/75/0.05) was subsequently developed to (35/65/0.05). Each eluted peak of radioactivity was diluted in assay buffer, assessed for its suitability in RIA and stored at 4^0C. The elution position of unreacted peptide was determined spectrophotometrically by

monitoring the u.v absorbance of each fraction at 214 nm. The specific activity of the preparation was calculated by the self displacement method.

Preparation Of An Antigenic Conjugate Of Neuromedin U8

Neuromedin U8 (400 nmol) and bovine serum albumin (BSA) (100 nmol) were dissolved in phosphate buffer (1ml, 60 mol/l pH=7.5). Glutaraldehyde (30 µmol) dissolved in the same buffer (1.5 ml) was then added drop-wise to the mixture over 15 minutes, stirring constantly. A trace of radiolabelled neurotensin was added in order to monitor the degree of conjugation. The mixture was left stirring overnight at 4^0C. At the end of this period, an aliquot (20 µl) was fractionated by gel permeation chromatography (Sephadex G-50 superfine, 1x100 cm) eluted with phosphate buffer at 4 ml/hour at 4^0C. The ratio of void volume (conjugated tracer) to total radioactivity represented the degree of incorporation. The conjugate was lyophilized and stored at -20^0C.

Immunization

Lyophilized conjugate was dissolved in tap water (6ml) and emulsified with equal volumes of Freund's complete adjuvant. The mixture was administered to 8 rabbits. Each rabbit received four subcutaneous injections, one

into each groin and axilla. Animals were boosted two months later with approximately 50 µg neuromedin U8 coupled to BSA in Freund's incomplete adjuvant and bled from an ear vein 7 days after this injection. Boosts were administered regularly thereafter at 6-8 week intervals.

Antiserum Titre

Antisera were tested to determine their titre (the reciprocal of the dilution required to give 45-55% binding of neuromedin U8 tracer) and sensitivity. Serial dilutions (tenfold) of each antiserum were made in assay buffer (60 mmol/l phosphate buffer pH=7.4 containing 10 mmol/l EDTA and 0.3% bovine serum albumin) to which 50 µl neuromedin U8 tracer was added (1800-2400 cpm, 1-2 fmol). To a duplicate set of tubes, synthetic neuromedin U8 (25 fmol) was added in order to assess the displacement at each dilution. Assays were incubated for 4-5 days at 4^0C. At the end of this time, bound and free neuromedin U8 tracer was separated by adding 250 µl dextran-charcoal slurry (0.4mg: 4mg w/w to each tube suspended in 60 mmol/l phosphate buffer containing EDTA and 0.3% gelatine) and the tubes centrifuged for 15-20 minutes (2,500 g_{av}) at 4^0C. The radioactivity in both the resultant pellet (free) and supernatant (bound) was counted in a 16 well gamma counter for 3 minutes. By counting a total of over 4000 counts the error

attributed to counting was estimated to be less than 1%.

Antiserum Specificity

The specificity of the chosen neuromedin U antiserum (YA7) was studied by attempting to displace neuromedin U8 tracer with more than 100 pmol/tube of various other peptides. The cross reactivity, was defined as [x/y x 100%] where x is the concentration of synthetic neuromedin U8 and y is the concentration of the substance tested required to decrease the binding of neuromedin U8 tracer by 50%.

Assay Details

Neuromedin U8 standards were gravimetrically prepared, dissolved in a solution of lactose (50 g/l), citric acid (2g/l), BSA (25 g/l), L-cysteine hydrochloride (1g/l), aprotinin ($8x10^5$ KIU/l), and formic acid (0.1 mol/l) and lyophilized. Each standard was stored at -20^0C and reconstituted in assay buffer immediately before use.

Samples (2-700 µl) were added to assay buffer (60 mmol/l sodium potassium phosphate, pH=7.4, containing 10 mmol/l EDTA and 0.3% (v/v) BSA) followed by 50 µl neuromedin U8 tracer (1800-2400 cpm) and 50 µl diluted neuromedin U antiserum. Each assay contained at least one standard curve containing synthetic neuromedin U8 standard (2.5-

250 fmol) and a volume of medium equivalent to that added to each sample tube. After a 4-5 day incubation at 4^0C, free neuromedin U8 tracer was separated by adsorption onto a slurry of charcoal (4mg/tube) coated with 10% dextran (mol wt 70,000), centrifuged and the radioactivity in the resultant supernatant and pellet counted as outlined above. Assay reproducibility was evaluated by measuring 3 samples to which exogenous neuromedin U8 was added in concentrations of 8, 20 and 40 fmol/tube in 8 consecutive assays. Within-assay precision was evaluated by measuring 8 replicates of each of these samples in a single assay.

To determine the optimal buffer for use in this assay, 60 mmol/l phosphate (pH 7.4), 50 mmol/l acetate (pH 5.0) and 50 mmol/l veronal (pH 8.1) buffer containing 0.3% BSA w/v were tested. A standard curve was constructed in each buffer and the binding following a 4-5 day incubation at 4^0C examined as above. The effect of BSA in phosphate buffer was also examined.

The amount of dextran coated charcoal slurry required for the separation of bound and free antigen was tested. Over the range of 2-64 mg charcoal/tube the effect of doubling additions was examined upon the non-specific (blank), zero and antibody excess binding as well as assessing its effect upon assay sensitivity with the inclusion of a single concentration of neuromedin U8

standard (25 fmol).

The effect of incubation time at 4°C upon the assay parameters described above was also examined. Assay incubations were terminated after 1, 2, 3, 4, 5, 6 and 21 days and the resultant antibody binding determined.

Results And Discussion

^{125}I NmU8

Reversed phase HPLC fractionation of iodination products consistently produced two peaks of radioactivity. An initial peak eluting in the void volume represented unreacted ^{125}I, whilst the second eluting at fraction 42, represented ^{125}I NmU8 (Figure 2: 5). Unreacted neuromedin U8 was clearly separated from the tracer (fraction 24) when monitored by u. v absorbance at 214nm. More than 90% of this product bound antisera YA7 when it was present in excess. In the absence of antisera, non-specific binding of the tracer was always less than 10%. The specific activity of the neuromedin U tracer was calculated to be greater than 65 Bq/fmol. The integrity of the label preparation was examined by addition of 10 fold less and 10 fold more tracer to an antiserum dilution curve (approximately 0. 1 and 10 fmol respectively) (Figure 2: 6).

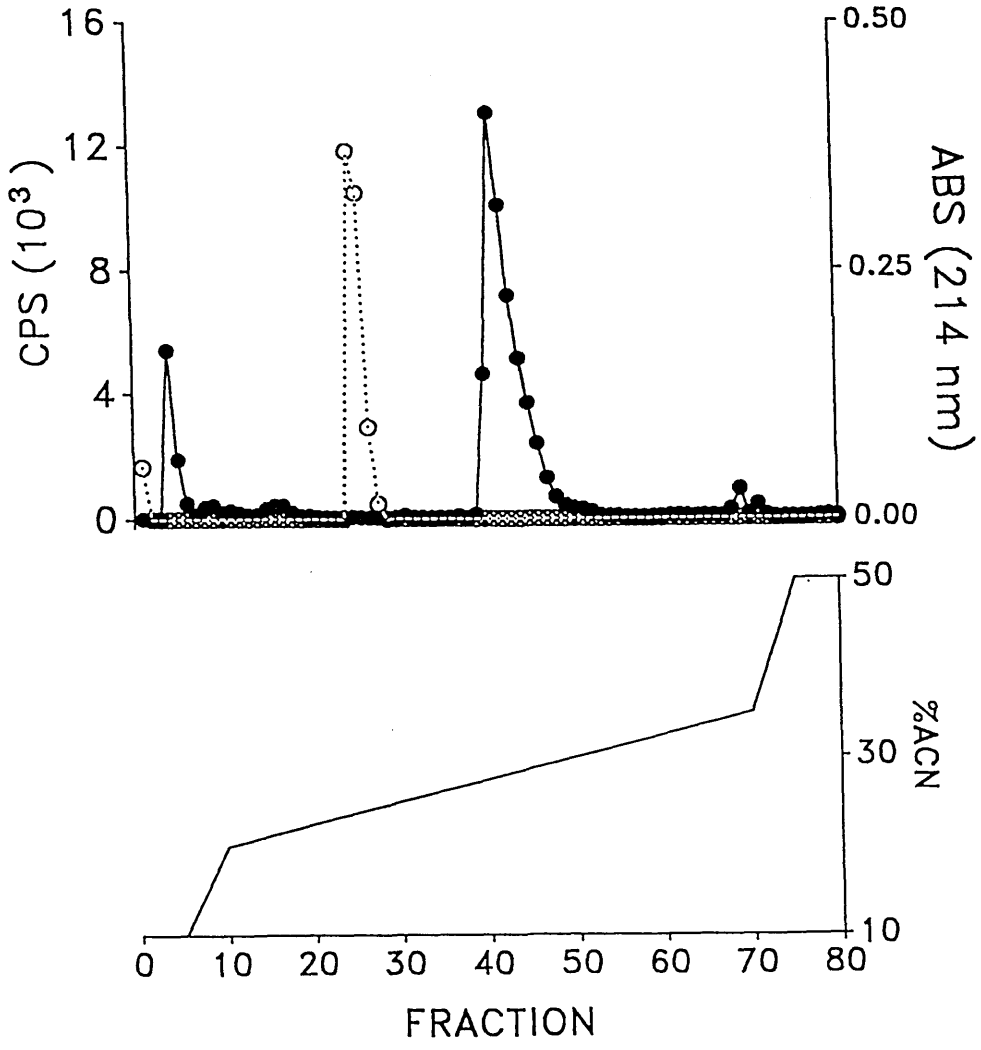


Figure 2:5 Representative reversed phase HPLC purification profile of ^{125}I-NmU 8 on a Techsil C_{18} column (0.4x250mm). The upper panel shows the elution position of unreacted and incorporated ^{125}I (solid line) and the elution position of the unreacted peptide (dotted line). the lower panel represents the acetonitrile gradient used.

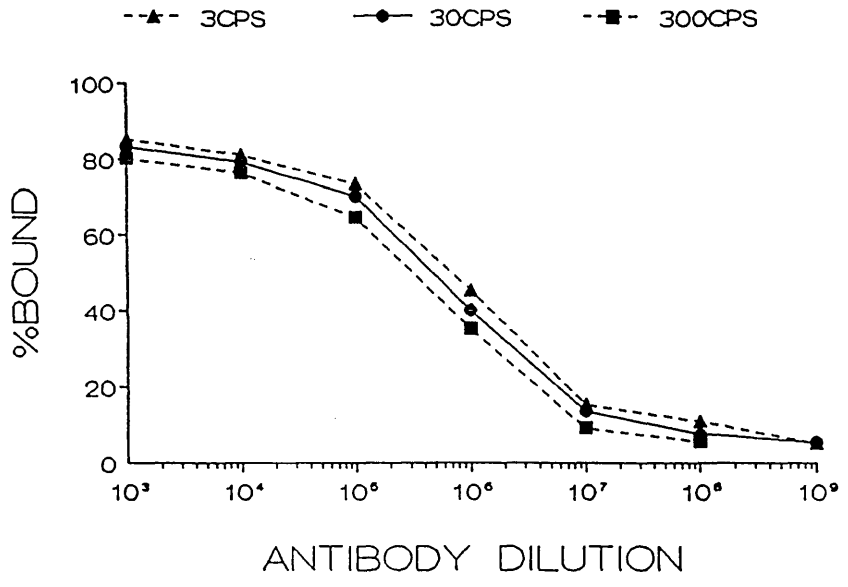


Figure 2: 6 Assessment of the tracer preparation by comparative analysis of the binding at three dilutions to sequential dilutions of antiserum.

Antisera

Two out of eight rabbits immunized produced antisera to neuromedin U of a quality suitable for radioimmunoassay. Whilst antiserum (YA7) failed to cross-react with any other peptide (Table 2:1) the other (YA4) partially cross-reacted (0.1%) with pancreatic polypeptide and was therefore not used for subsequent studies. At a final dilution of 1:10,000 antiserum YA7 detected changes of 4 fmol neuromedin U8/tube with 95% confidence limits and although it cross-reacted fully with neuromedin U25, it only cross reacted 5% with the C-terminal pentapeptide (NmU_{21-25}). The addition of 37 fmol reduced the zero binding by 50% (^{125}I NmU8 IC_{50}) (Figure 2:7). The affinity constant of antiserum YA7 was determined by self-displacement to be 7.34×10^{10} l/mol (Figure 2:8). This antiserum was used in all further studies described in this thesis.

Assay Details

Figure 2.9 shows the dependency of the assay upon the buffer conditions used. Three buffers were tested, all contained 0.3% BSA. Incubation in acetate buffer (pH=5.0) resulted in only non-specific binding. Successful assays could potentially be performed in either phosphate (pH=7.4) or veronal (pH=8.2) buffers although the assay was less sensitive in veronal buffer.

Having established the preference for phosphate buffer, the effect of BSA was examined (Figure 2.9 lower panel). No difference was observed between any of the BSA concentrations tested. Omission of BSA in the buffer however, produced low zero binding and a "hump" in the standard curve.

Neither the zero percent binding nor the slope of the standard curve (range 2.5-250 fmol) was affected by the addition of up to 20 µl of 0.5 mol/l acetic acid (data not shown). Separation of bound and free tracer was achieved using dextran coated charcoal (250 µl per tube). Varying the amount of charcoal added per tube caused little change in the binding observed for additions of 2-8 mg but the addition of greater amounts dramatically decreased antibody binding (Figure 2:10). For all subsequent assays 4mg charcoal/assay tube was therefore used.

At 4^{0}C, antiserum binding increased with time up to 6 days (Figure 2:11). Optimal assay sensitivity (the difference between zero binding and 25 fmol neuromedin U8/tube) was observed on days 4 and 5. After 21 days the antibody binding decreased slightly, sensitivity dropped and the nonspecific binding increased.

Table 2:1 Peptides and proteins examined for cross-reaction with antisera YA-7 at a final dilution of 1:10,000

Peptide or fragment	% Cross-reaction
Neuromedin U25	100
Neuromedin U25 (21-25)	5
Neuromedin B	<0.001 upto 100 pmol/tube
Neuromedin C (GRP-10)	
Bombesin	
Somatostatin	
Neurotensin	
Neuromedin N	
Gastrin	
Cholecystokinin	
Vasoactive intestinal polypeptide (VIP)	
Human pancreatic polypeptide	
Porcine pancreatic polypeptide	
Peptide tyrosine tyrosine (PYY)	
Neuropeptide tyrosine (NPY)	
TRH	
Growth hormone	
TSH	
LH	
Prolactin	
ACTH	
Substance P	<0.001 upto 10 pmol/tube
Peptide histidine isoleucine	
Calcitonin gene related peptide	
Glucagon	
Bovine pacreatic polypeptide	
Avian pancreatic polypeptide	
Neurokinin A	
Secretin	

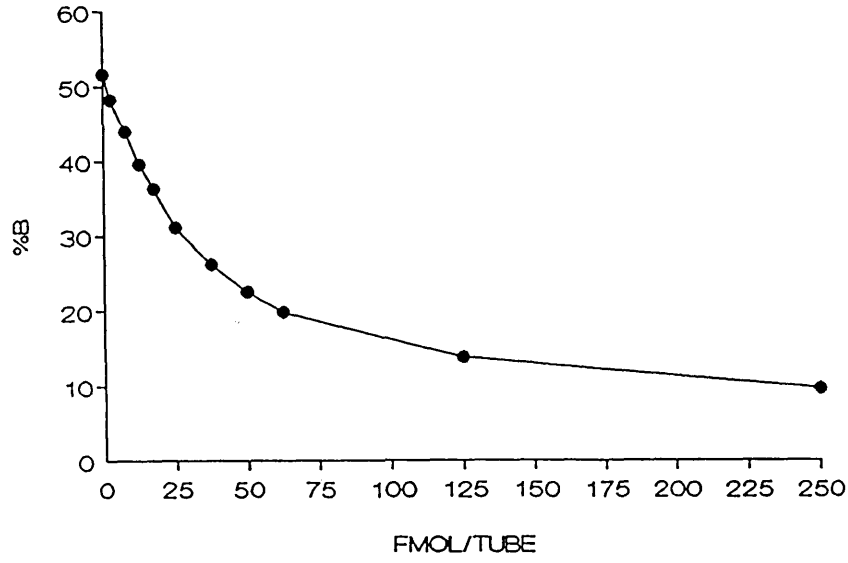


Figure 2:7 A typical neuromedin U standard curve using antiserum YA7. Antibody binding is decreased by the addition of synthetic neuromedin U8 standard. A fixed amount of ^{125}I NmU8 tracer (1 fmol) was added per assay tube.

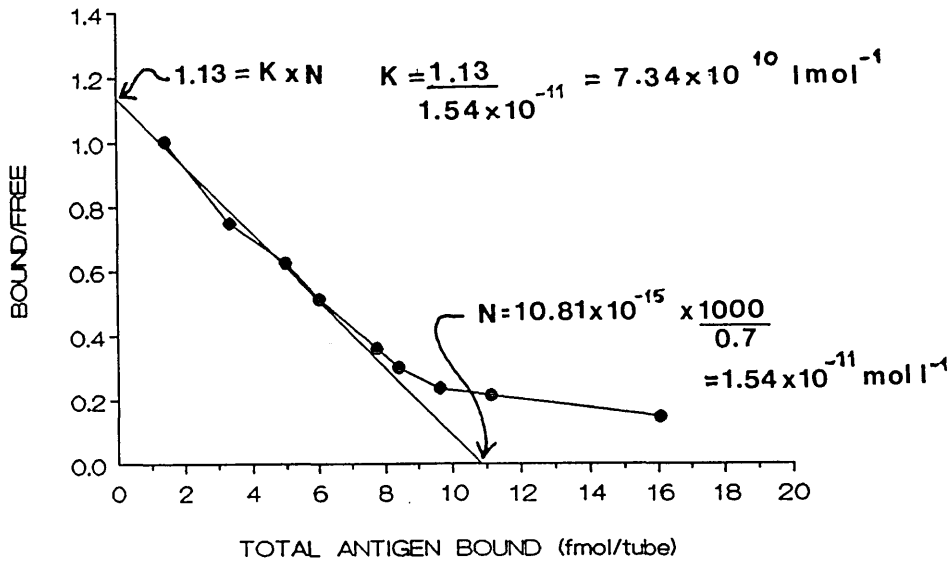


Figure 2: 8 A Scatchard plot. The intercept on the vertical (Y) axis is K (affinity constant) x N (concentration of antibody binding sites). The intercept on the horizontal (X) axis is N.

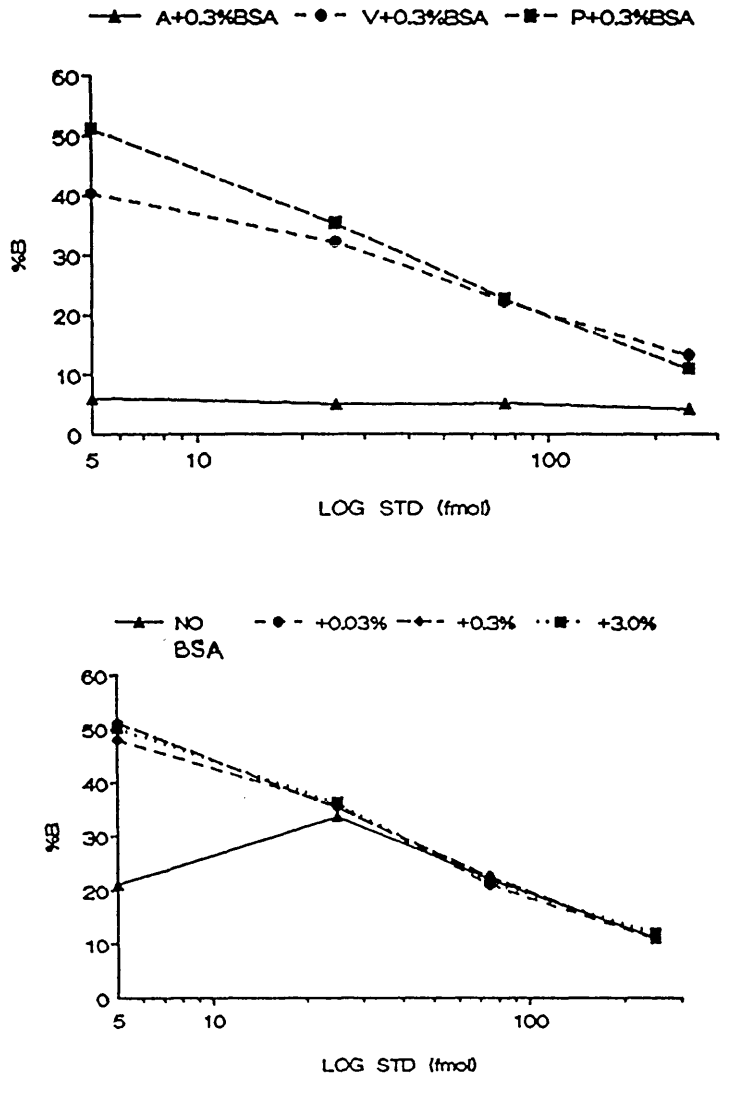


Figure 2: 9 The effect of different buffers A-acetate, V-veronal and P-phosphate (upper panel) and the effect of various additions of BSA to phosphate buffer (lower panel) upon the standard curve.

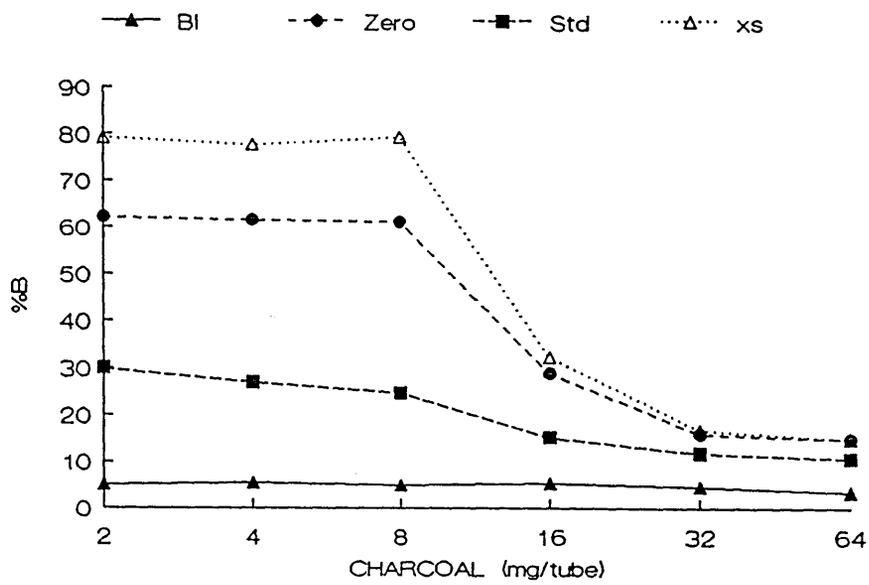


Figure 2:10 The dependence of non specific (Bl), zero (Z), displacement (Std) and excess (XS) binding upon the quantity of dextran coated charcoal added per assay tube.

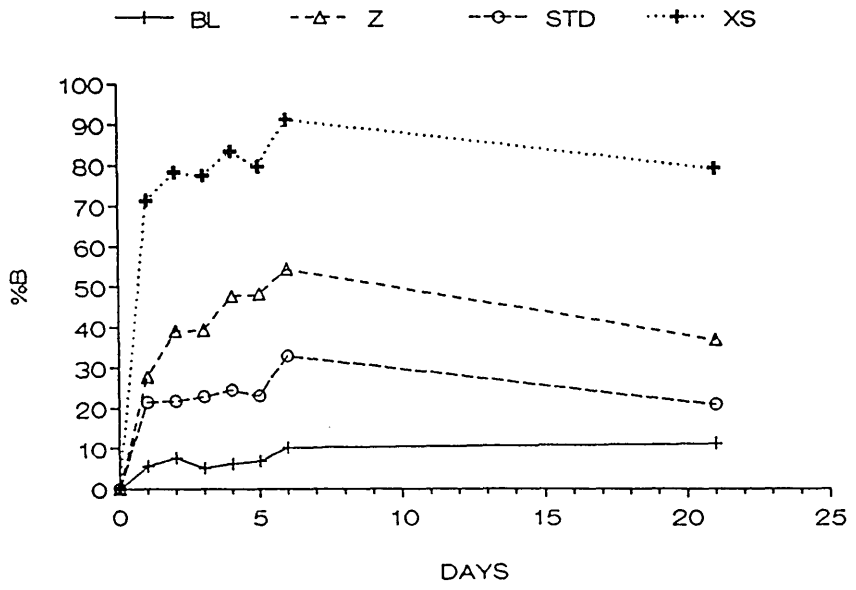


Figure 2:11 The effect of incubation time upon the non specific binding (Bl), zero binding (Z), displacement (Std) and excess antibody binding (XS).

Using optimal incubation and separation conditions, the interassay coefficient of variation over the range 8-40 fmol/tube was less than 13%. The intra assay variation over the same range of standards was less than 7%.

General Discussion

Radioimmunoassay offers a highly sensitive and specific technique to measure substances present in biological fluids in very small amounts (femtomoles 10^{-15}mol). In principle the system is simple, although RIA is not without its problems and limitations. The most important of these is perhaps the fact that RIA is based upon recognition of tertiary structure rather than the biological activity of the substance.

Often only a small number of amino acid residues act as the antigenic determinant for antibody binding. These residues can be either directly adjacent to each other (continuous epitope) or they can be at a distance brought into close proximity only when the substance adopts its tertiary structure (discontinuous epitopes). Thus precursors, fragments as well as unrelated substances having a similar sequence to the antigenic determinant may also be recognised by the antibody thereby adding to the total immunoreactivity quantified. Such problems occur when attempting to estimate cholecystokinin (CCK) and gastrin. These two peptides

share a common carboxyl-terminal pentapeptide sequence (Rehfeld, 1982). Antibodies raised to one of these peptides may therefore cross-react with the other. The measurement of cholecystokinin exemplifies a further problem since different antisera may also show varying cross-reaction with the variety of molecular forms which occur naturally. Apart from specific interference, RIA is also susceptible to non-specific interference from factors such as high salt concentrations, lipids and excess protein which alter the antigen-antibody interaction. In addition, problems such as marked inter or intra-assay variation, non parallelism and poor peptide extraction protocols may produce further artifacts.

The radioimmunoassay described in this chapter allows the quantification of neuromedin U-like immunoreactivity in tissue extracts. The antiserum (YA7) used in this thesis fully cross reacts with neuromedin U25 and appears to be highly specific. Although the precise antigenic determinant which the antibody recognises is unclear due to the lack of available fragments, it does not fully cross react with the carboxyl-terminal pentapeptide. Due to the presence of a tyrosine residue, the iodination of neuromedin U8 did not prove to be a problem. High specific activities (greater than 60 Bq/fmol) were consistently obtained. This reflected the good separation of the tracer from unreacted peptide by

reversed phase HPLC. Both the chloramine T iodination and the subsequent separation of products are rapid with the entire procedure being completed within two hours.

There is no ideal assay system. Different techniques will possess different intrinsic characteristics. However, the important criterion of a well validated radioimmunoassay must be its usefulness in the biochemical and physiological study of the substance to be examined.

CHAPTER 3

EXTRACTION AND CHROMATOGRAPHIC CHARACTERIZATION OF NEUROMEDIN U-LIKE IMMUNOREACTIVITY

Introduction

During the course of their studies, Bayliss and Starling outlined details of a technique which simultaneously prevented the degradation of the hormone secretin and facilitated its solubilization from tissue. They discovered that whilst proteolytic enzymes became denatured, the bioactivity of their mucous membrane preparation was unaffected by boiling in either acid or water (Bayliss and Starling 1902). A similar conclusion was later reached by Edkins (1906) and Blair et al (1961) concerning the thermostability of gastrin. Consequently, the protocols of Jorpes and Mutt (1961) for the purification of secretin and Gregory and Tracy (1964) for the purification of gastrin, incorporate a step which involves boiling. Other methods of peptide extraction do exist, but only those based on treatment with aqueous acid/organic solvent solutions have resulted in the successful isolation of essentially pure peptides. Insulin (Banting et al, 1923), pancreatic

glucagon (Staub et al, 1953) and bovine neurotensin (Kitabgi et al, 1976) were all purified in this way.

Chromatography

Early peptide purification protocols were based upon selective adsorption/precipitations followed by fractionation on columns filled with materials such as Sephadex® (Pharmacia, Uppsala Sweden). Since the early 1980s however, many of these methods have been superseded by HPLC. This method offers the potential of superior separations, better reproducibility and a reduction in analysis time.

Gel Permeation Chromatography

Gel permeation chromatography became an established laboratory technique in 1959 with the introduction of Sephadex, a beaded form of dextran gel. Separations are made both simply and rapidly on the basis of apparent molecular weight. Molecules which are larger than the pores of the Sephadex pass through the bed with the mobile phase and are eluted first. Smaller molecules penetrate the gel particles to varying degrees and consequently elute later. The fraction of the gel volume available for a given solute (Kav) is used to standardise and summarise the behaviour of the solute on the column and is derived using the method of Laurent

and Killander (1964)

$$K_{av} = (V_e - V_0) / (V_t - V_0)$$

where:

V_e = observed elution volume

V_0 = column void volume

V_t = total column volume

Whitaker (1963) amongst many, concluded that for globular proteins the elution volume was exponentially related to the molecular weight. Such reports led many groups to use gel permeation chromatography in order to estimate the molecular weight of peptides and proteins. It soon became evident however, that the elution coefficient (K_{av}) of a molecule is also dependent upon its shape and its state of aggregation. Attempts to overcome these problems by the addition of urea or guanidine hydrochloride to the column buffer have only met with limited success. It appears that such estimations are only accurate when the substance of interest is covalently modified and the chromatography performed in an organic solvent (Meredith, 1984).

Ion Exchange Chromatography

The basic principle of ion exchange chromatography is that separations are achieved on the basis of the net charge carried by solute molecules. The technique is capable of separating two molecules with even small charge differences. An ion exchange resin consists of an insoluble matrix to which charged groups are covalently bound. Such groups are associated with mobile counter ions which can be reversibly exchanged with other ions of the same charge. Positively charged exchangers have negatively charged counter ions (anions) for exchange and are termed anion exchangers. Negatively charged exchangers have positively charged counter ions (cations) and are termed cation exchangers. Separation is achieved by first applying the sample. Unbound sample is then washed off with the starting buffer. The bound substances are then eluted from the column on the basis of their different attraction to the exchanger. This interaction is dependent upon both the ionic strength and pH of the mobile phase.

Reversed Phase HPLC

In recent years, microparticulate, stationary phases such as octadecyl-silica have offered a rapid and efficient means for the separation of peptides and proteins by HPLC. Retention on non-polar sorbents is

primarily due to van der Waals forces developed between the solutes and hydrocarbons covalently attached to the surface of the stationary phase. Consequently, substances are eluted using less polar, organic solvents in order of increasing hydrophobicity.

Studies of peptide retention time on such systems have led to the publication of various indices predicting elution orders, based on amino acid composition (Meek, 1980). To date, such data does not, take factors such as conformation into account. This is highlighted by the fact that isomeric compounds can be separated using this technique (Molnar and Horvath, 1977).

Separations using reversed phase HPLC are dependent upon the following factors:-

(i) Sorbent

Bonded silica materials typically fall into three major catagories namely polar (e.g unbonded silica), non-polar (e.g octadecyl and phenyl) and ion exchange (anionic and cationic). The choice of appropriate sorbent is governed by the chemical nature of the substance to be separated. Octadecyl silica (C_{18}) is, however, probably the most often used in peptide research.

(ii) Solvent

Not only is it important to choose the appropriate solvent for the sorbent employed but the initial and final solvent composition and gradient used must be determined. Typical organic solvents used for HPLC include acetonitrile and methanol.

(iii) Ion Pairing Agents and pH

On reversed phase HPLC, acidic and basic peptides are often separated with poor peak shapes at neutral pH due to partial solute ionization. One solution to this problem is to suppress ionization and fractionate compounds in the unionized state. Acidification of the solvent with acids such as trifluoroacetic acid (TFA), suppresses the ionization of acidic peptides and can provide counter ions for ionic species. In this way neutral, acidic and basic components can be fractionated by the same system.

(iv) Temperature

Studies have shown that as the temperature is increased, peptide and protein retention decreases due to increasing solute solubility in the mobile phase. Resolution also improves due to an accelerated transfer of the solute between the mobile and the stationary

phase (Guo et al, 1986).

Solid Phase Extraction

Solid phase extraction is an extention of HPLC methodology allowing rapid sample preparation. Disposable extraction cartridges such as the Sep Pak® series (Waters Assoc, Milford USA) contain a small ammount of bonded silica material and are utilized to extract specific compounds from a complex sample. A clean, concentrated extract is produced which simplifies subsequent analysis.

Aim Of This Study

This chapter has two goals. The first is to establish the optimal condition for the extraction of neuromedin U-like peptides for subsequent studies. The second is to evaluate the degree of molecular heterogeneity of neuromedin U like peptides present in the central nervous system and gastrointestinal tract of four mammalian species.

A. Extraction Of Neuromedin U-Like Immunoreactivity From Rat Gastrointestinal Tract

Materials And Methods

The small intestine was removed from five male Wistar rats immediately following sacrifice, washed in saline, blotted carefully, diced and weighed. Tissue pieces were then extracted in 10 volumes of the following solutions:

(i) boiling distilled water
(ii) boiling 0.2 mol/l NaOH
(iii) boiling 0.2 mol/l HCl
(iv) boiling 0.5 mol/l acetic acid
(v) cold acid/ethanol (0.2 mol/l HCl/eth 1:3 v/v)
(vi) cold acetic acid
(vii) cold distilled water

For extraction in media (v) (vi) and (vii), tissues were homogenized in 5 volumes of solution at 4°C for 2 minutes, centrifuged at $1500g_{av}$ and the resultant pellet re-homogenized with an equal volume of the extraction medium. After centrifugation, both supernatants were pooled. To remove the ethanol from tissue extract (v), this sample was lyophilized overnight and resuspended with buffer immediately prior to assay.

To assess if any degradation of solubilized neuromedin U had occured during the extraction procedure, neuromedin U25 tracer was added together with tissue to each solution and extracted as above.

Extraction Efficiency

A portion of ileum from five adult Wistar rats was removed and extracted in 10 volumes of boiling 0.5 mol/l acetic acid for 15 minutes as above. When cool, the extract was centrifuged at $1500g_{av}$ for 10 minutes and the supernatant decanted and frozen. An equal volume of fresh acid was added to the pellet and the tissue re-extracted as above. This process was repeated for a total of three times.

Radioimmunoassay

The tissue content of neuromedin U like immunoreactivity was measured using the radioimmunoassay described in chapter 2. Neuromedin U25 tracer was prepared by the chloramine T procedure also described in that chapter using synthetic neuromedin U25 (Bachem, Torrance, USA).

Chromatography

For analysis by gel permeation chromatography, tissue extracts and synthetic standards were loaded onto a

column of Sephadex G-50 Superfine (0.9x100cm) and eluted with 60 mmol/l phosphate buffer (pH7.4) containing 0.3% bovine serum albumin (BSA) and 200 mmol/l sodium chloride at a flow rate of 10ml/hour. The columns were calibrated with dextran blue (mol wt $2x10^6$), horse heart cytochrome C (mol wt 12,384) and $Na^{125}I$. Trace ammounts of all three markers were included in all column runs to confirm the consistency of successive runs. The fractions (2ml) were assayed for neuromedin U-like immunoreactivity and the position of each immunoreactive peak expressed in terms of its elution co-efficient (K_{av}).

For analysis by reversed phase fast protein liquid chromatography (FPLC), tissue extracts were filtered (0.2μm) and loaded onto a C_{18} column (PepRPC, Pharmacia) eluted at 1ml/min with acetonitrile/water /trifluoroacatic acid (TFA) (0/100/0.05) using a Pharmacia FPLC® system (2 P-500 pumps, 1 MV-7 motorised valve injector, 1 LCC-500 solvent programmer and a 500ul injection loop). After 5 minutes, a linear gradient was developed to acetonitrile/water/TFA (40/60/0.05) over 40 minutes. The gradient was then increased to acetonitrile/water/TFA (60/40/0.05) in 5 minutes where it remained for a further 5 minutes. Fractions (1ml) were collected, lyophilized and reconstituted with buffer prior to assay.

Results

The chromatographic examination of neuromedin U 25 tracer following each extraction protocol is shown in figure 3: 1. The results reveal that only boiling in acid (C) and (D) retained the integrity of the neuromedin U tracer (H). Both extraction procedures which involved water (A) and (G), generated a less hydrophobic molecular form. Boiling in aqueous sodium hydroxide (B) generated a very broad peak, eluting around the position of the intact tracer. Extraction in acid/ethanol (E) generated multiple molecular forms as did extraction in cold acetic acid (F).

Extraction in cold water was found to generate the highest levels of neuromedin U immunoreactivity (Table 3. 1). Boiling acid was the next most efficient followed by extraction in cold, acidified media. Boiling tissue at a neutral or basic pH extracted low levels of neuromedin U-like immunoreactivity.

Characterization of the endogenous neuromedin U-like immunoreactivity extracted by each protocol, using gel permeation chromatography is shown in figure 3: 2. Extraction in boiling water (A), hydrochloric (C) and acetic acid (D) as well as cold acid/ethanol (E) all produced a single molecular form of immunoreactivity (Kav=0. 69) which had a smaller apparent molecular

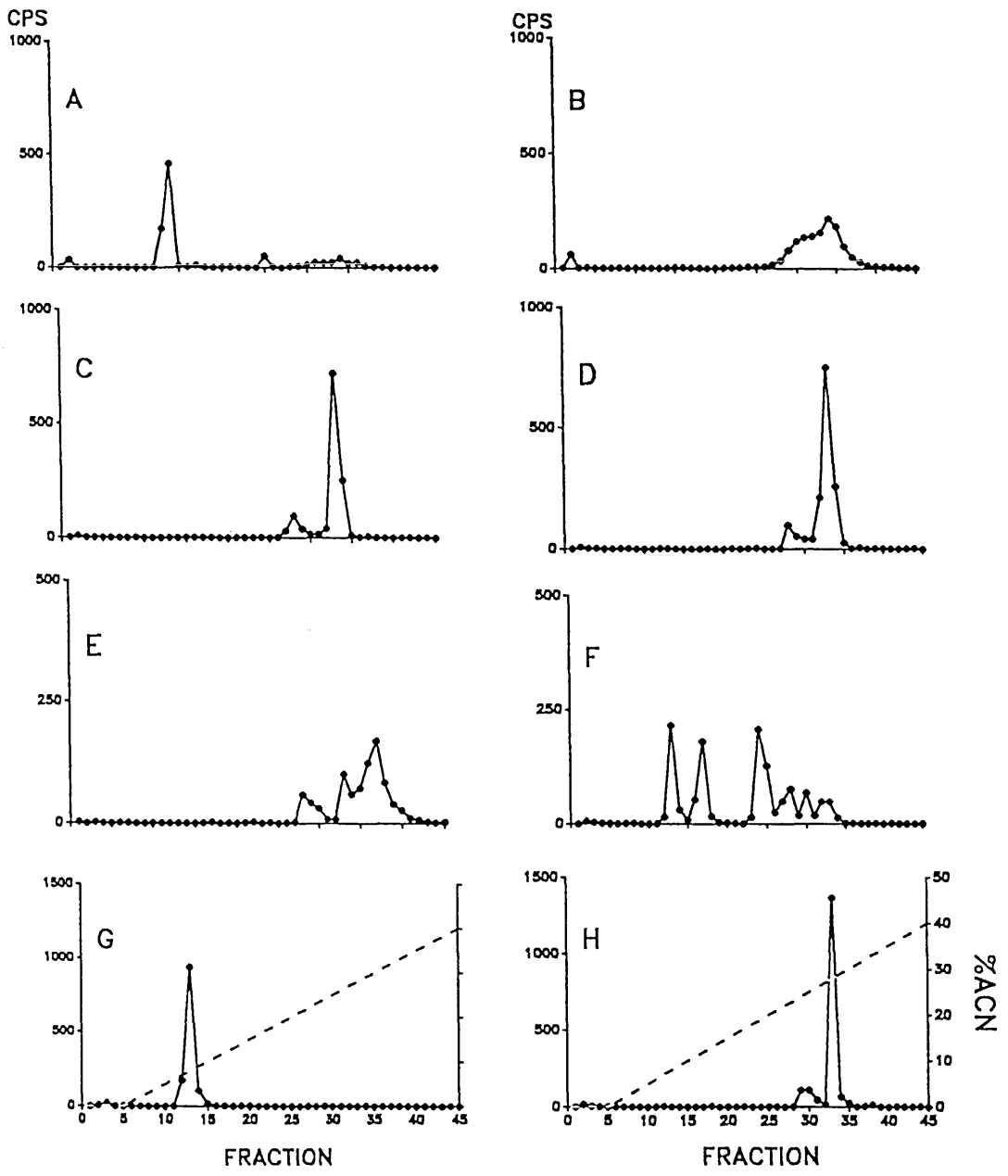


Figure 3: 1 Characterization by reversed phase FPLC of neuromedin U25 tracer recovered following extraction using various conditions. A- boiling water, B- boiling 0.2 mol/l NaOH, C- boiling 0.2 mol/l HCl, D- boiling 0.5 mol/l acetic acid, E- acid/ethanol, F- cold acetic acid, G- cold water, H- no treatment.

Table 3:1 The effect of different methods upon the extraction of neuromedin U-like immunoreactivity from rat gastrointestinal tract (mean±SEM, n=5).

		NmU-LI (pmol/g)
Boiling:	H_2O	7.5±0.8
	0.2 mol/l NaOH	2.0±0.2
	0.2 mol/l HCl	81.5±7.4
	0.5 mol/l acetic acid	83.6±7.4
Cold:	HCl/ethanol	53.0±7.7
	0.5 mol/l acetic acid	43.0±16.8
	H_2O	631.2±180.0

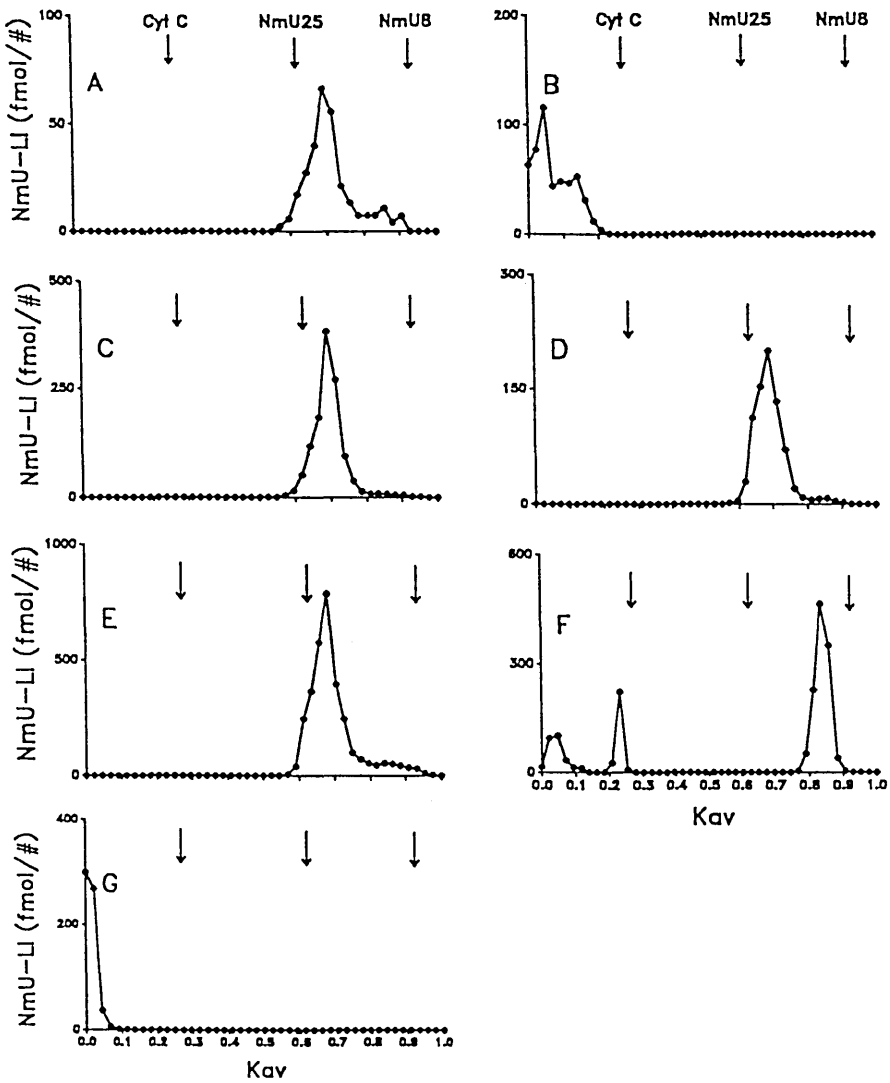


Figure 3: 2 Characterization by gel permeation chromatography of endogenous neuromedin U-like immunoreactivity extracted from rat small intestine using various extraction techniques. A- boiling water, B- boiling 0.2 mol/l NaOH, C- boiling 0.2 mol/l HCl, D- boiling 0.5 mol/l acetic acid, E- acid/ethanol, F- cold acetic acid, G- cold water. Arrows indicate the elution positions of horse heart cytochrome C (Cyt C), neuromedin U25 (NmU25) and neuromedin U8 (NmU8).

weight than synthetic porcine neuromedin U 25 (Kav=0.62) but greater than neuromedin U8 (Kav=0.92). Extraction in boiling 0.2 mol/l sodium hydroxide (B) produced two molecular forms of immunoreactivity which eluted close to the void volume (Kav=0.05 and 0.14) indicating large molecular weight, cross-reacting material. Extraction in cold acetic acid (F) produced a predominant molecular form of neuromedin U immunoreactivity with a smaller apparent molecular weight (Kav=0.84) than either synthetic neuromedin U25 or the neuromedin U immunoreactivity extracted by boiling in the same medium (D)(Kav=0.69). In addition, two minor molecular forms were also extracted by this method, one eluting close to cytochrome C (Kav=0.23) and the other close to the void volume (Kav=0.05). Extraction in cold water produced only void volume immunoreactivity (G). All column recoveries were in excess of 80%.

Characterization of endogenous neuromedin U immunoreactivity in rat ileum by reversed phase HPLC revealed that boiling water (A), hydrochloric (C) and acetic acid (D) extract a single molecular form of neuromedin U immunoreactivity eluting at fractions 34-35 (Figure 3:3). Boiling in aqueous sodium hydroxide (B) also produced a single molecular form which was slightly less hydrophobic eluting at fraction 32. Extraction in cold acid/ethanol (E) generated two molecular forms, the

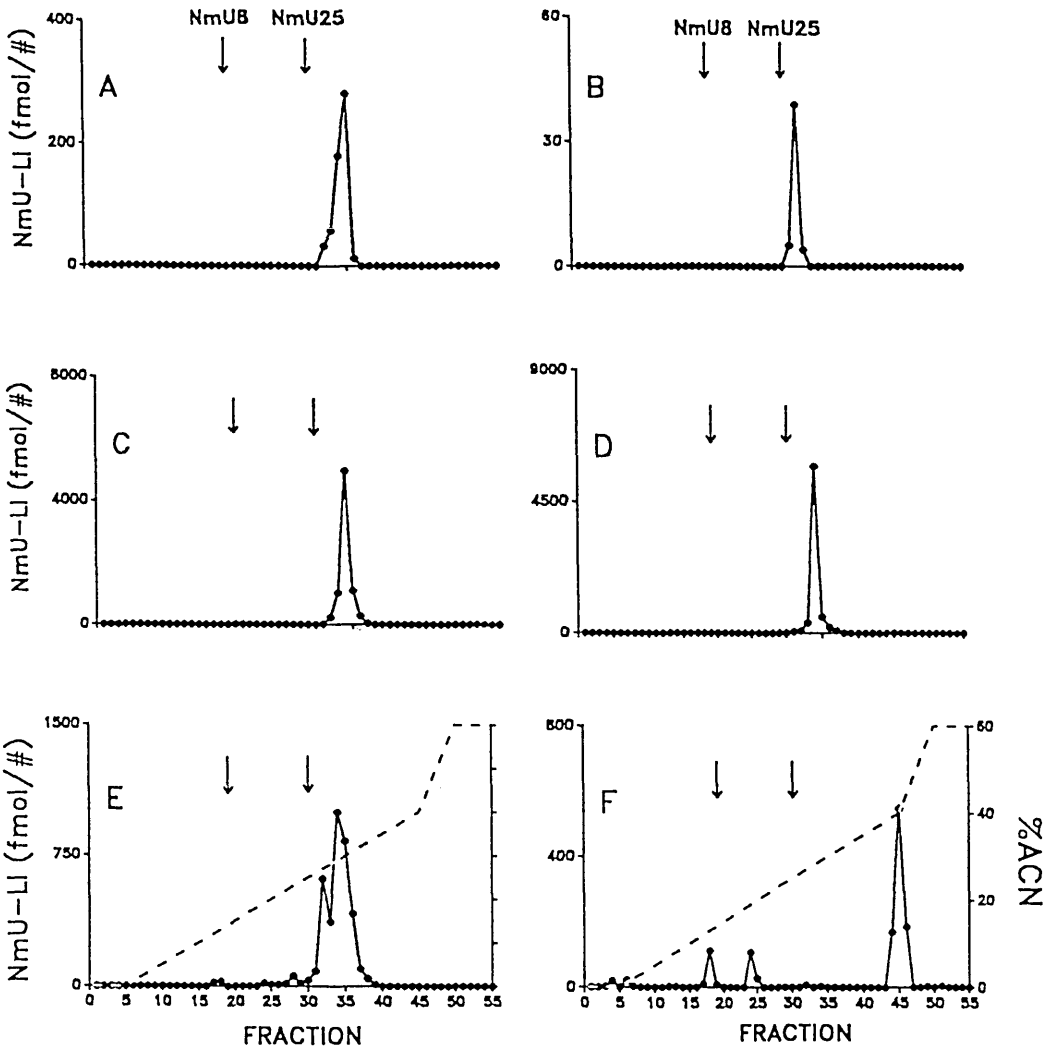


Figure 3:3 Characterization by reversed phase FPLC of neuromedin U-like immunoreactivity extracted from rat gastrointestinal tract using various conditions.
A- boiling water, B- boiling 0.2 mol/l NaOH, C- boiling 0.2 mol/l HCl, D- boiling 0.5 mol/l acetic acid, E- acid/ethanol, F- cold acetic acid. Arrows indicate the elution positions of synthetic neuromedin U25 and neuromedin U8.

Table 3:2 Assessing the efficiency of extracting neuromedin U-like immunoreactivity from rat gastrointestinal tract using boiling 0.5 mol/l acetic acid (mean±SEM, n=5).

	NmU-LI (pmol/g)	% Total Immunoreactivity
First extraction	76.5±7.9	81.8±3.8
Second extraction	15.1±3.1	16.0±3.0
Third extraction	1.3±0.5	1.3±0.6
Fourth extraction	0.9±0.3	0.9±0.4

first eluting at fraction 32, with the predominant form eluting at fraction 34. Cold acetic acid (F) extracted three molecular forms of immunoreactivity, the predominant one being more hydrophobic than any other molecular form noted thus far (fraction 45). Two minor components were also found at fractions 18 and 24. No neuromedin U-like immunoreactivity was recovered from the cold water extracts on this system. All other recoveries were in excess of 87%.

Sequential tissue extraction using 0.5 mol/l acetic acid revealed that the initial boiling procedure extracted 82% of the total extractable content of neuromedin U (Table 3.2).

Discussion

The aim of this study was to determine the optimal conditions under which endogenous neuromedin U-like peptides were extracted from tissue. In order to evaluate the degree of degradation or structural modification incurred during the extraction of endogenous neuromedin U, neuromedin U25 tracer was added to tissue at the start of each extraction procedure. Subsequent conclusions are made however on the assumption that the tracer and endogenous peptide behave identically.

The results of this study demonstrate that only boiling in acid preserved the integrity of the tracer (Figure 3: 1). All other extraction methods resulted in alterations in its retention time on reversed phase FPLC columns indicating that structural modification had occurred. Treatment with either cold or boiling water produced a less hydrophobic form of the tracer which is likely to represent a stable fragment. Treatment with boiling sodium hydroxide resulted in a broad, ill defined peak, whereas acid/ethanol and cold acetic acid treatment resulted in heterogenous profiles.

When the levels of endogenous neuromedin U immunoreactivity were quantified in the various rat gut extracts, the highest concentrations were found in cold water extracts (Table 3: 1). Subsequent chromatographic examination, however, revealed that all the immunoreactivity present eluted in the void volume on gel columns (Figure 3: 2 G). In addition, no immunoreactivity could be recovered following reversed phase FPLC. Consequently, the immunoreactivity solubilized using this extraction method is regarded as spurious, representing large molecular weight material which interfers with the assay.

The extraction methods which gave the second highest recovery of neuromedin U immunoreactivity were those which involved boiling tissue in acid. Extraction by

boiling in either 0.2 mol/l hydrochloric or 0.5 mol/l acetic acid liberated a single molecular form of immunoreactivity from rat gut (Figure 3:2 and Figure 3:3, plates C and D). Extraction using cold acid/ ethanol also produced a relatively high recovery from the tissue although FPLC analysis revealed the presence of two molecular forms (Figure 3:3 E). The first co-eluted with the immunoreactivity extracted by boiling in acid whilst the second was less hydrophobic. Considering the heterogenous profile obtained following fractionation of neuromedin U25 tracer treated in this way (Figure 3:1 E) degradation of the extracted peptide is likely to occur using this procedure. Thus the less hydrophobic material detected following FPLC analysis probably represents an immunoreactive fragment of the larger molecular form.

Although extraction using cold acetic acid produced a form of neuromedin U immunoreactivity which had a smaller apparent molecular weight than that obtained using boiling acid, it was more hydrophobic when characterized by reversed phase FPLC analysis (Figures 3:2 and 3:3 plate F).

Extraction in boiling water and sodium hydroxide produced a very poor recovery of neuromedin U peptides (<10% of that obtained using boiling acid). Whilst boiling in water appeared to extract the same molecular

form as by boiling in acid, boiling in aqueous sodium hydroxide extracted large molecular weight material, eluting near the void volume on gel columns (Figure 3:2 B). This material may represent precursor forms of neuromedin U. Paradoxically however, only a single peak of neuromedin U immunoreactivity was found following FPLC fractionation which eluted close to synthetic porcine neuromedin U25. Further studies are now required to clarify its identity.

It is not clear why extraction in acid is such an efficient method for the solubilization of basic peptides when compared to water alone. It has been noted however that the majority of cellular proteins carry a negative charge at physiological pH values and may therefore bind basic peptides by electrostatic interaction (Jorpes 1968). Upon lowering the pH, the charge of the proteins would become positive and this interaction abolished.

In conclusion, boiling in either 0.2 mol/l hydrochloric or 0.5 mol/l acetic acid is optimal for the extraction of neuromedin U-like peptides from tissue. Consequently, all tissues were extracted in 0.5 mol/l acetic acid for all subsequent studies. Although higher levels of immunoreactivity were detected by radioimmunoassay following extraction with cold water, this was the result of non specific assay interference.

B. Chromatographic Characterization Of Mammalian Neuromedin U-Like Peptides

Materials And Methods

Tissues from rat and guinea pig were dissected following sacrifice and extracted immediately. Porcine tissue was collected at an abattoir following sacrifice and transported on ice to the laboratory where a more precise dissection was made. The total time from tissue collection to extraction did not exceed 2 hours. Histologically normal human ileum was obtained surgically following intestinal resection and human brain was collected at post-mortem. The time following death was approximately 24-48 hours.

Tissue Extraction

Dissected specimens were weighed, diced and immersed into polypropylene tubes containing boiling 0.5 mol/l acetic acid (approximately 10ml/g of tissue) and boiled for 10-15 minutes. The tubes were then stored at -20^{0}C. Extracts were also assayed at various dilutions to examine whether they displaced tracer in parallel to synthetic neuromedin U8 standard.

Before fractionation, extracts were applied to disposable C_{18} cartridges (Sep-Pak, Waters Associates).

Each cartridge was activated with 10ml acetonitrile containing 0.05% TFA (v/v) and washed with water (10ml). After application of the sample, the cartridge was eluted with a further 10ml of water containing 0.05% TFA, momentarily dried using 15-20ml of air and then eluted with 2ml of acetonitrile/water/TFA (60/40/0.05). This eluate was then lyophilized overnight prior to reconstitution with running buffer before each column run. Peptide recovery from this system was determined using synthetic neuromedin U25 and was found to be in excess of 85%.

Chromatography

Tissue extracts were fractionated by gel permeation chromatography as described in the methods section in the first part of this chapter.

For analysis by reversed phase HPLC, tissue extracts and synthetic standards were loaded onto a 5um Techsil C_{18} column (3.9x300mm)(HPLC Technology, Macklesfield, UK) eluted with acetonitrile/water/TFA (20/80/0.05) at 1ml/minute using a Waters HPLC system (Waters Associates Inc. Milford, USA) (2 model 6000A pumps, 1 model 660 gradient programmer, 1 model U6K injector and a 2ml injection loop). After 10 minutes a linear gradient was developed to acetonitrile/water/TFA (40/60/0.05) over 2 hours where it remained for a further 10 minutes.

Fractions (2ml) were collected, lyophilized overnight and reconstituted in buffer before assay.

Results

The concentration of neuromedin U-like immunoreactivity present in each tissue studied is shown in Table 3.3. Extracts from pig and rat gut all demonstrated displacement curves parallel to the porcine neuromedin U8 standard (Figure 3.4). Figure 3.5 shows the molecular forms of neuromedin U-like immunoreactivity from extracts of pig, rat, human and guinea pig tissue following gel permeation chromatography on Sephadex G-50. A single molecular form of neuromedin U-like immunoreactivity was evident in pig (Figure 3.5 A and B), rat (Figure 3.5 C and D) and human (Figure 3.5 E and F) central nervous system and gut. Only the neuromedin U-like immunoreactivity present in porcine tissue co-eluted with neuromedin U-25 standard (K_{av}=0.62). The immunoreactivity present in rat (K_{av}=0.67) and human tissue (K_{av}=0.68) eluted later.

Two molecular forms of neuromedin U-like immunoreactivity were found in guinea pig tissue extracts (Figure 3.5 G and H) (K_{av}=0.67 and 0.95). The earlier eluting peak appeared to be of a similar apparent molecular weight to the neuromedin U like peptides detected in rat and human tissue but the

predominant, later eluting peak, co-eluted with synthetic neuromedin U8 standard (K_{av}=0.96). All gel permeation column recoveries were greater than 70%.

Fractionation of rat and human tissue by reversed phase HPLC also demonstrated the presence of a single predominant molecular form of immunoreactivity (Figure 3.6 C-F)(Fraction 43 and 44 respectively). Profiles of neuromedin U-like immunoreactivity in human tissue extracts also revealed an additional peak which was not evident following gel permeation chromatography (Figure 3.6 E, fraction 24 and Figure 3.6 F, fraction 28). Analysis of guinea-pig tissue extracts by HPLC revealed two neuromedin U like peptides. The major form present in guinea pig spinal cord (Figure 3.6 G, fraction 27) and ileum (Figure 3.6 H, fraction 28) co-eluted with synthetic porcine neuromedin U8 standard (fraction 27). The second more hydrophobic molecular form of guinea pig neuromedin U (fraction 38) was indistinguishable from the molecular form of neuromedin U-like immunoreactivity present in porcine tissue (Figure 3.6, A and B) and synthetic porcine neuromedin U25 standard (fraction 38). All HPLC column recoveries were greater than 85%.

Table 3:3 Concentrations of neuromedin U-like immunoreactivity in rat, porcine, human and guinea pig ileum and CNS (n=5-6). Results are expressed as mean±SEM; n=5-6

Species	Tissue	Concentration±SEM (pmol/g)
Rat	Sacral spinal cord	61.0±9.5
	Ileum	182.0±6.0
Pig	Sacral spinal cord	26.6±6.7
	Ileum	13.2±1.0
Human	Cerebral cortex	3.5±0.3
	Ileum	11.1±4.1
Guinea Pig	Sacral spinal cord (Dorsal horn)	32.2±5.5
	Ileum	33.0±6.0

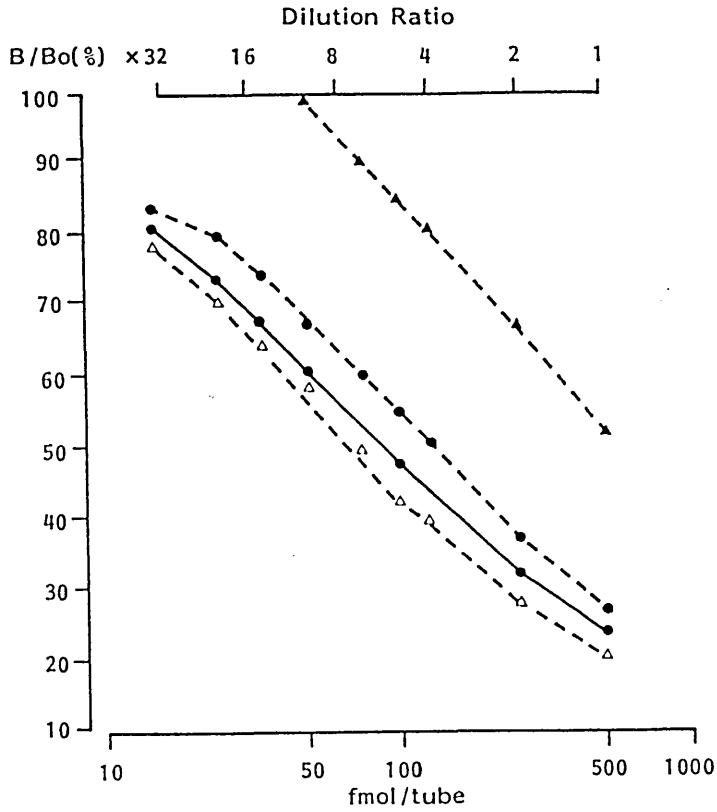


Figure 3: 4 A standard curve of synthetic porcine neuromedin U8 (closed circles) and serial dilution curves (dotted lines) of rat ileal (open triangles), rat duodenal (closed circles) and porcine ileal (closed triangles) extracts in the neuromedin U assay.

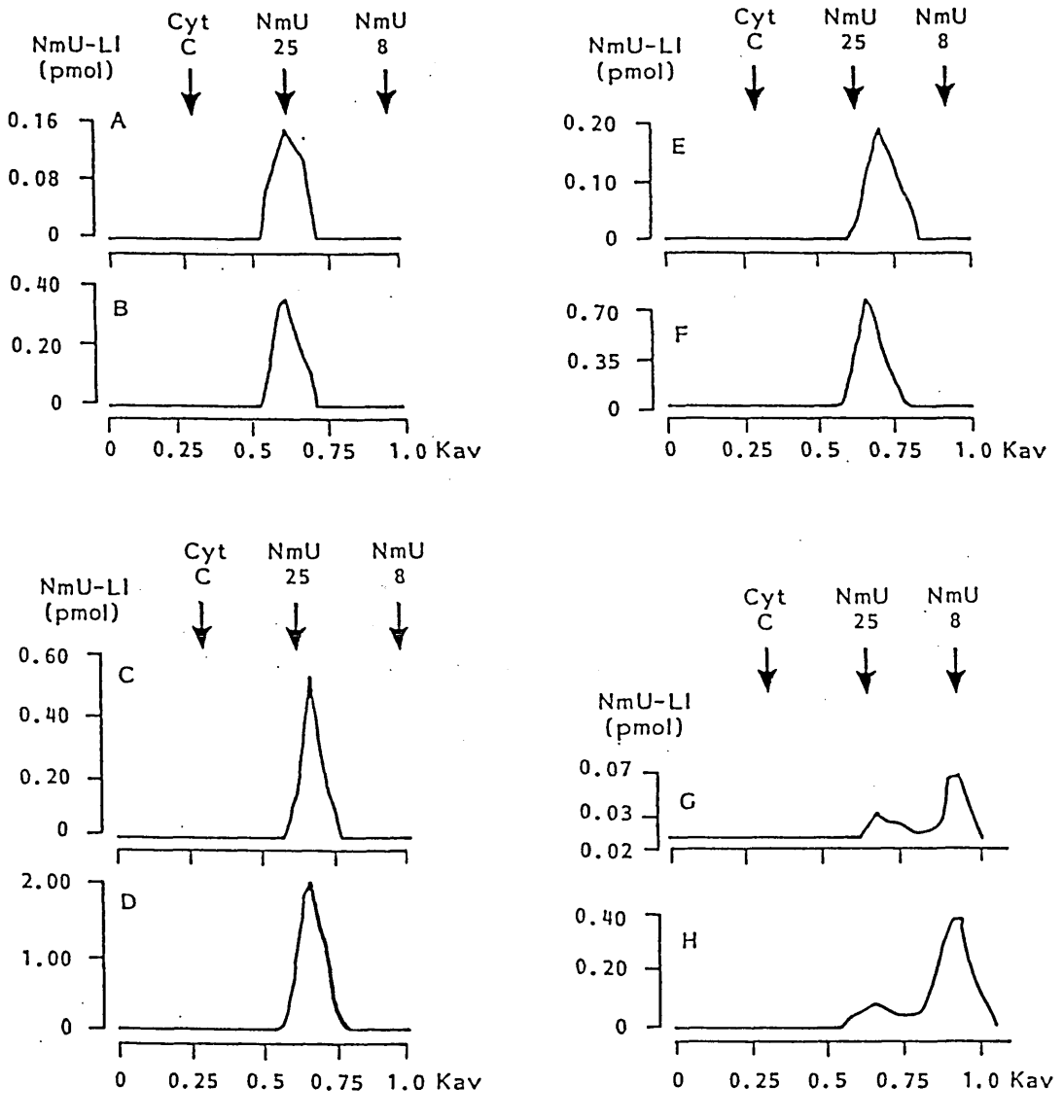


Figure 3:5 Representative gel permeation profiles of neuromedin U-like immunoreactivity in semi-purified and concentrated tissue extracts. (A) porcine spinal cord; (B) porcine ileum; (C) rat spinal cord; (D) rat ileum; (E) human brain; (F) human ileum; (G) guinea pig spinal cord and (H) guinea pig ileum.

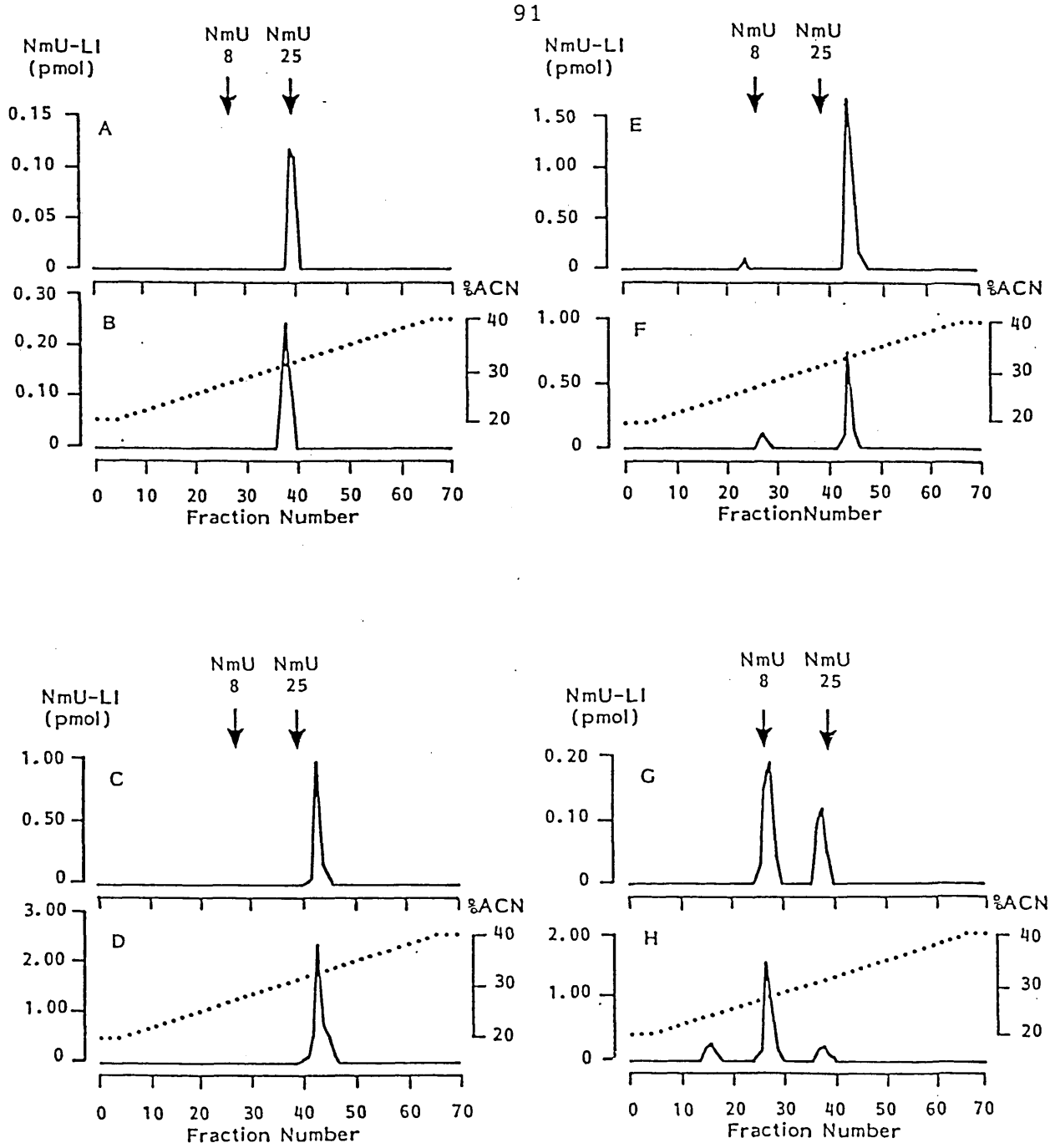


Figure 3:6 Representative reversed phase HPLC profiles of neuromedin U-like immunoreactivity in (A) porcine spinal cord; (B) porcine ileum; (C) rat spinal cord; (D) rat ileum; (E) human brain; (F) human ileum; (G) guinea pig spinal cord and (H) guinea pig ileum.

Discussion

The results of this study describe the immunoreactive molecular forms of pig, rat, human and guinea pig neuromedin U. Contrary to the description of its isolation (Minamino et al, 1985b) we were unable to detect a molecular form of neuromedin U in porcine spinal cord corresponding to neuromedin U8. Since the antisera used in the radioimmunoassay was raised to neuromedin U8, these findings are unlikely to be due to poor antibody cross-reactivity. Gel permeation profiles indicate that rat, human and the large molecular form of neuromedin U present in guinea pig tissue is of a comparable, but smaller molecular weight than porcine neuromedin U25 (Figure 3.5). Although this proposed molecular heterogeneity could be confirmed by reversed phase HPLC analysis for rat and human tissue, the results obtained for guinea pig tissue were not so clear (Figure 3.6).

In contrast to the results reported above, Augood et al (1988) has described the presence of at least 5 molecular forms of neuromedin U-like immunoreactivity in rat and guinea pig tissue. Despite this finding, they also concluded that molecular forms equivalent to the porcine peptides do not exist in rat tissue. One explanation for the multiplicity of molecular forms found by this group could be the comparatively lengthy

extraction protocol they used. Interestingly, samples extracted in acetic acid failed to dilute in parallel with synthetic neuromedin U 8 standard in their assay. The results reported in this chapter show that such extracts do dilute in parallel in our assay system (Figure 3:4). The answer to this discrepancy may lie in the fact that their antibody cross reaction to synthetic neuromedin U 25 was more than 10 fold higher than to neuromedin U 8. This, coupled with the multitude of molecular forms present in such extracts, could explain their failure to obtain parallel curves.

Extensive studies on the biosynthesis of many neuropeptides and peptide hormones have shown that they are cleaved from larger prohormones (Loh, 1985). Recent advances in molecular cloning techniques have greatly accelerated the task of identifying and determining the primary structure of these prohormones (Comb et al, 1982). The patterns of amino acid sequences indicative of processing sites are thus slowly becoming established. The majority of prohormones and proneuropeptides have pairs of basic amino acid residues flanking the region to be cleaved. The most common of such pairs is the Lys-Arg sequence although cleavage at Arg-Lys, Arg-Arg and Lys-Lys also occurs. The list of sites at which precursor cleavage occurs at single basic residues is also growing (Schwartz, 1986; Schwartz, 1987). It is also clear that whilst a given prohormone

may have many potential cleavage sites, only a limited number are utilized. Our current understanding of why one site should be chosen over another is poor, although some patterns are emerging. For example, a nearby proline residue can frequently be found when cleavage occurs at a single arginine residue (Schwartz, 1986). The lack of consistent sequence similarity around homologous processing sites suggests that the endopeptidases involved recognize some unknown component of secondary/tertiary structure.

Once peptides are liberated from their precursor they may then undergo "trimming" of the basic residues from their C- and N-termini by the action of carboxypeptidase B- and aminopeptidase B-like enzymes respectively. Other enzymes can further modify the product by amidation of the C-terminus, an event signalled by a glycine residue on the N-terminal side of the basic amino acid pair (Eipper et al, 1985), acetylation, glycosylation, sulphation or phosphorylation.

Inter and intra-species molecular heterogeneity is a feature often encountered in the study of regulatory peptides (Rehfeld, 1982). Differences in elution position or the finding of multiple peaks of immunoreactivity using various fractionation methods can be attributed to the extention /deletion of known peptide sequences (macroheterogeneity), amino acid

residue substitution/ post-translational modification (microheterogeneity) or a combination of both. Such modification possibly reflects the fact that the entire peptide sequence is often not required for biological activity (see chapter 8). This would leave the remainder of the molecule open to modification thereby allowing the generation of variants which may prove advantageous to the organism. An additional aspect could be the adaption of molecules to novel target organ receptor structures.

Whatever the mechanism or consequence, whenever molecular heterogeneity is evident, caution is needed when quantifying the concentration of such variant forms by radioimmunoassay. Furthermore, particular care should always be taken to converse in terms of "peptide-like immunoreactivity" since the technique of radioimmunoassay is based on structural similarity and the true identity of the cross-reacting material can only be ascertained following its purification and sequence analysis.

CHAPTER 4

DISTRIBUTION OF NEUROMEDIN U-LIKE IMMUNOREACTIVITY

Introduction

Following the purification and characterization of neuromedin U8 and neuromedin U25 from porcine spinal cord, the radioimmunoassay described in chapter 2 was used to study the tissue distribution of neuromedin U-like immunoreactivity (Domin et al 1987, Domin et al (2), in press). These studies demonstrated that neuromedin U immunoreactivity is not only present in the mammalian spinal cord but also in several regions of the brain, throughout the gastrointestinal tract, in the pituitary and thyroid glands and the urogenital tract. Interestingly, higher concentrations of neuromedin U immunoreactivity were found in the gastrointestinal tract than in the central nervous system. Shortly thereafter, Honzawa et al (1987) documented a detailed study of the histochemical localization of neuromedin U-like peptides in the rat brain. Studies in the rodent pituitary gland revealed neuromedin U immunoreactivity to be colocalized with corticotropin (ACTH) in the

corticotropes of the anterior and intermediate lobes (Steel et al, 1988). In man, corticotropic cells of the anterior lobe and the anterior border of the posterior lobe were also intensely stained. Co-storage with ACTH was also found in corticotropin secreting tumours. The localization of neuromedin U immunoreactivity in the rat hypothalamus, was also described in this report.

In the rat and guinea pig gastrointestinal tract, neuromedin U immunoreactivity has been localized to nerve fibres which appear to be mainly intrinsic in origin (Ballesta et al, 1988; Augood et al, 1988).

This chapter describes the distribution of neuromedin U-like immunoreactivity in various mammalian species. In addition, the distribution of neuromedin U immunoreactivity was examined in the frog Rana temporaria as part of a study investigating the origins of mammalian regulatory peptides in lower vertebrates. A comprehensive chromatographic examination of the neuromedin U-like immunoreactivity found in the corresponding tissue extracts is also presented.

Materials And Methods

Tissues

Adult Wistar rats (n=>6) and Duncan Hartley guinea pigs (n=8) were killed by cervical dislocation and exsanguinated by cardiac puncture. Tissues were dissected, weighed, diced and extracted by immersion in 10 volumes of boiling 0.5 mol/l acetic acid for 10-15 minutes. The extracts were then cooled to room temperature, frozen on dry ice and stored at -20°C before assay. Porcine tissue was collected at an abattoir immediately following death, the pieces placed on ice and transported to the laboratory for a more precise dissection. All tissue pieces were extracted as above. The total time from death to extraction did not exceed 3 hours.

Adult male and female frogs (*Rana temporaria*) (n=8) were fasted overnight prior to sacrifice. Tissues were immediately dissected and extracted in boiling 0.5 mol/l acetic acid as above. Particular care was taken upon handling in order to minimise the possibility of contaminating internal organs with stored peptides in the skin.

Mucosa was separated from the underlying muscular layers of the rat small intestine by scraping sections of

stretched tissue tangentially with a glass slide as described by Hutchinson et al (1981). Both mucosa and muscle were then weighed and extracted as above or fixed in formalin. For histological examination, formalin fixed tissue was embedded in wax, sectioned (5μm) and stained with haematoxylin and eosin (McManus and Mowry, 1960) before microscopic examination.

Radioimmunoassay

The tissue content of neuromedin U-like immunoreactivity was quantified using the radioimmunoassay described in chapter 2. Somatostatin- and peptide YY-like immunoreactivity was also measured using the radioimmunoassays described by O' Shaughnessey (1982) and Adrian et al (1985) respectively. All results were expressed in terms of pmol/g wet weight of tissue and quoted as the mean and standard error of the mean (SEM).

Chromatography

Fractionation of tissue extracts by gel permeation chromatography and reversed phase HPLC was performed as previously described in chapter 3. The retention times of synthetic neuromedin U8, neuromedin U25 and the recently purified rat neuromedin U (custom synthesis IAF Biochem, Quebec, Canada; for details of purification see chapter 5) were used to calibrate each column.

Results

A. The Central Nervous System

Figure 4: 1 shows the content of neuromedin U-like immunoreactivity in various regions of the brain in the four species studied. Immunoreactivity was detected in many regions with particularly high amounts in the hypothalamus, amygdala, septum, nucleus accumbens and medulla oblongata. Whilst a detailed dissection of the frog brain was not possible, the concentration of neuromedin U immunoreactivity in the entire brain was 5 times that of any region of the rat brain and 10-20 times greater than that of the guinea pig and pig.

Neuromedin U-like immunoreactivity was also found in the spinal cord of all three mammalian species studied (Figure 4: 2). Higher levels of immunoreactivity were detected in the dorsal than in the ventral horn, a feature most prominent in the pig. Furthermore, there was a general trend for the concentrations to increase towards the sacral region.

Although relatively high concentrations of neuromedin U-like immunoreactivity were found in the dorsal root ganglia of both rodent species, it was not present in this area of the pig spinal cord.

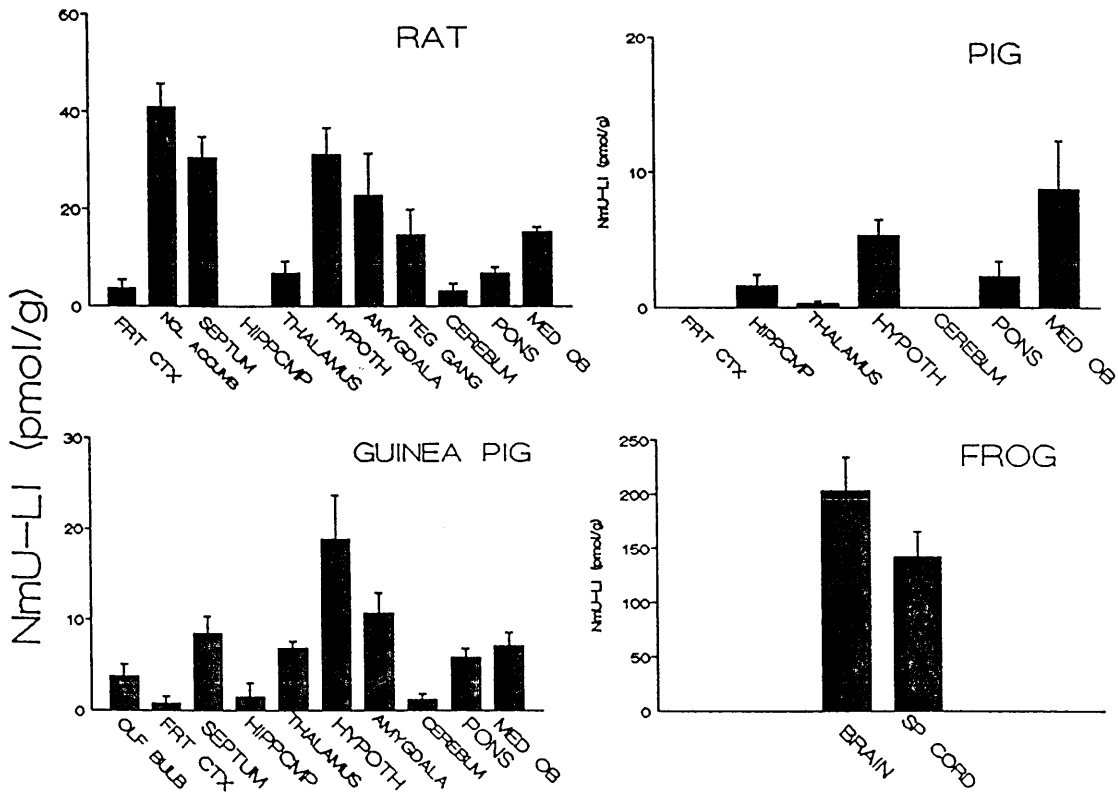


Figure 4:1 Distribution of neuromedin U-like immunoreactivity in the rat, pig, guinea pig and frog brain. Results are expressed as mean±SEM; n=>6

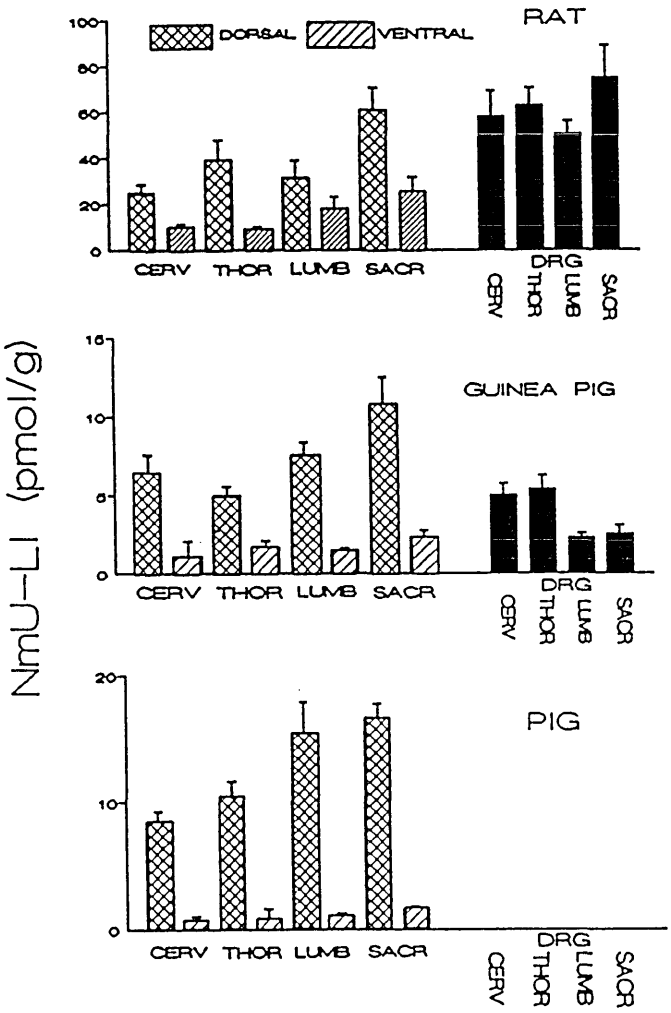


Figure 4: 2 Distribution of neuromedin U-like immunoreactivity throughout the rat, guinea pig and pig spinal cord and dorsal root ganglia.

Results are expressed as mean±SEM; n=>6

Chromatographic characterization of porcine (Figure 4:3) and rat (Figure 4:4) hypothalamic (A and B) and spinal cord (C and D) extracts revealed a single molecular form of immunoreactivity coeluting with synthetic neuromedin U25 and rat neuromedin U respectively. There was no evidence of neuromedin U8 in extracts of porcine spinal cord (see chapter 3). Guinea pig hypothalamic extracts (Figure 4:5 A and B) contained a predominant molecular form which co-eluted with synthetic rat neuromedin U following fractionation on a gel permeation column, but eluted closer to neuromedin U25 following separation upon reversed phase FPLC. Fractionation of guinea pig spinal cord (Figure 4:5 C and D) revealed two molecular forms, following gel permeation analysis. The first peak (Kav=0.67) co-eluted with that present in the hypothalamus, the second (Kav=0.9) which was also present in small amounts in the hypothalamus had a smaller apparent molecular weight which was slightly greater than neuromedin U8. Frog brain (Figure 4:6 A and B) and spinal cord (Figure 4:6 C and D) contained two molecular forms of immunoreactive neuromedin U of which a smaller molecular form predominates. Neither of these frog neuromedin U like peptides coeluted with the porcine standards following gel permeation chromatography.

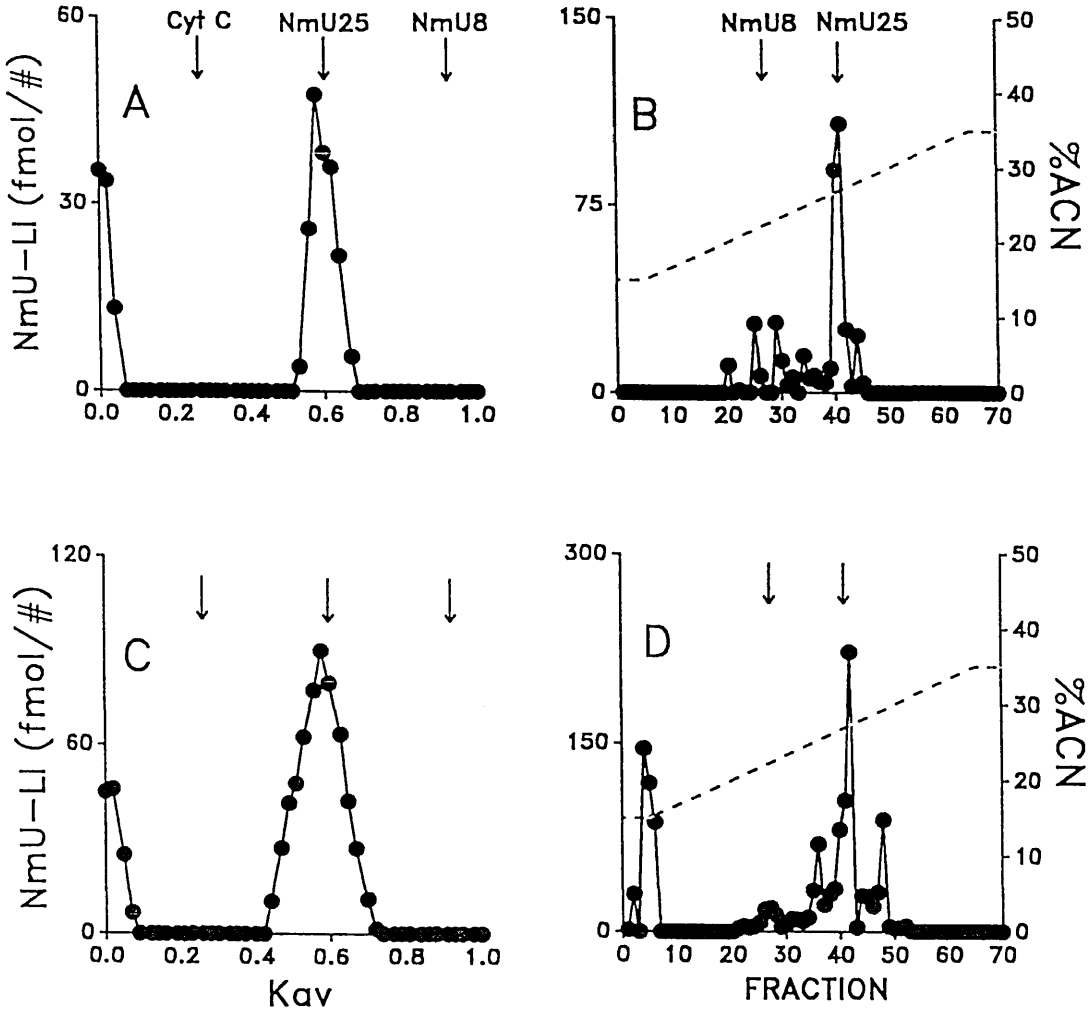


Figure 4:3 Chromatographic characterization of neuromedin U-like immuoreactivity in extracts of pig hypothalamus (A) and spinal cord (C) by gel permeation chromatography and reversed phase FPLC (B and D respectively). Arrows indicate the elution positions of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (NmU25) and synthetic neuromedin U8 (NmU8).

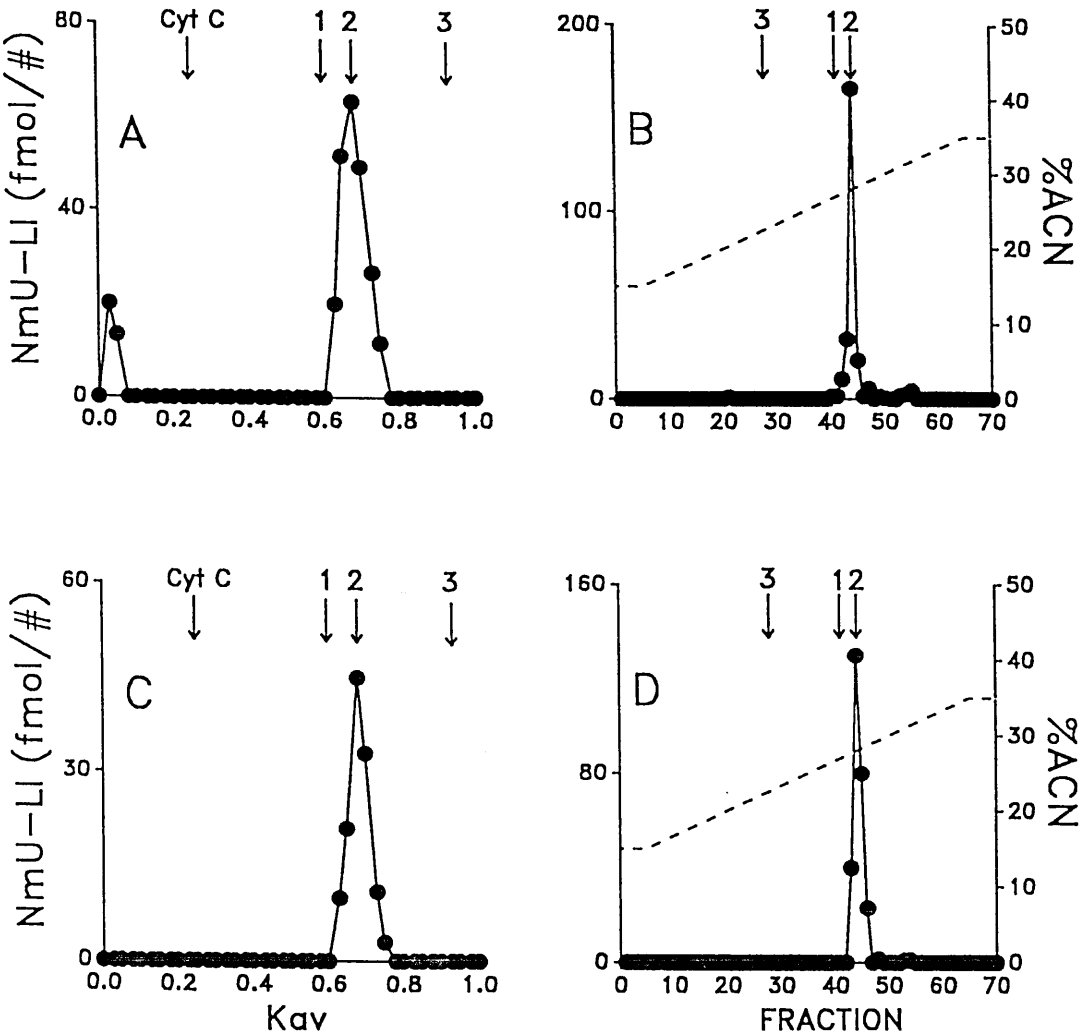


Figure 4: 4 Chromatographic characterization of neuromedin U-like immunoreactivity in extracts of rat hypothalamus (A) and spinal cord (C) by gel permeation chromatography and reversed phase FPLC (B and D respectively). Arrows denote the elution position of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

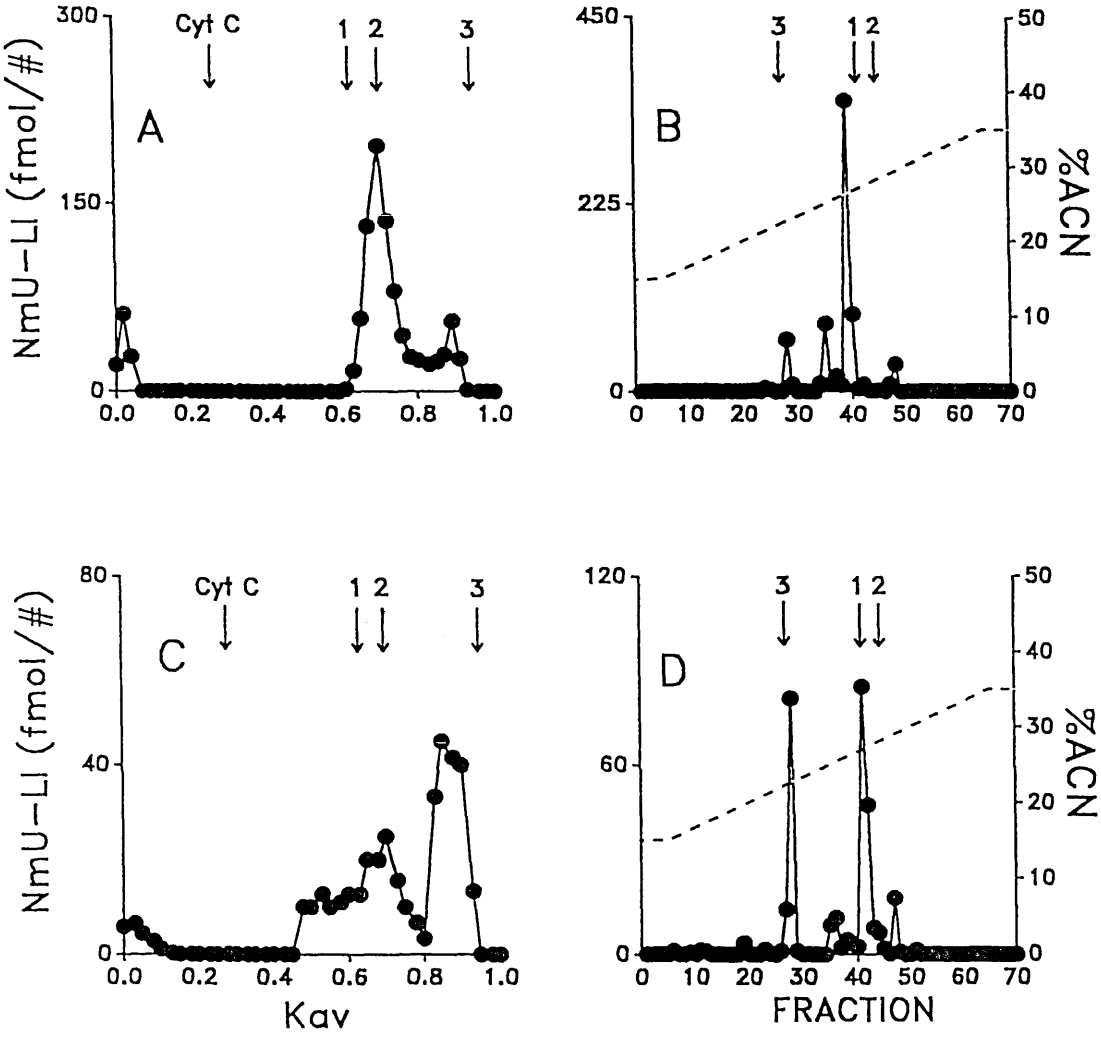


Figure 4:5 Chromatographic characterization of neuromedin U-like immunoreactivity in extracts of guinea pig hypothalamus (A) and spinal cord (C) by gel permeation chromatography and reversed phase FPLC (B and D respectively). Arrows denote the elution position of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

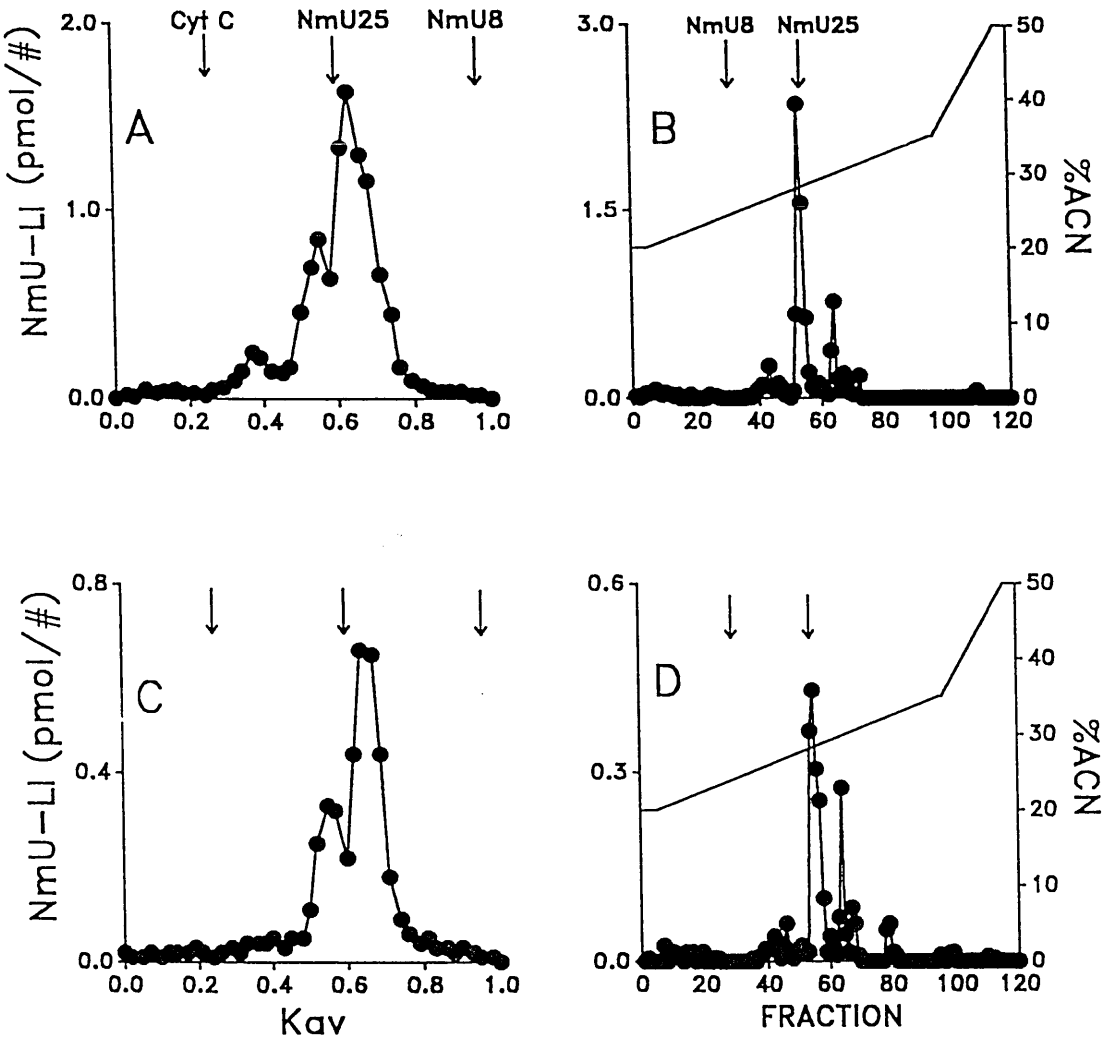


Figure 4: 6 Chromatographic characterization of neuromedin U-like immunoreactivity in extracts of frog brain (A) and spinal cord (C) by gel permeation chromatography and reversed phase HPLC (B and D respectively). Arrows indicate the elution positions of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (NmU25) and synthetic neuromedin U8 (NmU8).

B. The Gastrointestinal Tract

In all species studied, the highest concentrations of gastrointestinal neuromedin U immunoreactivity occurred in the small intestine (Figure 4: 7). Whilst in rat and frog there was little difference along this region, in pig, concentrations were higher in the duodenum and jejunum compared to the ileum and in the guinea pig this distribution was reversed.

Separation of rat small intestine into muscle and mucosal fractions revealed that neuromedin U-like immunoreactivity was entirely restricted to the muscle layer (Figure 4: 8). The distribution of the putative hormone peptide YY established the separation of endocrine cells from the underlying regions. This separation was also confirmed by histopathological examination which revealed the removal of mucosa to the level of the muscularis mucosae (Figure 4: 9).

Fractionation of porcine gut extracts on a gel column and subsequent assay revealed a single peak of neuromedin U immunoreactivity which coeluted with synthetic neuromedin U25 (Figure 4: 10). This was further resolved into two components following analysis by reversed phase FPLC (Figure 4: 11). Of the two forms detected, only one coeluted with neuromedin U25. The second form eluted earlier but was more hydrophobic than

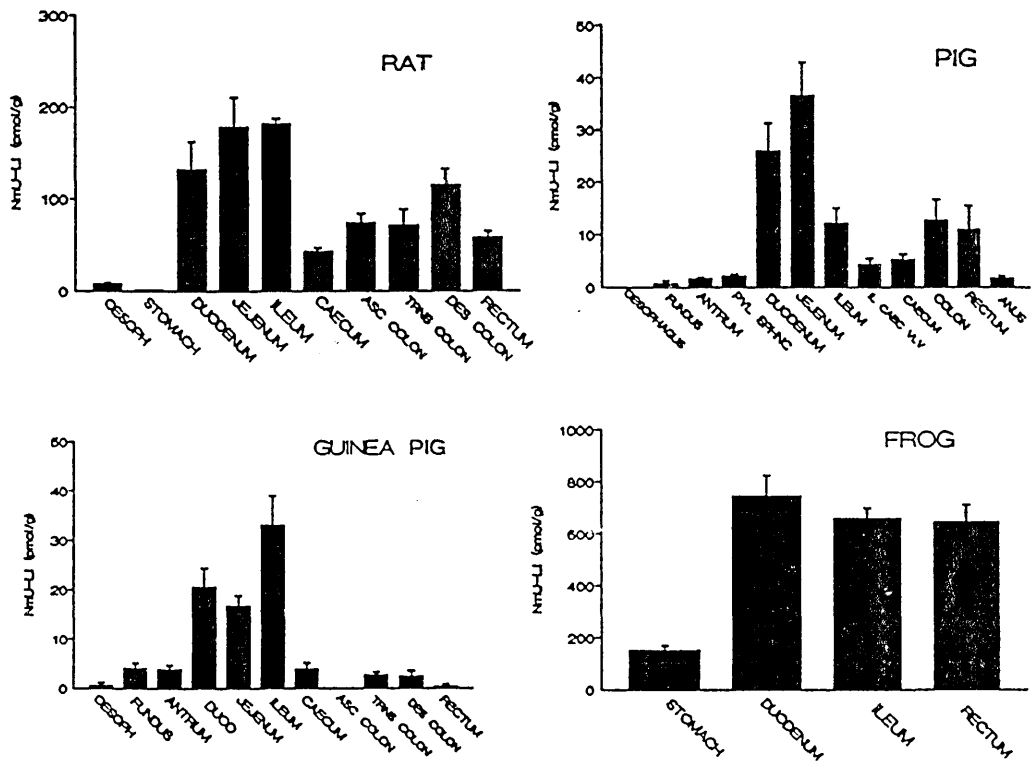


Figure 4:7 Distribution of neuromedin U-like immunoreactivity along the rat, pig, guinea pig and frog gastrointestinal tract.

Results are expressed as mean±SEM; n=>6

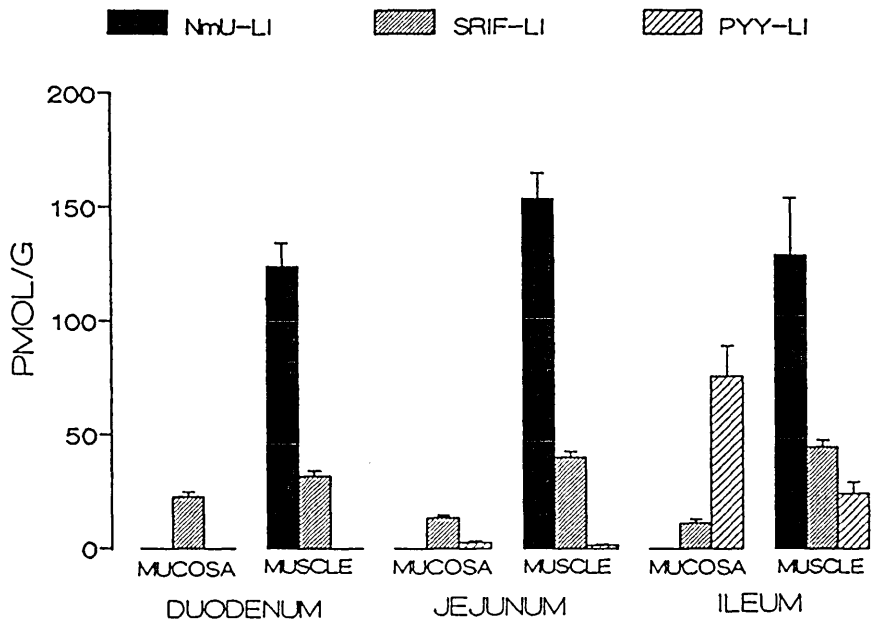


Figure 4: 8 Quantitation of neuromedin U-, somatostatin- and peptide YY-like immunoreactivity in acid extracts of rat gastrointestinal mucosa and muscle. Results are expressed as mean±SEM; n=8

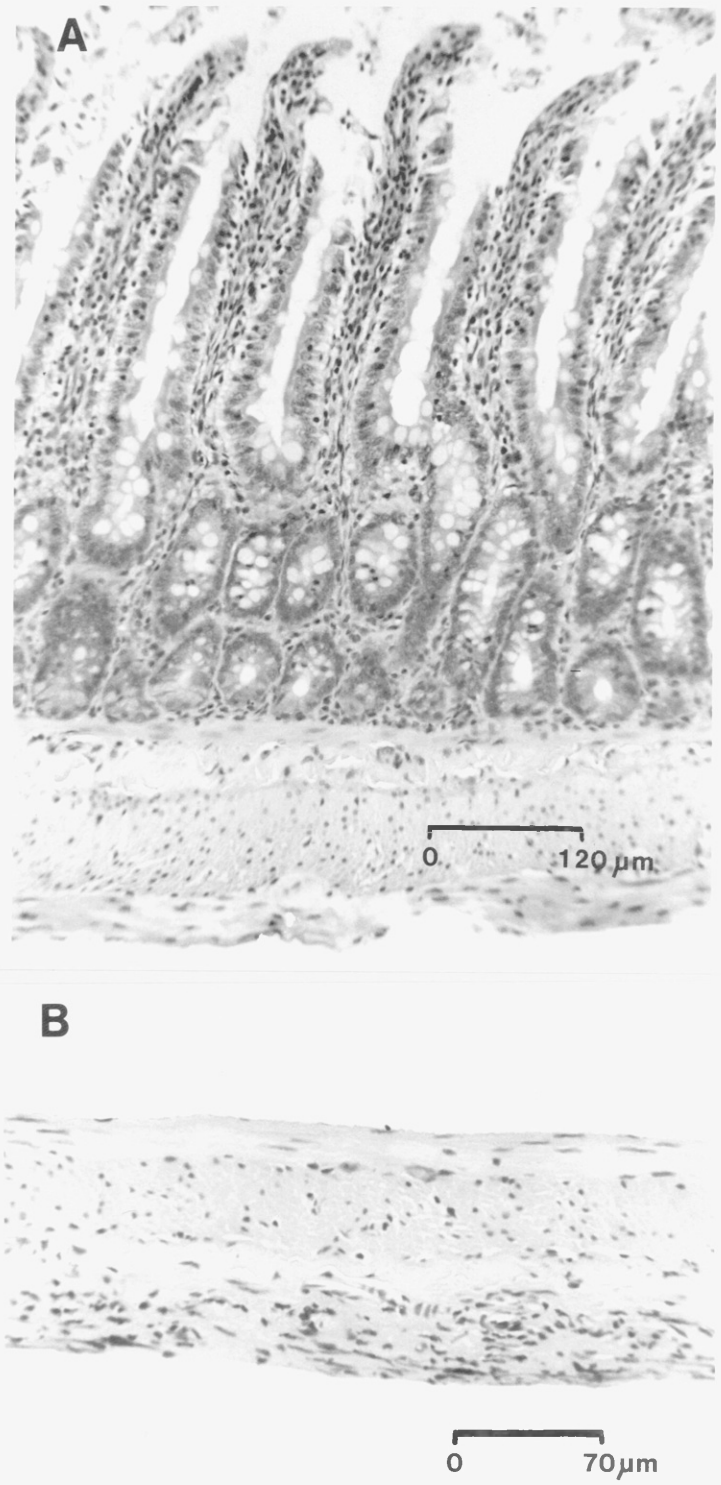


Figure 4:9 Histopathological examination of rat small intestine before (A) and after (B) scraping of the mucosa as described above. Both sections were stained with H&E.

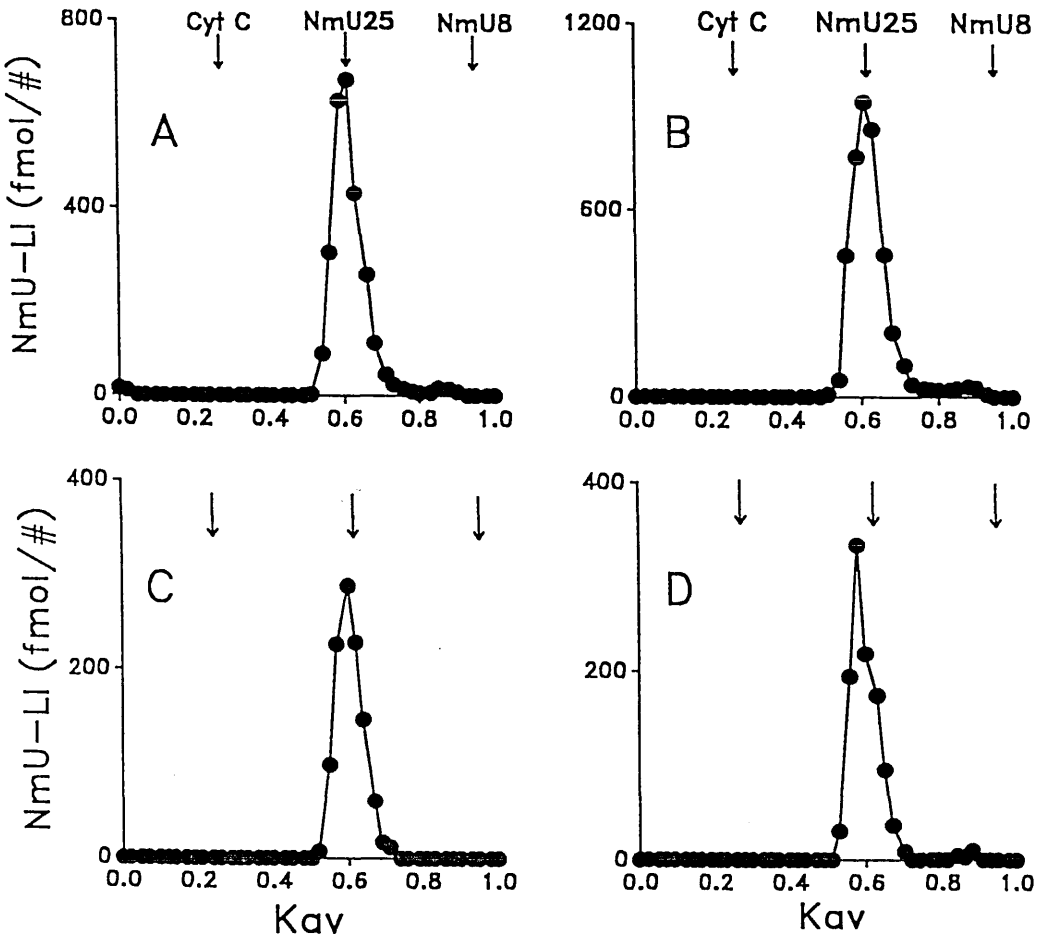


Figure 4:10 Gel permeation analysis of neuromedin U-like immunoreactivity in extracts of pig duodenum (A), jejunum (B), ileum (C) and colon (D). Arrows indicate the elution positions of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (NmU25) and synthetic neuromedin U8 (NmU8).

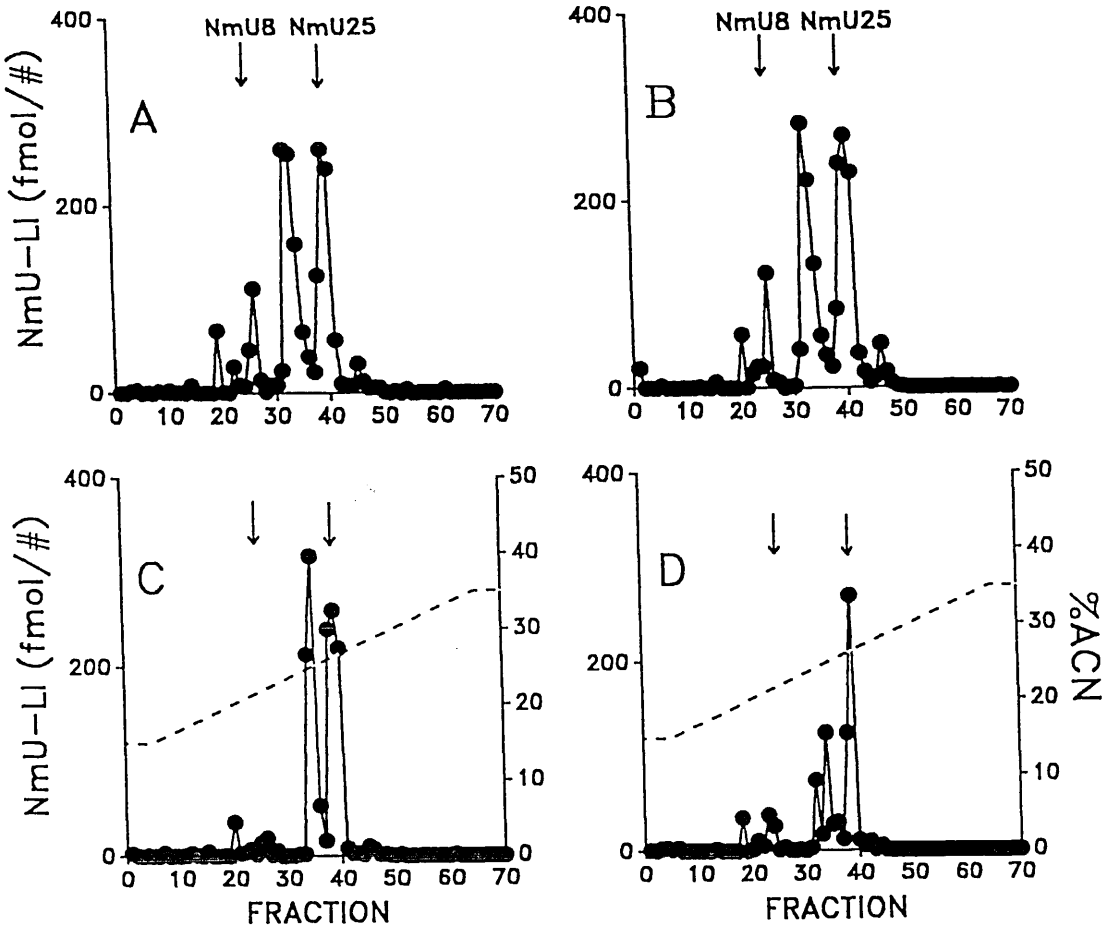


Figure 4:11 Reversed phase FPLC analysis of neuromedin U-like immunoreactivity in extracts of pig duodenum (A), jejunum (B), ileum (C) and colon (D). Arrows indicate the elution positions of synthetic neuromedin U25 (NmU25) and synthetic neuromedin U8 (NmU8).

neuromedin U8. Rat gut extracts contained one molecular form of immunoreactivity which was indistinguishable from rat neuromedin U standard (Figures 4:12 and 4:13). Guinea pig gut extracts contained two molecular forms of neuromedin U-like immunoreactivity following gel permeation chromatography (Figure 4:14). One form (Kav=0.68) had an apparent molecular weight similar to rat neuromedin U. The predominant molecular form present in guinea pig gut however eluted later (Kav=0.9) indicating a smaller molecular weight, although slightly greater than neuromedin U8 (Figure 4:14). Reversed phase FPLC fractionation confirmed the presence of two immunoreactive forms, similar to neuromedin U8 and neuromedin U25 (Figure 4:15). Frog gut extracts (Figure 4:16) contained a predominant molecular form of immunoreactivity which eluted ahead of neuromedin U25 on gel columns (A and C) but slightly later than neuromedin U25 following reversed phase HPLC analysis (B and D) .

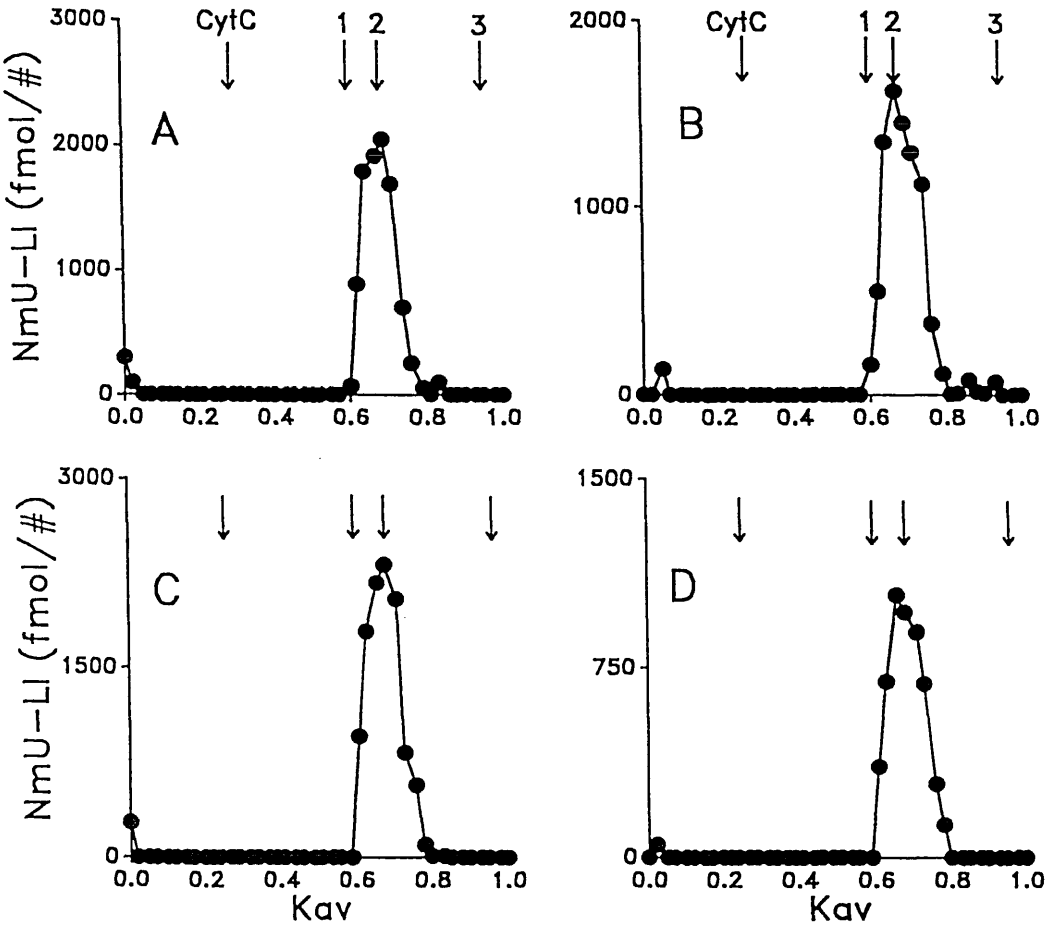


Figure 4:12 Gel permeation analysis of neuromedin U-like immunoreactivity in extracts of rat duodenum (A), jejunum (B), ileum (C) and colon (D). Arrows denote the elution position of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

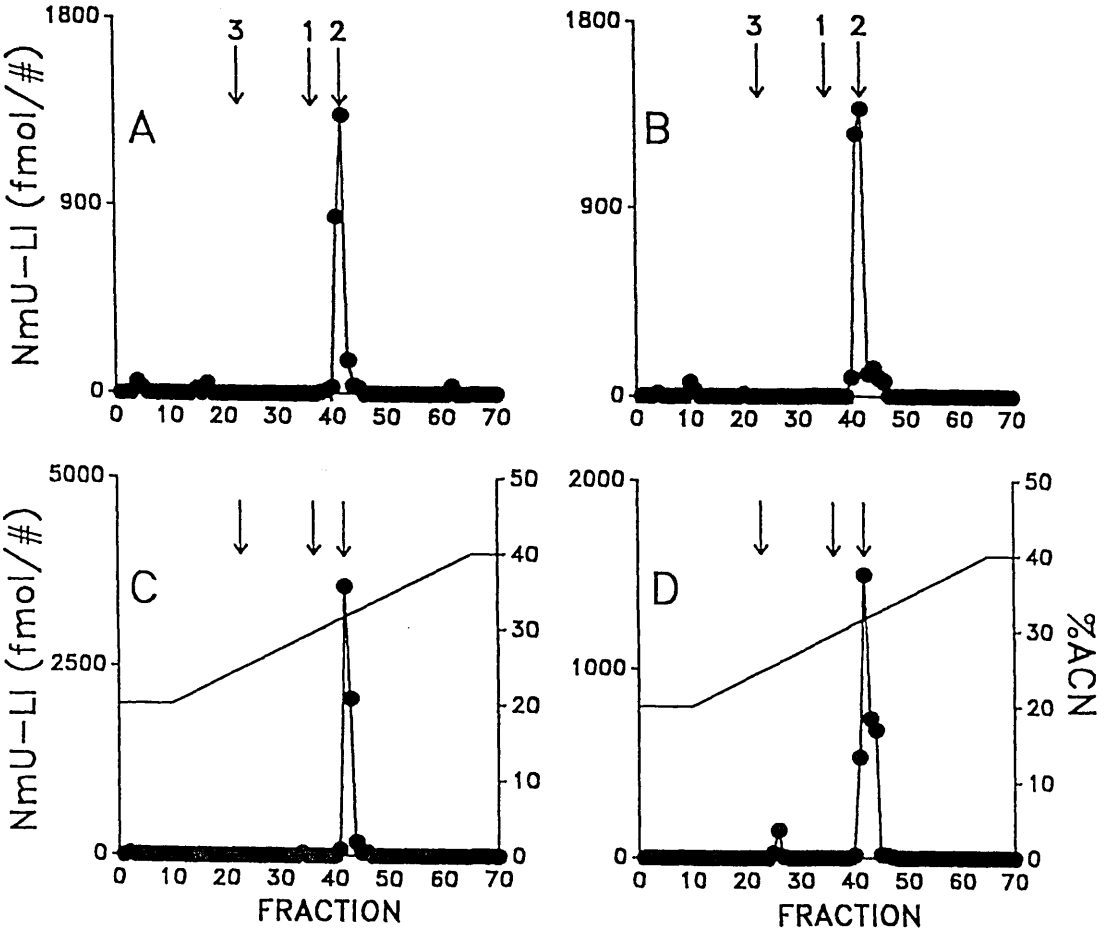


Figure 4:13 Reversed phase HPLC analysis of neuromedin U-like immunoreactivity in extracts of rat duodenum (A), jejunum (B), ileum (C) and colon (D). Arrows indicate the elution position of synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

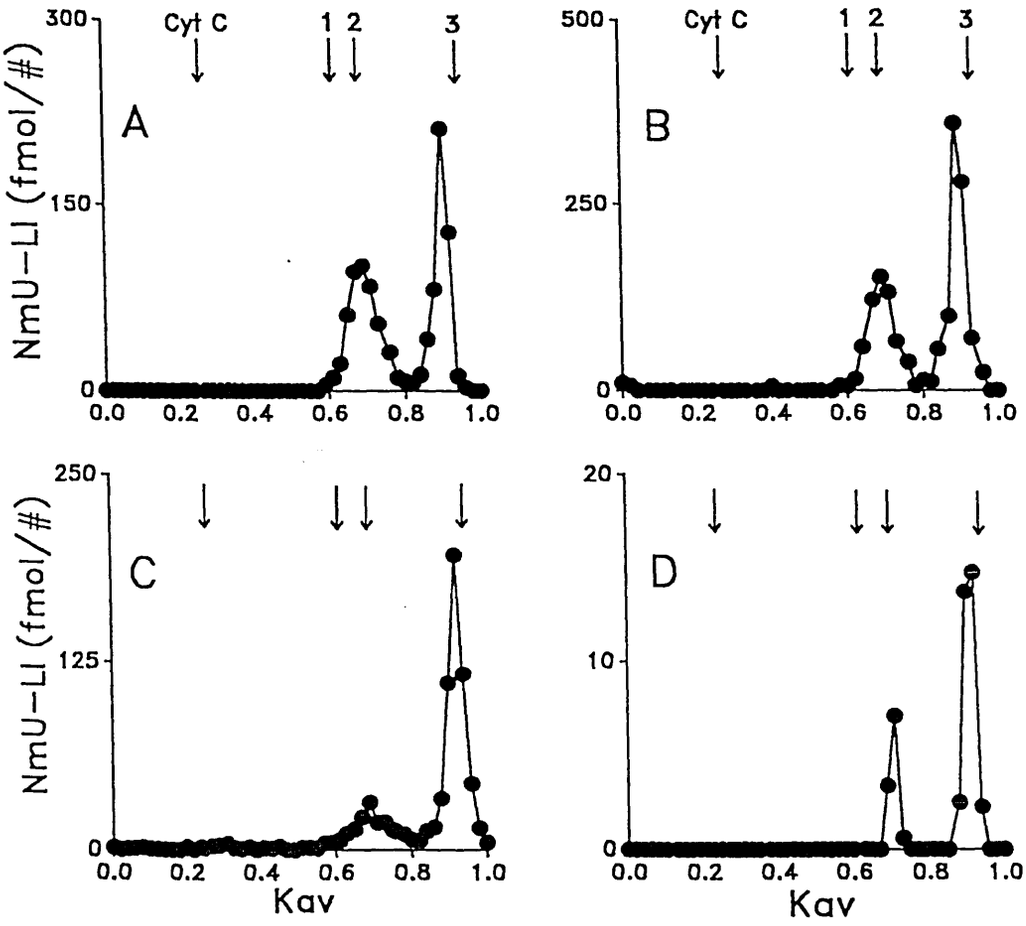


Figure 4: 14 Gel permeation analysis of neuromedin U-like immunoreactivity in extracts of guinea pig duodenum (A), jejunum (B), ileum (C) and colon (D). Arrows denote the elution position of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

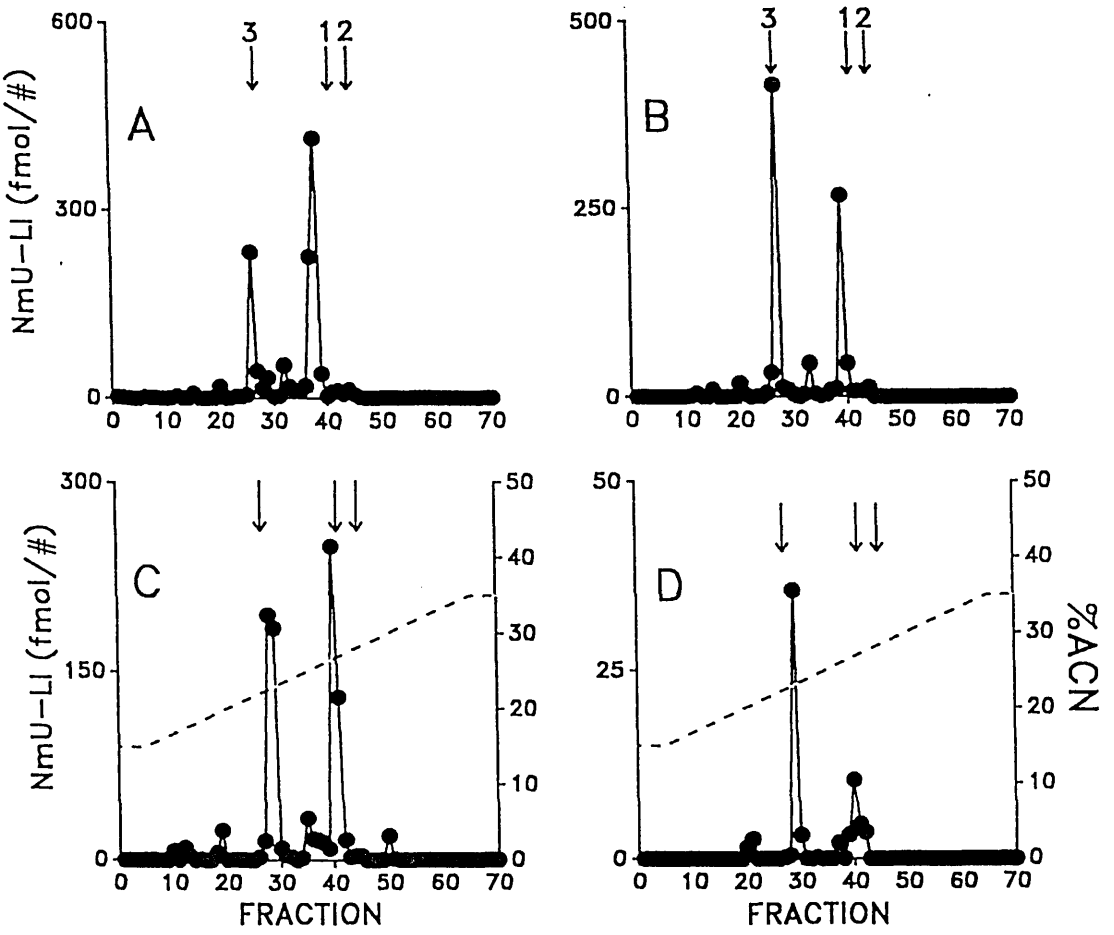


Figure 4:15 Reversed phase FPLC analysis of neuromedin U-like immunoreactivity in extracts of guinea pig duodenum (A), jejunum (B), ileum (C) and colon (D). Arrows indicate the elution position of synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

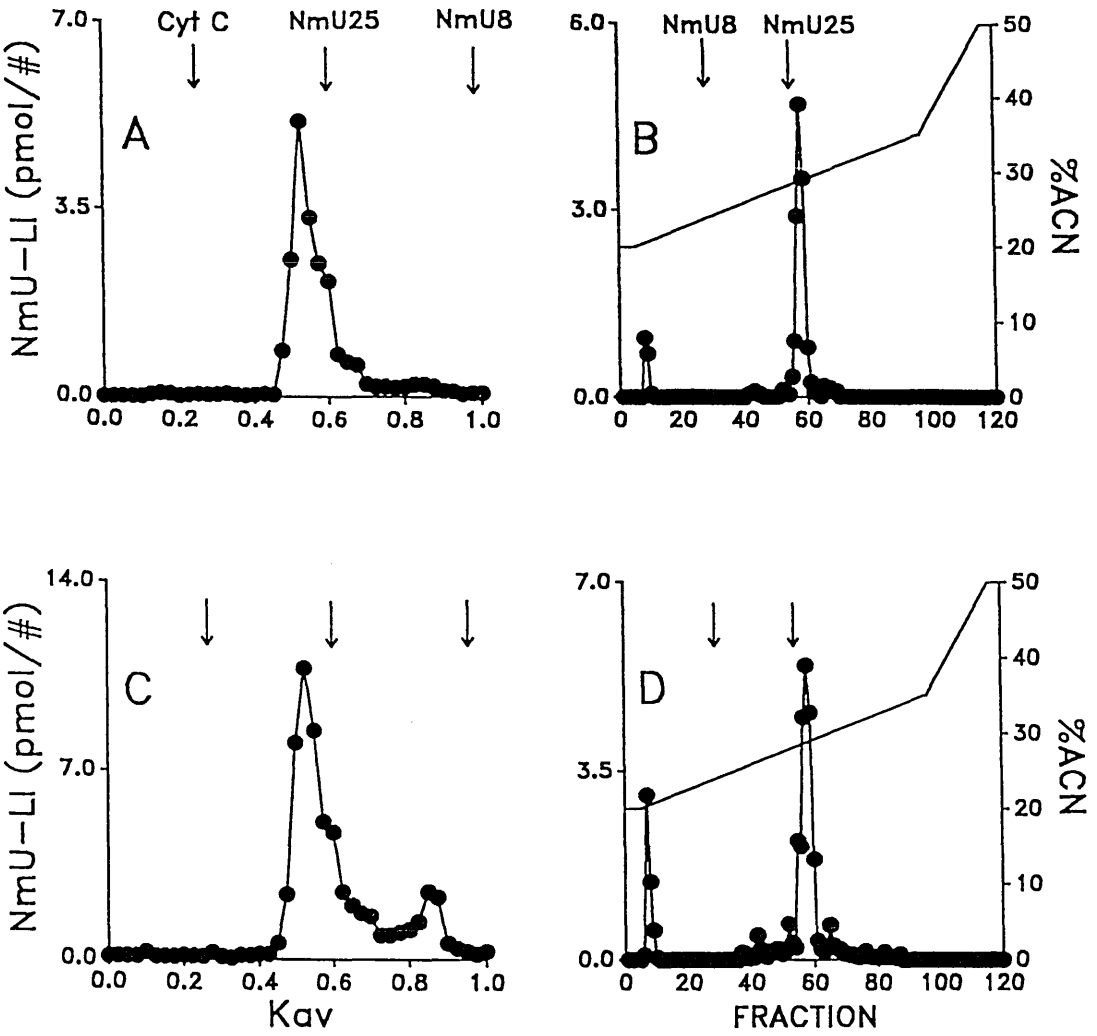


Figure 4:16 Analysis of neuromedin U-like immunoreactivity in extracts of frog stomach (A and B), and frog ileum (C and D) by gel permeation chromatography (A and C) and reversed phase HPLC (B and D). Arrows indicate the elution positions of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (NmU25) and synthetic neuromedin U8 (NmU8).

C. The Pituitary Gland, Thyroid Gland And Urogenital Tract

Neuromedin U-like immunoreactivity was detected in the pituitary gland of all three mammalian species studied (Figure 4: 17). Separation of the rat anterior and posterior/intermediate lobes showed that the immunoreactivity was predominantly present in the anterior lobe. Fractionation of rat pituitary extracts revealed a single molecular form of immunoreactivity which coeluted with rat neuromedin U standard (Figure 4: 18 A and B). Extracts of guinea pig pituitary also contained a predominant form of immunoreactive neuromedin U which, like the larger form present in both guinea pig CNS and gut, eluted close to synthetic rat neuromedin U (Figure 4: 18 C and D). A second, smaller form was also detected eluting ahead of neuromedin U8 standard although it contributed little to the total immunoreactivity present.

Neuromedin U-like immunoreactivity was also detected in extracts of the rodent thyroid gland but it was absent in that of pig (Figure 4: 17). A single molecular form of neuromedin U immunoreactivity was found in extracts of rat thyroid which coeluted with synthetic rat standard (Figure 4: 19 A and B).

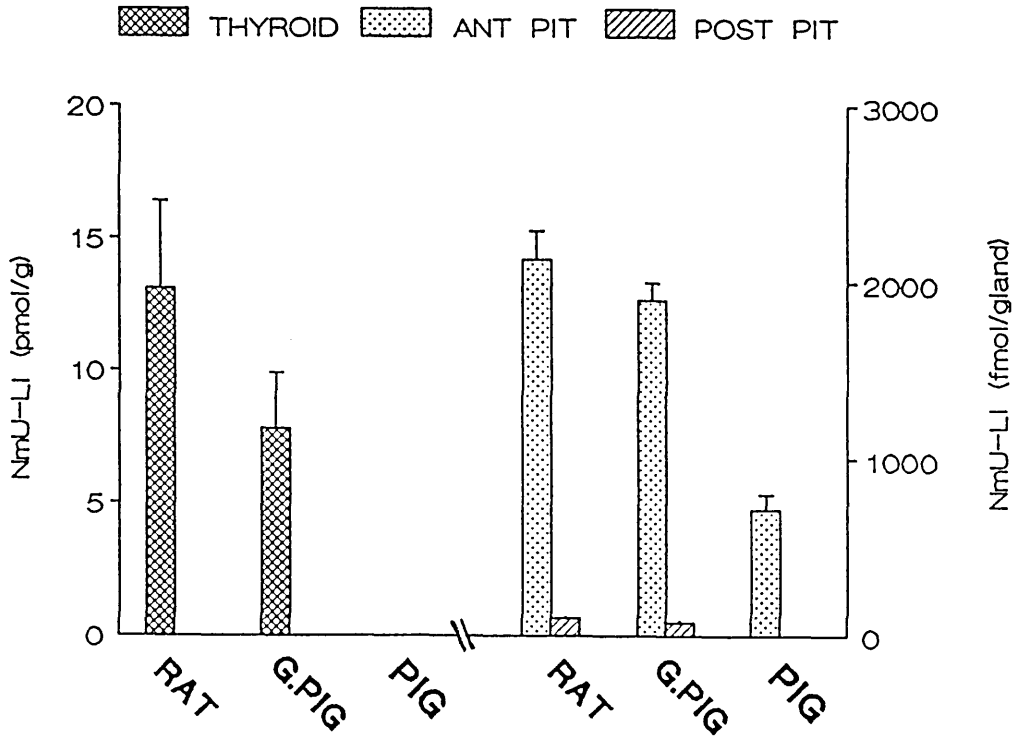


Figure 4:17 Concentrations of neuromedin U-like immunoreactivity in the rat, guinea pig and pig thyroid, anterior and posterior pituitary glands.

Results are expressed as mean±SEM; n=>6

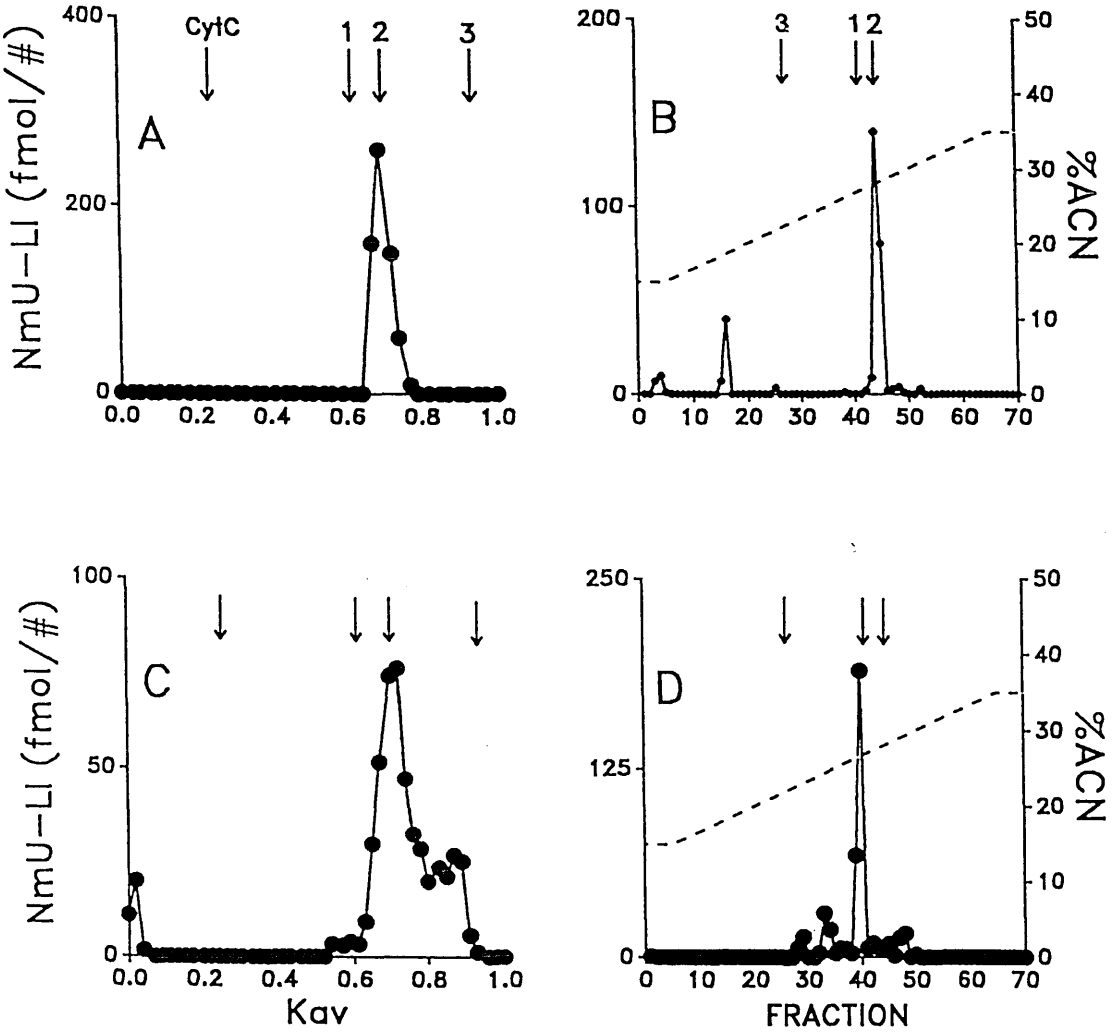


Figure 4:18 Chromatographic characterization of neuromedin U immunoreactivity in extracts of rat (A and B) and guinea pig (C and D) anterior pituitary by gel permeation (A and C) and reversed phase FPLC (B and D). Arrows denote the elution position of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

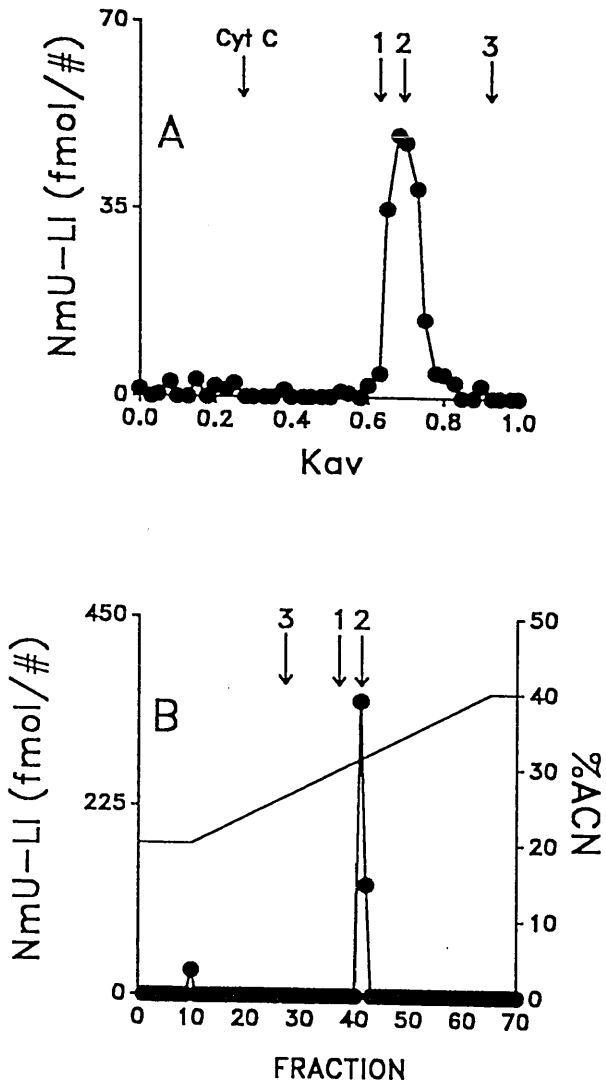


Figure 4: 19 Chromatographic characterization of rat thyroid gland extracts by gel permeation (A) and reversed phase FPLC (B). Arrows denote the elution position of horse heart cytochrome C (Cyt C), synthetic neuromedin U25 (1), synthetic rat neuromedin U (2) and synthetic neuromedin U8 (3).

Table 4:1 Distribution of neuromedin U-like immunoreactivity in the rat, pig, guinea pig and frog urogenital tract

TISSUE	RAT (pmol/g)	PIG (pmol/g)	GUINEA PIG (pmol/g)	FROG (pmol/g)
KIDNEY	-	-		-
URETER	8.8±2.4	-	2.8±0.3	
BLADDER:		-		23±7
DOME	2.8±0.8		1.9±0.2	
TRIGONE	3.9±0.3		1.6±0.2	
TESTIS	1.9±0.3			-
EPIDIDYMIS	3.6±1.1			
VAS DEFERENS	32.0±7.9			
SEMINAL VESICLES	-			-
PROSTATE	7.6±1.2			
PENIS	1.7±0.3			
OVARY	3.7±0.5	-	1.5±1.0	-
FALLOPIAN TUBE	9.3±1.4	-		
UTERUS	1.6±0.4	-	2.1±1.0	
URETHRA	8.7±1.3	-	2.0±0.2	
VAGINA	4.1±1.1	-	1.7±0.8	
OVIDUCT			13.1±4.1	

Results are expressed as mean±SEM; n=>6

- = undetectable

The presence of neuromedin U-like immunoreactivity in the urogenital tract was restricted to rodent species with the exception of the frog bladder (Table 4: 1). In the rat, highest concentrations were found in the vas deferens, ureter, fallopian tube and urethra.

General Discussion

This study has shown the presence and quantified the distribution of neuromedin U-like immunoreactivity in the central nervous system and a variety of peripheral tissues.

Within the central nervous system, neuromedin U-like peptides were identified in all species studied. Although present along the whole spinal cord, levels of neuromedin U immunoreactivity were higher in the dorsal horn and tended to show a sacral predominance characteristic of many neuropeptides including substance P, calcitonin gene related peptide and neuropeptide Y (Anand et al, 1983). Such a distribution has been attributed to the increase in ratio of grey to white matter although a specific local function may also exist. In the brain, high levels of immunoreactivity were detected in several regions, perhaps most notably in the hypothalamus, suggesting a possible involvement in the control of pituitary function. Augood et al

(1988) has also established the presence of neuromedin U-like peptides within the rodent brain by radioimmunoassay. Whilst there is general agreement regarding the general distribution between our two studies, the levels of immunoreactivity detected by his group were approximately ten-fold lower than those reported in this chapter. Their use of a very insensitive assay system (18 fmol/tube), an antiserum which appears to show a 10 fold greater cross-reaction with neuromedin U25 than with neuromedin U8 and a prolonged extraction protocol which appears to generate a number of smaller molecular forms may account for a good deal of this difference.

Immunohistochemical studies have confirmed the occurrence of neuromedin U-like peptides in the central nervous system. Honzawa et al (1987) produced a detailed study of the distribution of immunoreactive neuromedin U-like structures in the rat brain. They found a widespread distribution including the presence of pyramidal cells in the cerebral cortex and fibres running into the cerebellum. In both of these areas notably low levels of immunoreactive neuromedin U were found by radioimmunoassay as described in this chapter. Shortly thereafter, Steel et al (1988) described the presence of immunoreactive fibres in the hypothalamus and cell bodies in the arcuate nucleus . This work was expanded by Ballesta et al (1988) who produced a more

detailed immunocytochemical study describing the distribution of neuromedin U-like peptides throughout the entire rat brain. Whilst his work also showed a widespread distribution of immunoreactive fibres, they were notably absent in the cerebellum and only present in very low levels in the cortex. The differences between his results and those of Honzawa were attributed, by Ballesta, to differences in the specificity of the antisera used.

The gastrointestinal tract contains a variety of neurotransmitters such as acetylcholine, GABA and serotonin as well as a diversity of peptides which have been localized to the wall of the gastrointestinal tract (Costa and Furness, 1982). Our original finding identifying the presence of neuromedin U-like immunoreactivity in the mammalian gut has now been substantiated by other workers. Augood et al (1988) described low levels of neuromedin U immunoreactivity along selected regions of the entire rat gut and in guinea pig ileum. Despite the low concentrations reported, the general distribution described was in agreement with the findings presented in this chapter. In the rat small intestine, he observed immunoreactive neuromedin U in subpopulations of submucous and myenteric neurons and in immunoreactive fibres and terminals within the ganglia and mucosa. No endocrine cells were stained. A similar localization was

reported by Ballesta et al (1988) who also showed by chemical treatment and denervation experiments that the immunoreactive fibres were short and intrinsic, running in both oral and anal directions. In addition, some colocalization of neuromedin U immunoreactivity was observed with calcitonin gene related peptide (CGRP) in cells of both the submucous and myenteric plexus. The absence of neuromedin U in endocrine cells by both immunocytochemical studies substantiates the results reported in this chapter (Figures 4:8 and 4:9). It is interesting however that whilst both immunocytochemical studies report a dense innervation of the small intestinal mucosa, our mucosal scrapings were totally devoid of immunoreactive neuromedin U. This point requires further clarification and more closely controlled, collaborative studies.

The extended chromatographic examinations presented in this chapter are in agreement with those presented in chapter 3 with the exception of finding an additional molecular form of neuromedin U immunoreactivity in pig small intestine (Figure 4:11). The identity of this second molecular form is at present unknown, although it is of a similar molecular weight to neuromedin U25 since only a single immunoreactive peak was detected using gel permeation chromatography (Figure 4:10). One could speculate that it represents either a slightly truncated form or a molecular form containing modified amino acid

residues. It is not clear whether its detection is the result of the different chromatographic systems used for the two studies or whether it represents an artefact of tissue processing. This additional molecular form was however, consistently found along the entire gastrointestinal tract in the latter study.

Following the original description of neuromedin U immunoreactivity in the rat anterior pituitary by radioimmunoassay (Domin et al, 1987), Steel et al (1988) showed a co-localization of neuromedin U with ACTH in rodent and human corticotropes and an intense staining in the rodent neurointermediate lobe. No immunoreactivity was found in the posterior lobe. When the anterior and posterior lobes are separated by hand, the neurointermediate lobe always remains attached to the posterior lobe and cannot be dissected away with any confidence to be extracted in its own right (Lerner, 1981). The low levels of neuromedin U immunoreactivity detected in the posterior lobe by RIA may thus be attributed to the adherent neurointermediate tissue. Neuromedin U thus joins a growing list of regulatory peptides now localized to the anterior lobe of the pituitary gland. The physiological significance of these substances in the pituitary is at present unknown, although it has been postulated that they may act as local modulators of pituitary hormone secretion (Denef, 1986). Studies investigating the possible role of

neuromedin U in the anterior pituitary are described in chapters 6,7 and 8.

In the rodent thyroid gland neuromedin U has been localized to a sub-population of parafollicular C-cells (Domin et al (2), in press). This distribution is similar to that of somatostatin and CGRP but differs from a whole host of neuropeptides localized to intrafollicular and perivascular fibres (Huffman and Hedge, 1986). Further work is now required to determine the effect of neuromedin U upon thyroid gland function. In the urogenital tract the distribution of neuromedin U immunoreactivity is similar to that of neuropeptide Y but at lower concentrations (Adrian et al, 1984). In view of its ability to elicit smooth muscle contraction, neuromedin U may influence motility and transport along the viscera.

In conclusion, neuromedin U-like immunoreactivity is widely distributed in mammalian and frog tissue. The results presented in this chapter have demonstrated a localization in the pituitary gland, brain, spinal cord, gastrointestinal tract, thyroid gland and urogenital tract. The co-localization of neuromedin U immunoreactivity with ACTH requires further investigation with the aim of establishing a possible physiological role in the pituitary gland. The presence of neuromedin U in the gastrointestinal tract and its

localization to nerves, suggests that it may act as a neurotransmitter/ neuromodulator. Although neuromedin U has been shown to have no effect upon the contraction of the small intestine (Minamino et al, 1985b) pharmacological studies have demonstrated specific effects upon splanchnic blood flow (Sumi et al, 1987; Gardiner et al, 1989) and ion flux (Brown and Quito, 1988). Such biological activity is consistant with its histochemical localization which describes the absence of this peptide in fibres supplying the muscle and a presence in fibres supplying the submucosal arterioles. Further possible actions upon the gut and other tissues now await investigation.

CHAPTER 5

PURIFICATION AND SEQUENCE ANALYSIS OF RAT AND AN AMPHIBIAN NEUROMEDIN U

Introduction

Whilst mRNA sequencing has allowed the rapid characterization of the precursor proteins for some brain and gut peptides, the nature of the peptides subsequently stored in the tissue cannot be predicted using this technique. To elucidate the primary structure of novel bioactive peptides and to characterize any post-synthetic modifications, microisolation and microsequence analysis must be employed. Today, despite the publication of an untimely obituary (Malcolm, 1978), such techniques are still an essential area of peptide research.

The purification of many peptides and proteins is however hampered by two main obstacles; the first is that they undergo degradation by proteolytic enzymes and secondly they are often present in low concentrations when compared to the total protein content of the gland or organ. Consequently, data is often produced with nothing other than the very crudest attempts at

purification. Current methods of preparative peptide isolation are almost solely based on column chromatography. The most commonly used methods namely, gel permeation, ion exchange and reversed phase high performance liquid chromatography have already been described in chapter 3. Here, only points pertinent to preparative techniques will be introduced and the purification of two novel neuromedin Us described.

Monitoring Purification

Over many decades, endocrine physiologists have instructed peptide chemists on which substances to look for. A biological action was thus assigned to an extract and the causative agent sought thereafter. Purifications were therefore based on the ability of a substance to retain its biological activity throughout the procedure. Whilst this approach will continue to be used, other methods have now been adopted.

The Chemical Approach

Some workers have adopted a strictly chemical approach by purifying substances to homogeneity on the basis of their spectrophotometric properties. Potential biological activity is then investigated at a later date. The purification of pancreatic polypeptide is an example of this approach (Kimmel et al, 1975). Other

techniques are based upon detecting the presence of unique structural features indicative of biological activity. In this way, Kazuhiko Tatemoto has isolated numerous substances over the last decade. His method detects the presence of a carboxl-terminal alpha-amide, a structure, originally noted in oxytocin and vasopressin. Using this technique he has isolated the peptides peptide histidine isoleucine (PHI), peptide tyrosine tyrosine (PYY), neuropeptide tyrosine (NPY) and galanin each of which has been found to be biologically active (Tatemoto, 1982).

The Radioimmunoassay Approach

Having developed a radioimmunoassay to a substance, any intra- and/or interspecies molecular heterogeneity can be determined. Such variants can then be purified, and the purification monitored by radioimmunoassay. Naturally, this approach is dependent upon sufficient sequence similarity between the two substances to allow antibody recognition and the retention of antigenicity during the purification process. This approach may also be used to identify the presence of hypothetical structures predicted by mRNA sequencing.

Purity

The term purity is a relative one. From a biochemists viewpoint, a peptide purification should be continued until its amino acid sequence can be unequivocally determined. Although a sample may contain an appreciable quantity of impurities, it may still be possible to attempt sequencing work on such material if the substance of interest predominates. Most peptide purifications are monitored spectrophotometrically and purity is acknowledged when the material studied co-elutes with a corresponding, well resolved peak of u.v absorbance.

Problems Associated With Purification

When the purification is of a minor component within a mixture, the problem of column overloading may arise. If the proportion of contaminants is very large, the chromatographic behaviour of the minor components becomes distorted. Substances no longer emerge as discrete peaks, but tend to smear and are eluted in large volumes. Nevertheless, efficiency may sometimes be compromised in order to obtain a maximum through-put, provided that adequate resolution of the desired component from other impurities is still obtained. This differs from the analytical situation where maximum

efficiency in the minimum analysis time is the primary requirement.

Structural Analysis

The relative ease with which peptides and proteins can be sequenced today can be directly attributed to the early work of Sanger and Edman. The phenylisothiocyanate method devised by Edman in its many modified forms remains the mainstay of modern peptide chemistry (Edman, 1950).

(i) The Edman Procedure

In this technique, phenylisothiocyanate reacts with the free amino group of a peptide to yield the corresponding phenylthiocarbamoyl peptide (Figure 5:1). Following treatment with anhydrous acid the N-terminal residue is cleaved leaving the remainder of the molecule intact. The phenylthiocarbamoyl amino acid then cyclyses to the phenylthiohydantoin derivative which can be separated and identified by gas liquid chromatography. As the reaction proceeds in a sequential manner, consecutive amino acid residues are identified starting from the N-terminus. Today the entire process is automated. The method is not infallible however, since some peptides have a modified α-amino group of their N-terminal amino acid thereby prohibiting the reaction with the

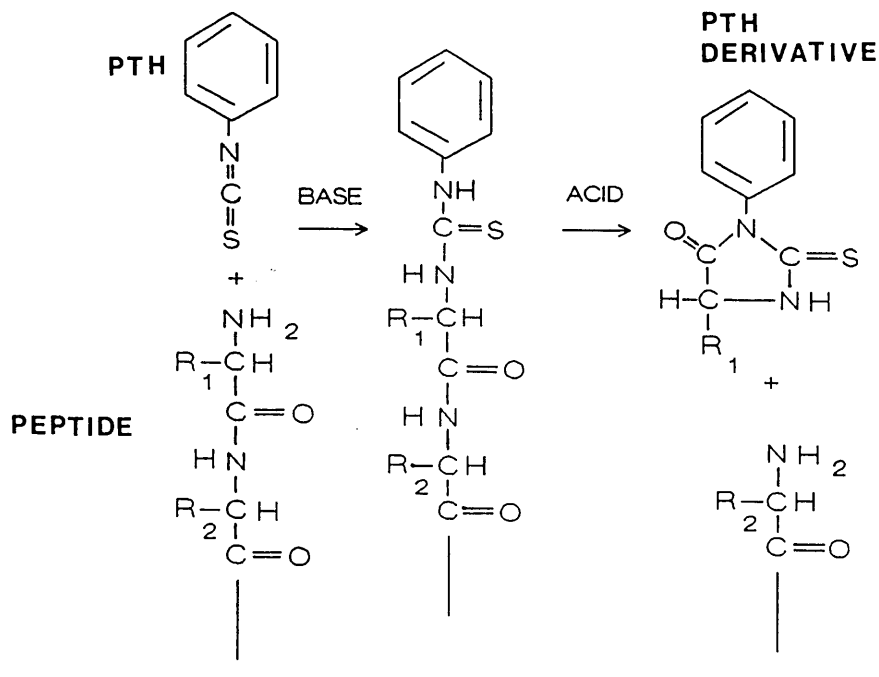


Figure 5: 1 Characterization of N-terminal residues by the Edman degradation.

phenylisothiocyanate residue. Modifications of this type can include cyclisation (eg gastrin and bombesin) and acetylation.

(ii) Amino Acid Analysis

Peptide bonds are hydrolysed by heating in acid (e.g 6 mol/l hydrochloric acid) at 100-120^{0}C for 10-24 hr usually under vacuo. The amino acid composition of a homogenous peptide can then be determined by quantitation of the liberated amino acids. This analysis is usually performed by an automated technique based on ion-exchange chromatography. Acid hydrolysis, however, results in the destruction of tryptophan, the loss of serine and threonine residues and the hydrolysis of the amide groups of asparagine and glutamine to yield aspartic and glutamic acids respectively.

(iii) Fast Atom Bombardment Mass Spectrometry

Mass spectrometry offers the capability to make accurate mass measurements with extremely high sensitivity. The method is based on the ionization of a molecule or the products of its cleavage and the subsequent quantitation of the resultant mass to charge ratios (m/z). Early mass spectrometric analysis of peptides involved their prior derivatization and needed great skill to interpret the resultant spectra (Morris et al, 1981). A more recent

modification has been the development of fast atom bombardment (Fab) analysis which amongst other things dispenses with the need to derivatize the starting material (Barber et al, 1981). Since the probability of any two fragments having an identical molecular mass is infinitesimally small, the products of a controlled reaction can be pieced together to offer the complete sequence. This method has certain advantages over those based on Edman degradation especially in cases of N-terminally blocked peptides and their mixtures.

The sample is dissolved in a non volatile solvent such as glycerol and applied onto a probe which is bombarded by a stream of neutral atoms (typically xenon and argon). Molecular ions are produced which are then accelerated though a magnetic field before passing through an ion deflector where the degree of their deflection from the straight line is related to their mass to charge ratio.

The Aim Of This Study

Chromatographic analysis revealed that the molecular form of neuromedin U-like immunoreactivity present in rat gut extracts consistently fails to co-elute with either synthetic neuromedin U8 or neuromedin U25 standards (Chapter 4 and Domin et al, 1986). Since the porcine forms are bioactive in the rat, the amino acid

sequence of rat neuromedin U was required to investigate the relative bioactivity of this peptide in the homologous species.

The discovery of extremely high concentrations of neuromedin U-like immunoreactivity in the gastrointestinal tract of the amphibian Rana temporaria (Chapter 4 and Domin et al (1), in press) raised the possibility that the primary amino acid sequence of the neuromedin U like peptide present could also be determined. With this information, the degree of sequence similarity between two molecules present in species which had diverged in evolutionary terms approximately 350 million years ago could be assessed. This chapter describes the isolation of both rat and frog neuromedin U and the determination of their primary structure.

Materials And Methods

(A) Isolation Of Rat Neuromedin U

Sample Preparation

The terminal ileum from 42 male Wistar rats was boiled in 10 volumes of 0.5 mol/l acetic acid for 15 minutes. The extract was then centrifuged (1,600g for 15 minutes) at 4^{0}C and the supernatant pumped (1ml/min) through

SepPak cartridges (Waters Associates, Milford USA) connected in series. Bound material was eluted from the cartridges using 70% v/v acetonitrile/water and lyophilized.

Chromatography

The lyophilized material was loaded onto a Sephadex G50 (fine) column (2.5x100cm) eluted with 1 mol/l acetic acid (25ml/hr) at 4°C. Fractions (4.5ml) were collected and assayed as described in chapter 2. The peak of neuromedin U-like immunoreactivity was then loaded at a flow rate of 2ml/min onto a semi-preparative reversed phase Supelcosil LC-18-DB column (1x25cm) (Supelco, Bellefonte, PA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) at 30°C. The solvent concentration was then elevated to acetonitrile /water/TFA (21/79/0.1 v/v) over 10 minutes followed by an increase to (49/51/0.1) over 70 minutes using a linear gradient. Absorbance was monitored at 214nm. Fractions (2ml) were collected and assayed for neuromedin U. The peak of immunoreactivity was then loaded onto an analytical Supelcosil LC-3DP column (0.5x25 cm) equilibrated with aqueous TFA 0.1% v/v at 30°C and a flow rate of 1.5 ml/min. The acetonitrile concentration was then raised to acetonitrile/water/TFA (21/79/0.1) over 10 minutes followed by an increase to (35/65/0.1) over 30 minutes. The single peak of

* Amino acid sequence analysis of rat neuromedin U was performed by Dr Mike Conlon, Clinical Research Group for Gastrointestinal Endocrinology of Max-Planck-Gesellschaft at the University of Gottingen, FRG.

† FAB mass spectrometric analysis of rat neuromedin U was performed by Prof Howard Morris, Dept of Biochemistry, Imperial College, London.

neuromedin U-like immunoreactivity was finally rechromatographed using a Vydac 218TP54 column (0.5x25cm) (The Separations Group, Hesperia, USA) equilibrated with acetonitrile/water/TFA (21/79/0.1) at 30°C and a flow rate of 1.5 ml/min. The concentration of acetonitrile was then elevated to (35/65/0.1) over 40 minutes.

Structural Characterization

Aliquots of purified rat neuromedin U were subjected to automated Edman degradation (Applied Biosystems, Model 470A gas-phase sequencer). The detection limit for phenylthiohydantoin (PTH) amino acids was 0.5 pmol. *

Fast atom bombardment mass spectrometry was performed on approximately 100 pmol of the peptide using a VG ZAB HF instrument equiped with an M-Scan Fab gun (Morris et al, 1981). Spectra were also run under the same conditions using synthetic canine motilin (m/z 2697) as a calibrating standard.†

Aliquots of the purified peptide were also subjected to chymotrypsin degradation. The peptide (100 pmol) was dissolved in 50 mM ammonium bicarbonate (100 µl, pH 8.4) and chymotrypsin added (1:50, w/w) at 37°C. The reaction was allowed to proceed for 3 hours after which time the sample was freeze dried. The chymotryptic digest was

submitted for analysis by Fab-MS as outlined above.

Results

Purification Of Rat Neuromedin U

The neuromedin U-like immunoreactivity from the rat ileal extract eluted as an immunoreactive peak from the Sephadex G50 column close to porcine neuromedin U25 standard (Figure 5: 2A). This immunoreactivity also eluted in a single fraction following fractionation on a semi-preparative C_{18} reversed phase HPLC column (Figure 5: 2B). Following rechromatography on an analytical diphenylmethyl- silylsilica column the neuromedin U like immunoreactivity emerged after 28 minutes (Figure 5: 3A). Rat neuromedin U was purified to near homogeneity on an analytical reversed phase C_{18} column (Figure 5: 3B). The immunoreactivity emerged as one peak of approximately 700 pmol.

Structural Analysis

Following analysis by automated Edman degradation it was possible to determine the amino acid sequence of residues 1-23. No trace of a phenylthiohydantoin (PTH) amino acid at cycle 24 was evident (Figure 5: 4).

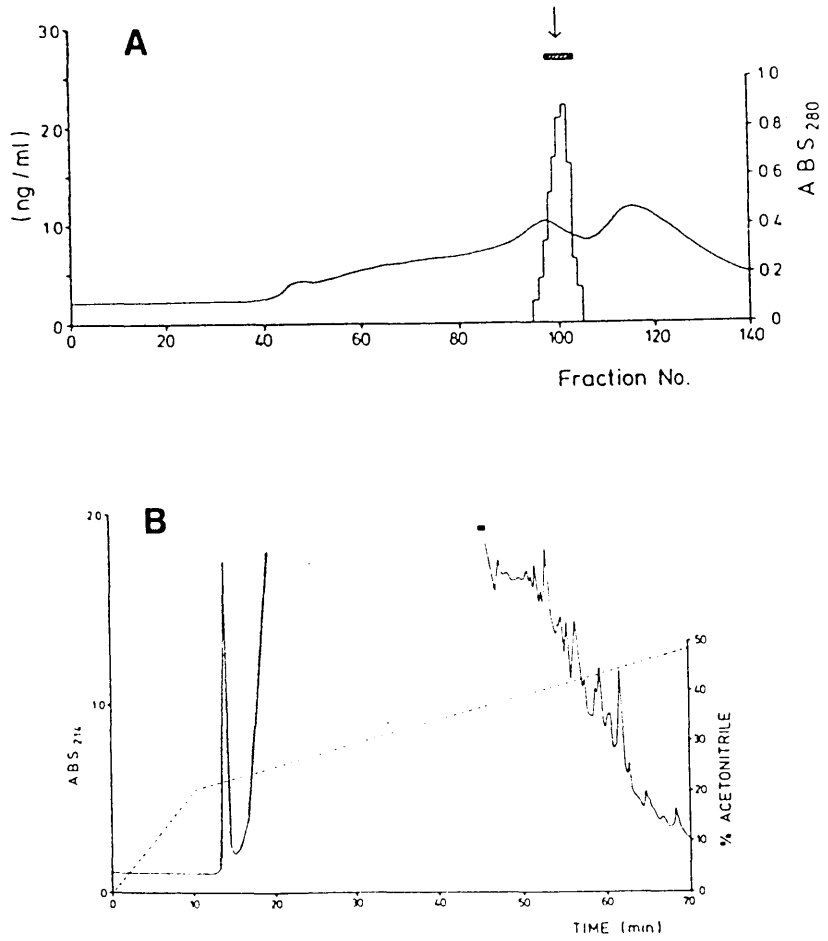


Figure 5.2 (A) Gel permeation chromatography (Sephadex G50) of rat ileal extract. The fractions containing maximum immunoreactivity, indicated by the bar, were pooled for subsequent analysis.

(B) Reversed phase HPLC on a semi-preparative C18 column (Supelcosil LC-18-DB, 1x25 cm) of the immunoreactive peak from Figure 5.2(A). The fraction (2ml) denoted by the bar contained 900 pmol of neuromedin U immunoreactivity.

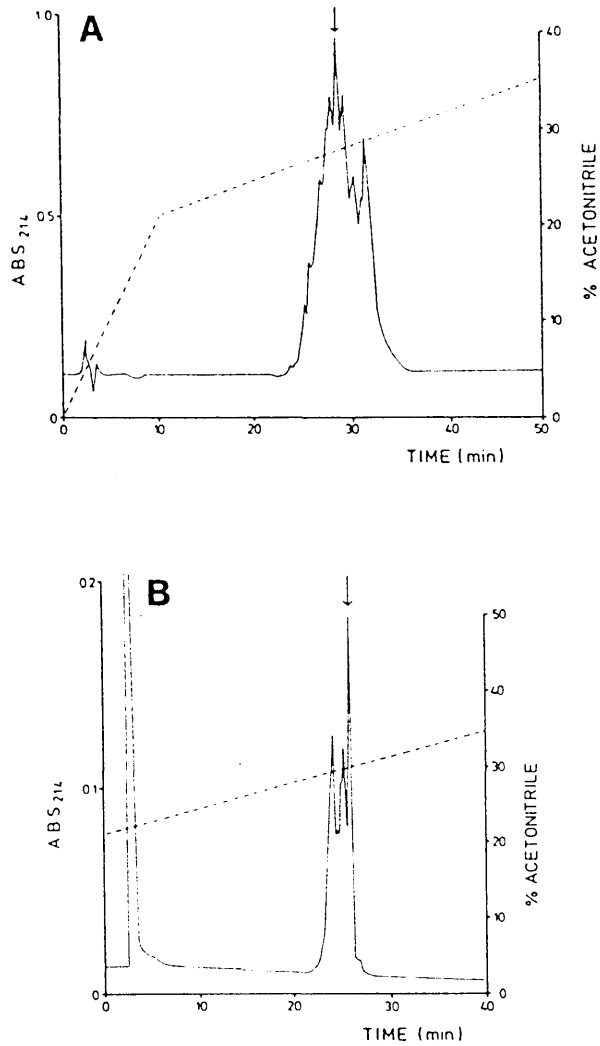


Figure 5.3 (A) Reversed phase HPLC on an analytical diphenylmethyl silylsilica column of the immunoreactive peak obtained in Figure 5.2(B). The peak denoted by the arrow contained 790 pmol of neuromedin U immunoreactivity.

(B) Purification of rat neuromedin U to near homogeneity (>90%) on an analytical C18 reversed phase HPLC column. The peak denoted contained approximately 700 pmol of immunoreactive neuromedin U.

The purified product was also subjected to Fab mass spectrometry. A prominent molecular mass ion was detected at m/z 2641+1 corresponding exactly to that predicted for the ∝-amidated form of the proposed sequence (Figure 5: 5A). Following limited chymotryptic digestion of rat neuromedin U, prominant mass ions were detected at m/z 948, m/z 801 and m/z 1859. These molecular mass ions corresponded to residues (17-23) amide, (18-23) amide and (1-17) respectively (Figure 5: 5B).

(B) Isolation Of Frog Neuromedin U

Tissue Preparation

The entire gastrointestinal tract was removed from 50 animals, washed and placed into ice cold, isotonic saline prior to extraction as described above. After cooling, the extract (200-250 ml) was filtered, mixed with diatomaceous earth (20g)(Sigma Chemical Co. USA) and refiltered.

Chromatography

Aliquots of the extract (10 ml) were loaded onto activated Sep-Pak C18 cartridges (Waters Associates). The bound material was eluted from each cartridge using 60% (v/v) aqueous acetonitrile and lyophilized. The

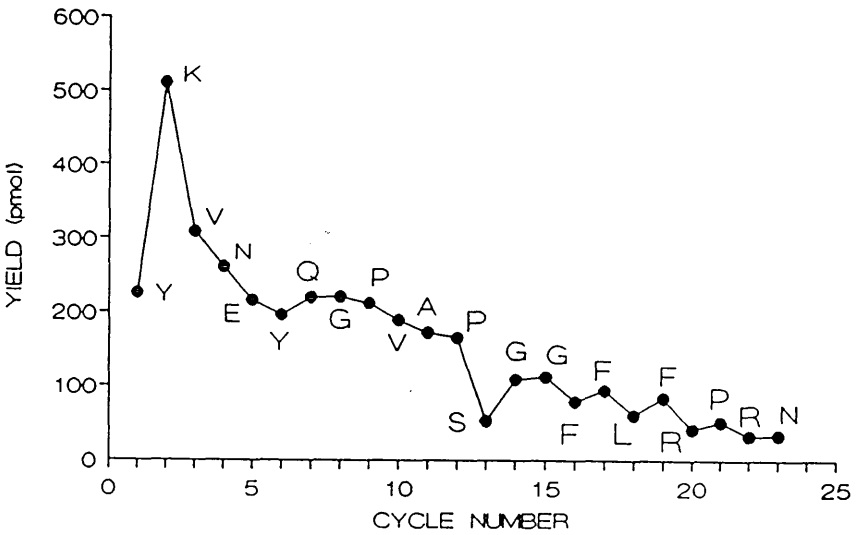


Figure 5: 4 Automated Edman degradation of neuromedin U from rat gut.

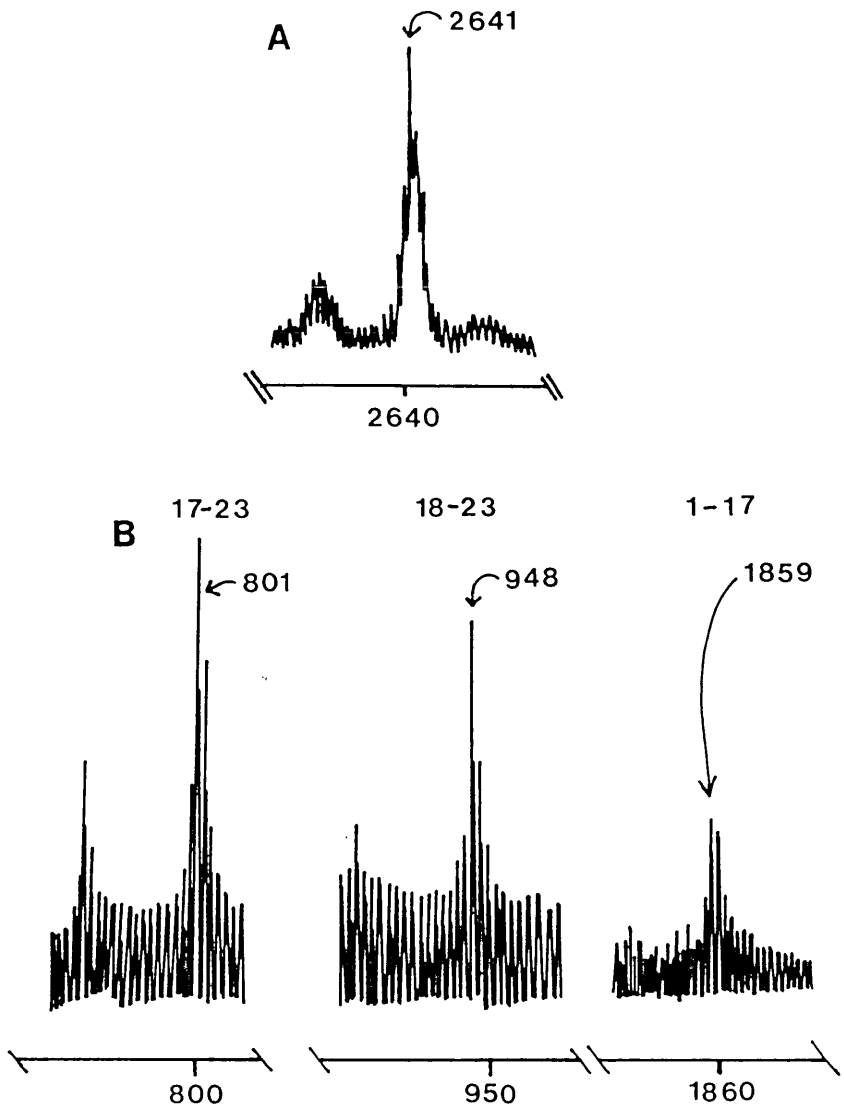


Figure 5.5 (A) Fab mass spectrum of rat neuromedin U. The spectrum was recorded on a VG ZAB HF instrument. Canine motilin was run seperately under identical conditions as a mass marker.

(B) Fab mass spectra of the products of a limited chymotryptic digest of rat neuromedin U.

samples were then reconstituted in water containing 0.1% TFA and loaded onto a poly fluorocarbon reversed phase column (Du Pont, Dreieich, W. Germany) and eluted with a linear gradient of acetonitrile /water/TFA (10/90/0.1) to (60/40/0.1) over 1 hour using a flow rate of 1ml/min. Fractions containing the peak of neuromedin U-like immunoreactivity were then concentrated and loaded onto a cation exchange column (Mono S HR 5/5, Pharmacia) eluted with 50 mM 2-(N-morpholino)-ethanesulphonic acid (MES) containing 20 % acetonitrile v/v pH=6.0. The sodium chloride concentration was raised to 0.3M over 1 hour using a linear gradient. The resultant fractions containing neuromedin U-like immunoreactivity were pooled and loaded onto a μ Bondapak reversed phase column (Waters Assoc) eluted with acetonitrile/ water/TFA (10/90/0.1). A linear gradient was developed to (40/60/0.1) over one hour where the gradient was held for a further 20 minutes. The immunoreactive peak was then loaded onto a Pep-RPC C_{18} column (Pharmacia) and eluted using a gradient of acetonitrile/ water/TFA (10/90/0.1) to (30/70/0.1) over 1 hour. The fraction containing the peak of neuromedin U-like immunoreactivity was submitted for structural analysis. All chromatographic procedures were monitored spectrophotometrically at either 280nm or 214nm.

* Amino acid sequence analysis of frog neuromedin U was performed by Dr Alaistair Aitken, Laboratory of Protein Structure, National Institute for Medical Research, Mill Hill, London.

Automated Sequence Analysis

Sequence analysis was carried out using Applied Bioscience 470A gas-phase and 477A pulsed liquid-phase peptide sequenators. The PTH-amino acids were analysed with an on-line Applied Bioscience 120A analyser. Data were collected and analysed on an Applied Bioscience 900A module calibrated with 25 pmol PTH-amino acid standards.*

Results

Preparative fractionation of the extract using a polymeric flurocarbon HPLC column yielded a single peak of neuromedin U immunoreactivity (data not shown). The immunoreactive peak was then loaded onto a cation exchange column eluted with a linear gradient of NaCl (Figure 5: 6A). The immunoreactive fraction was then subjected to reversed phase HPLC on a semi-preparative C_{18} column (Figure 5: 6B). The immunoreactive peak was purified to homogeneity using a second C_{18} column (Figure 5: 7). The yield of amphibian neuromedin U was 1.2 nmol and the entire amino acid sequence was determined with approximately 300-400 pmol of the peptide using a gas phase automated sequencer (Figure 5.8).

Frog neuromedin U was found to have striking sequence similarity to both neuromedin U 25 and rat neuromedin U.

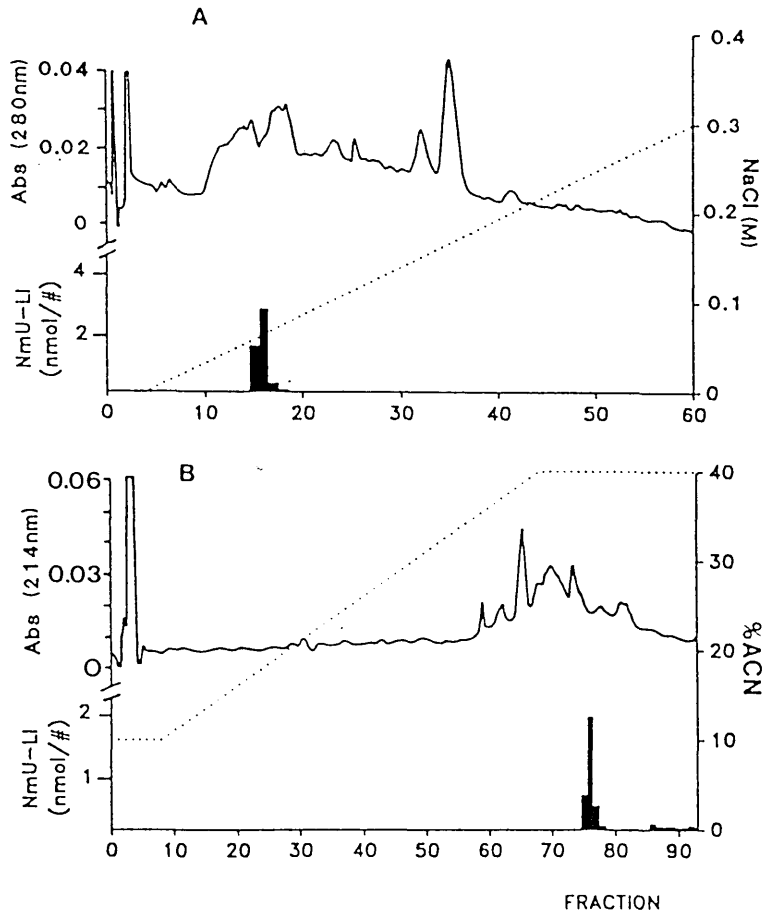


Figure 5: 6 (A) Cation exchange FPLC performed on a Mono S column eluted with 50 mM MES containing 20% ACN v/v pH=6. 0. A linear gradient of sodium chloride was developed at a flow rate of 1ml/minute.

(B) The immunoreactive peak obtained from Figure 5. 5A was columned on a semi preparative C_{18} reversed phase column.

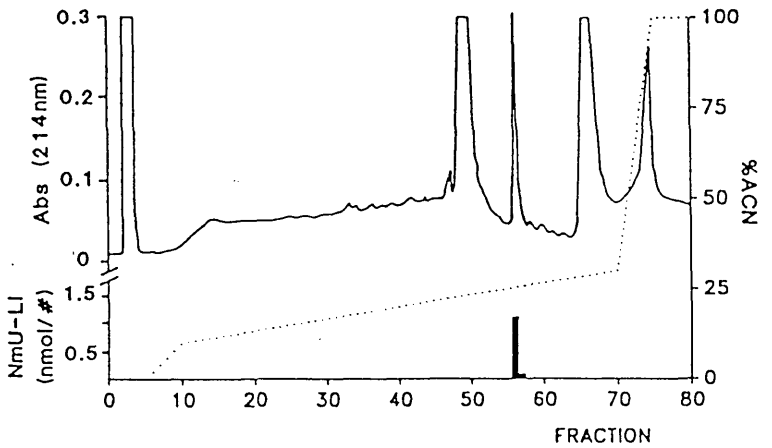


Figure 5.7 Final purification of frog neuromedin U by reversed phase HPLC using a Pep RPC C18 column. The immunoreactive peak was used for subsequent sequence analysis.

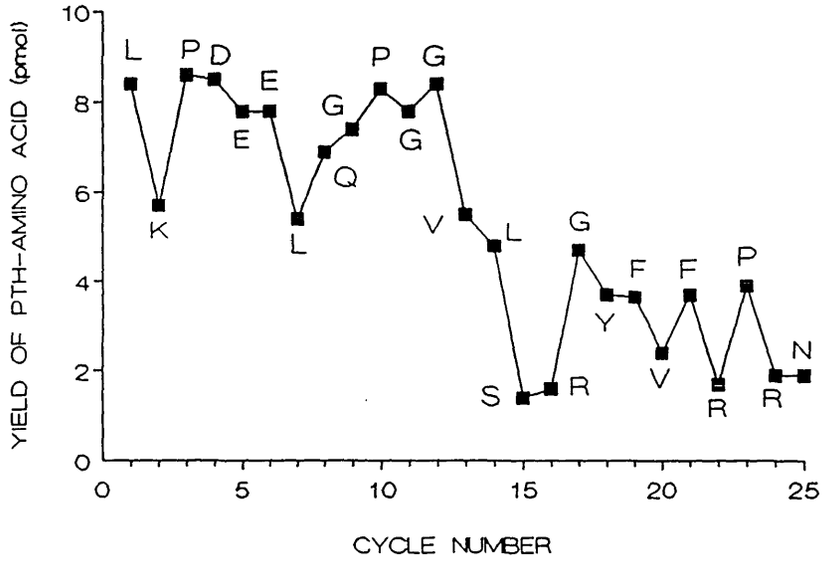


Figure 5.8 Results of automated Edman degradation of neuromedin U from frog gut.

We were unable to confirm analytically whether amphibian neuromedin U was C-terminally amidated. However, confirmation of this structure was infered by the degree of sequence similarity at the C-terminus with the rat and porcine forms and the indistinguishable chromatographic behaviour of the native peptide with the synthetic sequence amidated at its C-terminus (data not shown).

General Discussion

The results of this study have revealed that porcine, rat and frog neuromedin U are all highly related. The introduction of gaps into the rat peptide allowing maximum sequence alignment reveals that the positions of 11 amino acid residues are identical between all three species (44%) (Figure 5: 9).

Comparison of the rat peptide with porcine neuromedin U reveals that 14 residues are identical (56%) whilst the homology increases to 15 residues when the frog sequence is compared with that of pig (60%). Strong evolutionary pressure has evidently acted to conserve both the C-terminal region and certain residues present towards the N-terminus. The central region of these peptides however, appears to be more heterogenous. Since neuromedin U 8 displays all the biological actions of the extended form, the C-terminal region is clearly

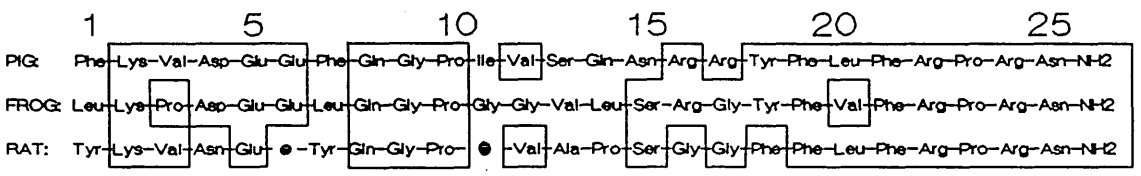


Figure 5: 9 Structural comparison of pig, frog and rat neuromedin U. Homologous sequences are boxed. Frog neuromedin U is shown to be amidated at its C-terminus although our studies were unable to confirm this. ● - signifies a gap introduced into the rat sequence to maximize stuctural similarity between the peptides.

responsible for conferring biological activity (Minamino et al, 1985b and Chapter 8). When a comparative examination of bioactivity is made however, the octapeptide is shown to be less potent than neuromedin U 25 in both contracting the uterus and in increasing blood pressure and is shorter acting. Thus, the N-terminal amino acid residues appear not to elicit but modulate the biological activity of these peptides.

Porcine neuromedin U contains two adjacent arginine residues at positions 16 and 17 which could be a site of endopeptidase cleavage (Loh Y P, 1985). Such a cleavage was originally proposed by Minamino et al to explain the occurrence of neuromedin U 8 in extracts of porcine spinal cord. This sequence however is absent from both the rat and frog forms of this peptide thereby making the generation of neuromedin U 8 like peptides in these species unlikely. This finding substantiates the chromatographic findings presented earlier in this thesis (Chapters 3 and 4). Augood et al (1988) also failed to detect endogenous neuromedin U 8, although the characterization of neuromedin U-like immunoreactivity in this study was problematical in other respects (Chapter 3).

All but three amino acid substitutions between the three peptides (positions 3, 11 and 13) can be explained by nucleotide point mutations occurring at the DNA level.

Contrary to the conclusion reached by Minamino et al (1988) not all of the amino acid replacements between the pig and rat sequences can be explained in terms of a single base substitution ie. position 13 Ala for Ser) and consequently the rat sequence is no more similar to neuromedin U 25 than that of the frog. Furthermore, the deletion of two amino acid residues within the rat sequence in positions 6 and 11 would necessitate the simultaneous deletion of three consecutive bases at both positions. Were this not the case, the resultant frame shift from a single or two site deletion would not only prohibit the high degree of sequence similarity observed at the C-terminus of the peptide but would also preclude any neuromedin U-like biological activity for the resultant form.

In conclusion, the primary amino acid sequences of rat and frog neuromedin U have been determined. Their high degree of sequence similarity indicates that this peptide may play an important, although not necessarily identical role in these diverse species.

CHAPTER 6

MANIPULATION OF ENDOCRINE STATUS AND ANTERIOR PITUITARY NEUROMEDIN U CONTENT

Introduction

The anterior pituitary gland is primarily composed of 5 classical cell types. Each of these are named on the basis of their ability to synthesise and secrete a particular hormone. Thus, somatotropes secrete growth hormone (GH), lactotropes secrete prolactin (PRL), thyrotropes secrete thyrotropin also termed thyroid stimulating hormone (TSH), gonadotropes secrete lutenising hormone (LH) and follicle stimulating hormone (FSH) and finally, corticotropes secrete the products of the pro-opiomelanocortin (POMC) gene including corticotropin (ACTH).

The role of the hypothalamus in the control of anterior pituitary function via the secretion of factors into the portal circulation was well established by the time the structure of thyrotropin releasing hormone (TRH) was determined in 1969 (Harris, 1955). In 1971, the decapeptide lutenising hormone releasing hormone (LHRH)

also termed gonadotropin releasing factor (GnRH) was sequenced. Two years later, somatostatin (SRIF), a tetradecapeptide which inhibited growth hormone release was isolated. By 1981, the stucture of ovine corticotropin releasing hormone (CRH or CRF) a 41 amino acid peptide which stimulates ACTH release was determined and shortly thereafter the structure of human growth hormone releasing factor (GRF) was ascertained (see Reichlin, 1987).

The concept of the hypothalamus-pituitary-target organ relationship thus became a reality and is represented by a model of feedback control mechanisms. In this model, the pituitary gland, under hypothalamic influence, secretes a hormone which in turn stimulates a target gland to release a second hormone. As the circulating levels of this target hormone rise, the release of the pituitary hormone is inhibited and its concentration falls. Consequently, the inhibition is removed and pituitary hormone secretion recommences. Inhibitory feedback mechanisms such as this have been identified for the pituitary-gonad, pituitary-thyroid and pituitary-adrenal axes. The inhibitory effect of the target hormones is exerted invariably at both the pituitary and hypothalamic level.

Other substances also alter anterior pituitary hormone secretion. These include established neurotransmitters

such as the catecholamines, serotonin, GABA, histamine, acetylcholine and dopamine (Neill, 1979). With the exception of dopamine, which is thought to be the physiological prolactin release inhibitory factor, the action of these neurotransmitters is thought to be restricted to the hypothalamus. Here, they appear to be involved in the relaying of incoming information from higher centres onto hypophysiotropic neurons.

Recently, much attention has also focused on how other peptides localized in the hypothalamus, but often better known for their wider distribution, influence pituitary hormone secretion. Thus, substance P has been shown to stimulate PRL and GH release and modulate LH secretion (Vijayan and McCann, 1979). Galanin elevates plasma GH and increases LH release in ovariectomized rats treated with exogenous estrogen and progesterone (Ottlecz et al, 1988). Gastrin releasing peptide (GRP) inhibits PRL secretion (Tache et al, 1979) and the structurally related neuromedin B inhibits the release of TSH (Rettori et al, 1989). Cholecystokinin stimulates ACTH secretion (Rehfeld, 1987) and neuropeptide Y increases the secretion of LH, GH and PRL (Chabot et al, 1988).

Initially, efforts were made to determine whether these hypothalamic peptides were secreted into the hypothalamo-pituitary-portal circulation to act directly on the pituitary or whether they, like the

neurotransmitters, exerted their effects solely at the hypothalamic level. It soon became clear that many peptides had different effects upon pituitary hormone secretion depending upon whether they were administered centrally, systemically or *in vitro*. Neurotensin exemplifies this since it acts to inhibit prolactin secretion when administered centrally and yet stimulates prolactin secretion when given systemically and *in vitro* (Vijayan et al, 1988). In addition, substance P stimulates PRL secretion when administered centrally, systemically and *in vitro* but it stimulates LH secretion when administered centrally and inhibits LH secretion when administered systemically (Vijayan and McCann, 1979). Since it is thought unlikely that centrally administered peptides reach the anterior pituitary and that many show a clear *in vitro* action, a direct effect of these substances was postulated upon pituitary hormone secretion *in vivo*.

In parallel to such physiological/pharmacological studies, many regulatory peptides were localized to the anterior pituitary itself, initially by radioimmunoassay and immunocytochemistry but more recently also by mRNA studies. Thus, galanin and vasoactive intestinal polypeptide (VIP) synthesis was shown in the anterior pituitary and a localization in lactotrophs proposed (Arnaout et al, 1986; Kaplan et al, 1988). Gastrin releasing polypeptide was found in the anterior lobe

(Major et al, 1983) as was neurotensin (Goedert et al, 1982). Neuropeptide Y has been localized to many cell types including gonadotropes, somatotropes, corticotropes and some lactotropes (Chabot et al, 1988). These studies show that there is often a correlation between the action of a regulatory peptide upon the secretion of a pituitary hormone and its localization within that cell type (ie neuromedin B and TSH (Steel et al, 1988), substance P and prolactin (Coslovsky et al, 1984), VIP and PRL (Morel et al, 1982).

The model of homeostatic control outlined above also contains a short loop feedback mechanism (Kakita and Odell, 1986). This involves pituitary gland secretions themselves modulating further pituitary secretion. One attractive hypothesis explaining the role of regulatory peptides within the pituitary gland is that they act as paracrine factors, co-ordinating such local inter- and intracellular communication.

Although high concentrations of neuromedin U-like immunoreactivity have been found in the anterior pituitary (Domin et al, 1987) and a co-localization with ACTH noted (Steel et al, 1988) nothing is known of its functional role in this tissue. Our initial studies revealed that whilst adrenalectomy produced changes in the anterior pituitary neuromedin U cell population, no corresponding change in its extractable content was

* Animals were supplied by Banfin and Kingman Ltd, Aldbrough, Hull, UK. Surgical gonadectomy and surgical adrenalectomy was performed by the supplier at source.

noted. Similar studies have shown however that the manipulation of endocrine status can lead to changes in pituitary hormones which are paralleled by the pituitary peptides involved in the control of their secretion (Steel et al, 1988, Goedert et al, 1982). In this chapter the results of studies aiming to establish a link between the levels of anterior lobe neuromedin U and alterations in the rat endocrine status are presented. Such studies begin to examine the possibility that neuromedin U may play a role in the local control of pituitary hormone secretion.

Materials And Methods

Adult control and surgically manipulated Wistar rats (body weight 180-200g) were caged in groups of four or five with an 13hr light, 11hr dark cycle and an ambient temperature of 22°C. Unless otherwise stated, animals received normal rat chow and were allowed water ad libitum.*

1. Estrogen Manipulated Female Rats

Five days following surgical castration, female rats received either replacement doses of 17β estradiol (2.5 µg/100g/day s.c.) dissolved in 100µl safflower oil or injections of vehicle alone. A hyperestrogenized group received 17β estradiol (1 mg/100g s.c on days 7 and 19).

Control animals had sham surgery, were also given daily injections of vehicle and were sacrificed during a period of diestrous as determined by daily vaginal smear examination during 2 consecutive cycles.

2. Chemically Castrated Female Rats

A depot preparation of the LHRH superagonist Zoladex® (ICI Pharmaceuticals Ltd, Macclesfield, UK) was administered s.c (1.8mg/100g) to groups of control and ovariectomized animals. A second group of intact animals received the anti estrogen tamoxifen citrate (Nolvadex, Aldrich Chemical Co. Ltd, Dorset, UK)(25µg/100g/day s.c) using safflower oil (0.2ml) as carrier. Treatment was continued for 28 days. Vaginal smears confirmed a state of acyclic diestrous in all treatment groups.

3. Testosterone Manipulated Male Rats

Castrated male Wistar rats received replacement doses of testosterone (250µg/100g/day) for 28 days by s.c injection using 0.2ml safflower oil as a carrier. Intact animals were given a high dose of testosterone (12mg/100g) s.c in 0.2ml safflower oil on days 1, 7, 14 and 21 and killed after 28 days. Control animals were injected with 0.2ml of the carrier alone for 28 days.

4. Adrenalectomy And Dexamethasone Treatment

Male Wistar rats were surgically adrenalectomized at source. A second group of animals were both surgically adrenalectomized and castrated. Both groups of animals were allowed 10 days to recover before their inclusion in the study and were given 0.9% saline in their drinking water. A third group of animals received daily s.c injections of dexamethasone (100µg/100g/day) for 28 days. A group of control animals as well as both sets of adrenalectomized animals received daily injections of the carrier (H_2O)for the entire treatment period. All animals were sacrificed between 9.00 and 10.00 hr.

5. Dopamine Agonists And Antagonists

Male Wistar rats (body weight 200-250g) received daily s.c injections (1ml) of either haloperidol (Serenace®, Searle Pharmaceuticals. Bray, Co. Wicklow, Eire) (250µg/100g per day), bromocriptine (Sandoz Products Ltd. Feltham, Middx U.K)(500 µg/100g per day) or vehicle (10% v/v ethanol/0.9% w/v NaCl). The treatment period lasted 9 days.

Tissue Extraction

All animals were killed by exanguination via cardiac puncture and the blood collected into lithium

heparinised tubes containing Trasylol® (2000 KIU) mixed and centrifuged. The resulting plasma was frozen immediately on dry ice and stored at -20°C. The anterior pituitary gland from each animal was separated from the neurointermediate lobe, weighed and extracted in boiling 0.5M acetic acid for 15 minutes as described in chapter 3. Extracts were cooled to room temperature, frozen and stored at -20°C until assay.

Radioimmunoassay

Neuromedin U-like immunoreactivity was quantified as described in chapter 2. Rat prolactin was measured using reagents supplied by Dr Parlow, UCLA Medical Centre, USA.

Results

Unless stated otherwise, the success of each endocrine manipulation was confirmed by quantitating pituitary target cell hormone mRNA (data not shown). All results are expressed as mean±SEM.

1. Ovariectomy And Estrogen

The effect of ovariectomy (OVX) and estrogen treatment upon anterior lobe wet weight and neuromedin U content is shown in figure 6:1. Although ovariectomy did not

alter the lobe wet weight (OVX 10.5±0.5mg, C 9.6±0.6mg), both the replacement dose of estrogen given to ovariectomised animals (REPL) and the high dose given to intact animals (E2) resulted in significant changes (REPL 26.2±1.3 mg; E2 21.4±1.9 mg)($p<0.001$).

Ovariectomy produced a slight, but not statistically significant fall in whole gland neuromedin U content (OVX 0.87±0.15, C 1.12±0.12 pmol/gland). Treatment of ovariectomized animals with replacement doses of estrogen and intact animals with high dose estrogen did produce a fall in whole gland neuromedin U levels (REPL 0.48±0.13 pmol/gland, E2 0.34±0.07 pmol/gland)($p<0.01$).

2. LHRH Analogue And Anti-Estrogen Treatment

The LHRH analogue Zoladex failed to alter the anterior lobe neuromedin U content when given to either intact (A 0.99±0.04 pmol/gland), or ovariectomized rats (OA 0.93±0.10 pmol/gland) compared to control animals (C 1.17±0.13 pmol/gland). Treatment with the anti-estrogen tamoxifen (T) however produced a significant fall in the anterior lobe neuromedin U content (T 0.71±0.08 pmol/gland)($p<0.01$)(Figure 6:2). Both the Zoledex and tamoxifen tretment regimes resulted in a statistically significant decreases in anterior lobe wet weight.

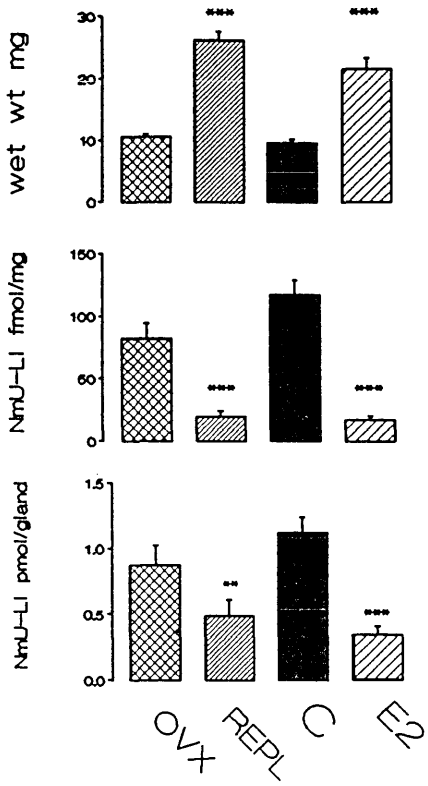


Figure 6: 1 The effect of ovariectomy (OVX), estrogen replacement therapy (REPL), and hyperestrogen treatment of intact animals (E2) upon anterior lobe wet weight (upper panel) and neuromedin U levels expressed in terms of wet weight (middle panel) and whole lobe content (lower panel).

** $p<0.01$, ***$p<0.001$ (C) signifies control group.

Results are expressed as mean±SEM; n=8

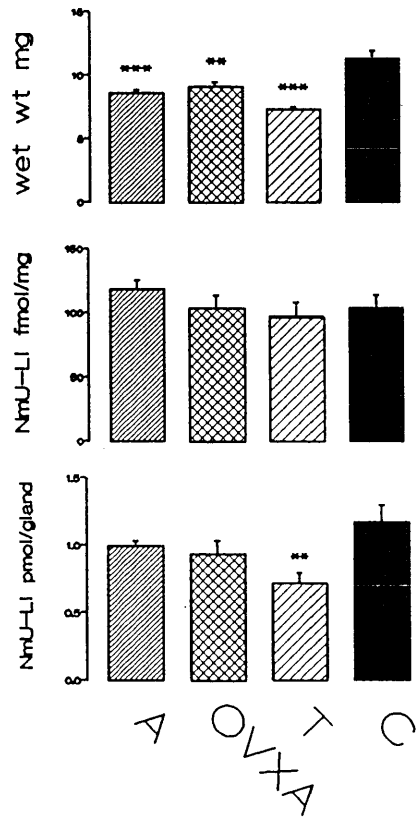


Figure 6: 2 Effect of LHRH super-analogue upon intact (A), and ovariectomized animals (OVXA), and tamoxifen treatment (T) upon anterior lobe wet weight (upper panel) and neuromedin U levels expressed in terms of tissue wet weight (middle panel) and whole lobe content (lower panel).

** $p<0.01$, *** $p<0.001$ (C) signifies control group.

Results are expressed as mean±SEM; n=8

3. Castration And Testosterone

Surgical castration in male rats failed to affect the anterior lobe content of neuromedin U immunoreactivity (C 2.09±0.12, CAST 1.89±0.23 pmol/gland) although it did increase the wet weight of the whole gland (Figure 6:3). Treatment with testosterone of either castrated animals at replacement doses (REPL) or to intact animals in high doses (TESTO) decreased the neuromedin U content of the whole gland (REPL 1.26±0.15 pmol/gland, TESTO 1.34±0.12 pmol/gland)($p<0.001$).

4. Adrenalectomy And Dexamethasone Treatment

Adrenalectomy produced a significant increase in the whole gland neuromedin U content (ADX 3.52±0.51 pmol/gland; C 1.86±0.15 pmol/gland)($p<0.01$) (Figure 6:4). When animals were both adrenalectomized and castrated, a significant fall in the whole gland neuromedin U content was observed (C 1.86±0.15 pmol/gland, ADX/CAS 1.22±0.13 pmol/ gland)($p<0.05$). A similar decrease was obtained in animals treated with the dexamethasone (C 1.86±0.15 pmol/gland, DEX 1.32±0.18 pmol/gland)($p<0.01$).

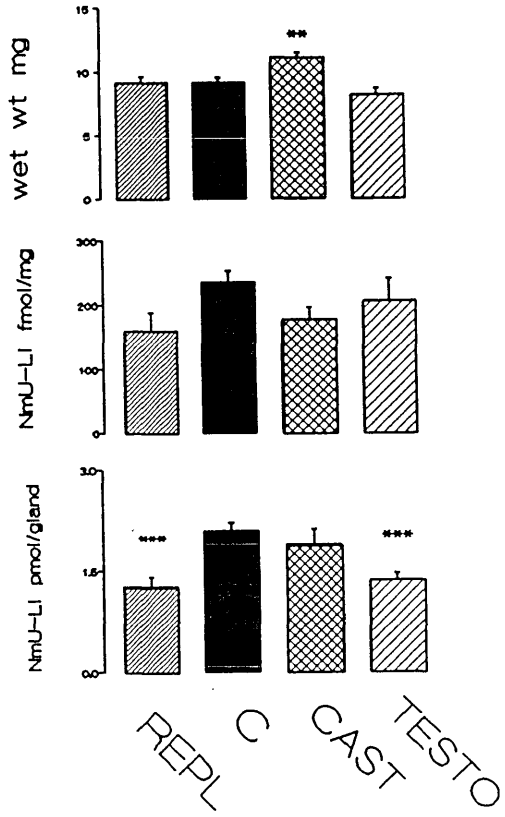


Figure 6:3 The effect of castration (CAST), testosterone relacement (REPL) and hyper-testosterone (TESTO) treatment of intact animals upon anterior lobe wet weight (upper panel) and neuromedin U levels expressed in terms of tissue wet weight (middle panel) and whole gland content (lower panel).
** $p<0.01$, *** $p<0.001$ (C) signifies control animals.

Results are expressed as mean±SEM; n=8

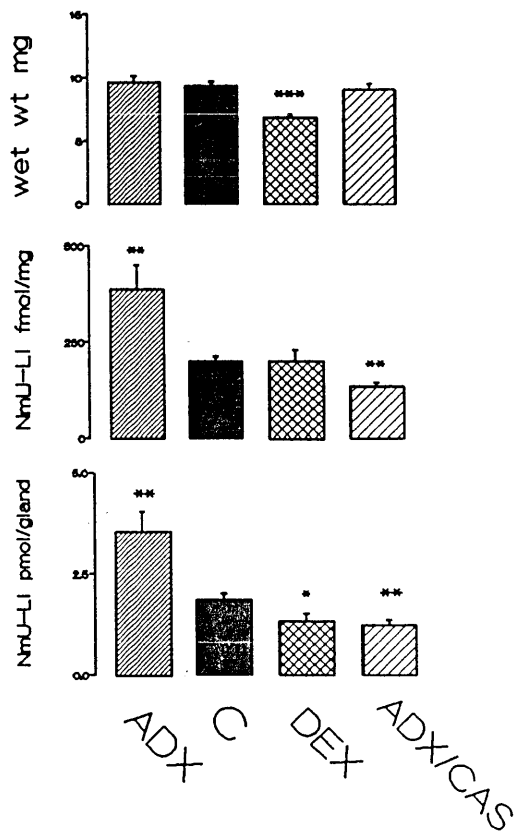


Figure 6:4 The effect of adrenalectomy (ADX), dexamethasone treatment (DEX) and combined adrenalectomy and castration (ADX/CAS) upon the wet weight of the male rat anterior pituitary (upper panel) and neuromedin U immunoreactivity expressed in terms of tissue wet weight (middle panel) and whole gland content (lower panel). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (C) signifies control animals.

Results are expressed as mean±SEM; n = 8

5. Dopamine Agonists And Antagonists

Treatment with bromocriptine and haloperidol produced levels of circulating prolactin which were significantly different to those achieved by injecting vehicle alone (Figure 6:5). Bromocriptine (BrCr), a dopamine agonist, suppressed circulating prolactin concentrations to below the detection limit of the assay (<3 μg/l) whilst haloperidol (Halop), a dopamine antagonist, produced an increase in serum prolactin (C 37±8 μg/l, Halop 65±12 μg/l. Despite the bromocriptine induced suppression of circulating prolactin, this treatment failed to change the extractable prolactin content of the anterior lobe (C 104±9 ng/gland; BrCr 100±9 ng/gland). Haloperidol treatment however, increased the pituitary prolactin content (Halop 154±6 ng/gland; $p<0.001$). Although both plasma and tissue prolactin concentrations were altered in this study, neither treatment altered the anterior lobe content of neuromedin U in these animals (C 4.23±1.03 pmol/gland; BrCr 3.75±0.48 pmol/gland; Halop 3.47±0.43 pmol/gland).

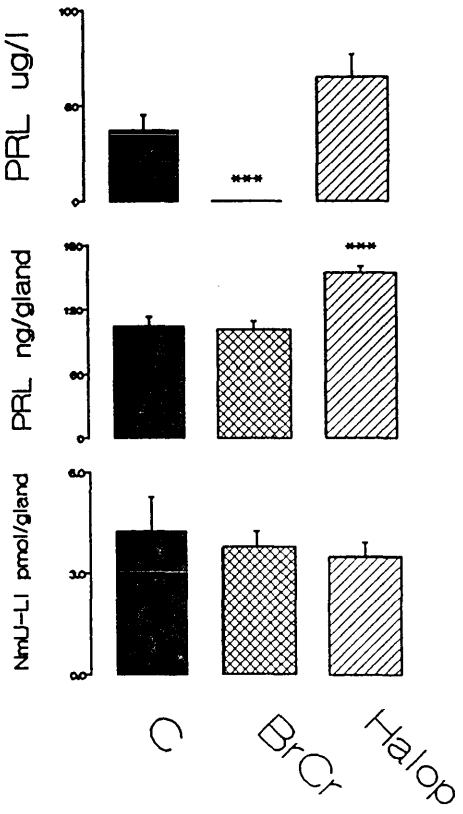


Figure 6:5 The effect of bromocriptine (BrCr) and haloperidol (Halop) treatment of male rats upon circulating prolactin concentrations (upper panel), anterior lobe prolactin content (middle panel) and immunoreactive neuromedin U content (lower panel). *** $p<0.001$ (C) signifies control animals

Results are expressed as mean±SEM; n=8

Discussion

1. Castration, Estrogen And Testosterone

The secretion of LH and FSH is controlled by a number of factors including hypothalamic LHRH and gonadal products including testosterone, estrogen, inhibin and activin which regulate LHRH release (Figure 6:6). The mechanism through which gonadal steroids exert their effects are complex and incompletely understood since they appear to influence both the hypothalamic oscillator regulating pulsatile LHRH release as well as affecting gonadotropes directly. Chronic gonadectomy, through the removal of negative feedback, produces both high circulating levels of LH and FSH as well as increasing their pituitary content (Clayton and Catt, 1981).

The results of the present study show that treatment of female rats with estrogen and male rats with testosterone markedly supresses the anterior pituitary content of neuromedin U. It is of interest, however, that castration was without effect in either sex upon the whole gland content of this peptide.

In females, the effects of chemical castration were also investigated. Zoladex is a LHRH superagonist which, when administered as above, acts to down regulate pitutary LHRH receptors. Consequently, the pulsatile release of

LHRH is abolished and both LH synthesis and secretion is decreased (Wildt et al, 1981). This treatment therefore interrupts the hypothalamic-pituitary-gonadal axis directly at the pituitary level as opposed to the gonadal level (at which surgery acts). Treatment of ovariectomized animals with this LHRH analogue results in an animal with relatively functionally isolated gonadotropes. Even under these conditions, no effect upon anterior lobe neuromedin U content was observed. The effect of tamoxifen in suppressing pituitary neuromedin U content is difficult to explain. The observed effect is not consistent with an anti-estrogen action, indeed, the drug appears to act as a partial estrogen agonist at the pituitary level. The action of tamoxifen is however both hormone and tissue specific (Furr and Jordan, 1984).

The mechanism by which estrogen suppresses neuromedin U content appears to be independent of the hypothalamic-pituitary-gonad axis since castration had no effect upon the neuromedin U levels in this tissue. It is possible that the effects attributed to estrogen and testosterone are indirect, acting through unidentified factors which are sensitive to gonadal status. Since testosterone is converted to estrogenic substances in males, the observed effects of testosterone upon anterior lobe neuromedin U content may represent the action of such metabolites (Paulsen, 1974).

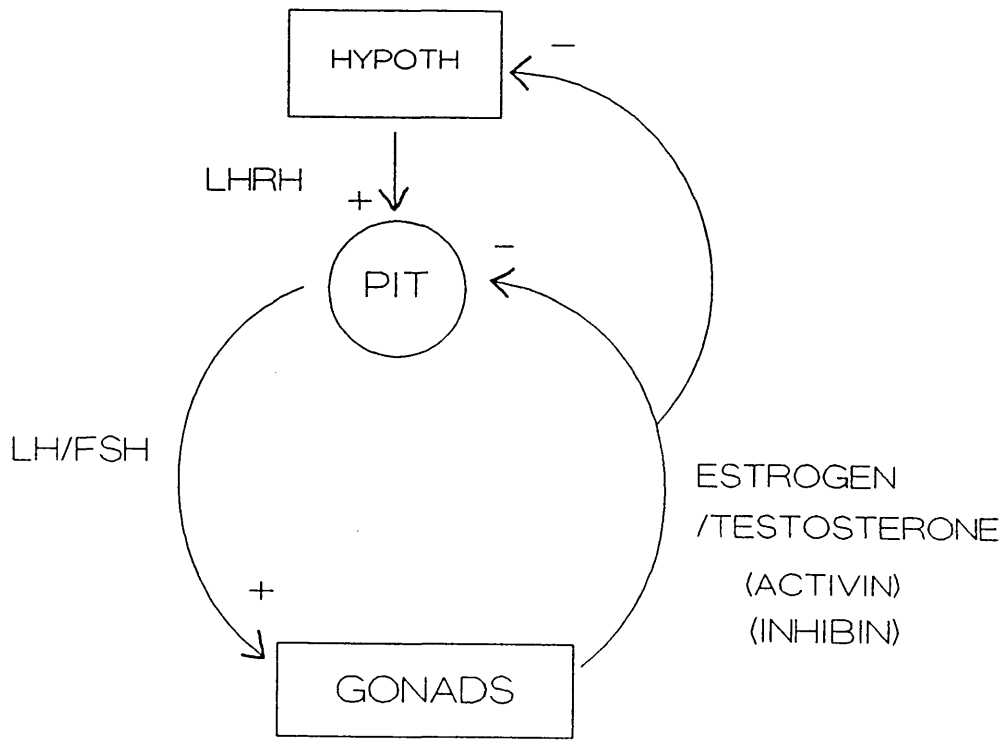


Figure 6: 6 A simplified diagrammatic representation of the hypothalamic-pituitary-gonadal axis.

To resolve this possibility, animals could be treated with dihydrotestosterone since this form is not converted to estrogen in the same way as testosterone.

The effect of estrogen upon pituitary peptide content has recently attracted much attention. Treatment with 17-β estradiol results in a dramatic elevation in the anterior pituitary content of both galanin and VIP (Kaplan et al, 1988; Prysor-Jones and Jenkins, 1988). Pituitary levels of substance P are also influenced by estrogen (Aronin et al, 1986). In addition, estrogen priming appears to influence the action of peptides in their regulation of pituitary hormone secretion. Thus, although neuropeptide Y (NPY) influences LH secretion in both male and female rats, the response is dependent upon the steroid hormone background of the animal. Whilst centrally administered NPY in ovariectomised rats inhibits LH release, pretreatment with estrogen reverses this effect and NPY has been found to facilitate LH secretion in male rats (Kalra et al, 1987). The effects of neurotensin upon LH secretion are also dependent upon the presence of estrogen (Akema et al, 1987). Consequently, in addition to thyroid hormones and glucocorticoids, gonadal steroids have been proposed to act as "permissive" hormones exerting profound effects upon the ability of cells to respond to a variety of other hormones (Malbon et al, 1988).

2. Adrenalectomy And Dexamethasone

Pituitary secretion of ACTH is primarily influenced by hypothalamic corticotropin releasing factor (CRF) secretion. ACTH then stimulates glucocorticoid production from the adrenal cortex. Glucocorticoids, in turn, exert a major inhibitory effect on ACTH release acting both at the hypothalamic level by tonically inhibiting CRF gene expression and also directly at the pituitary level (Jones and Hillhouse, 1976)(Figure 6: 7). Administration of glucocorticoids to animals thus lowers plasma ACTH concentrations. Adrenalectomy removes the feedback inhibition and consequently increases CRF stimulated ACTH secretion.

The results of this study have revealed that adrenalectomy increases the anterior lobe neuromedin U content in a manner similar to that reported for VIP (Matsumara et al, 1982). Conversely, dexamethasone suppresses its anterior lobe content. The increase in neuromedin U levels following adrenalectomy contrasts with an absence of effect following surgical castration as well as a shorter period of adrenalectomy (7 days) previously reported (Steel et al, 1988). The changes in neuromedin U content found in this study also parallel those of ACTH (Birnberg et al, 1983) and support the histochemical co-localization of these two molecules in corticotropes. Interestingly, simultaneous castration

reverses the increase in gland content following adrenalectomy. The effect of this combined treatment is very interesting since surgical castration alone had no effect upon anterior lobe neuromedin U content. Such combined treatment removes the site of both glucocorticoid and gonadal steroid synthesis and it indicates that a gonadal component, probably not testosterone, is required to facilitate the increase in neuromedin U content following adrenalectomy.

Chronic administration of glucocorticoids is known to result in a reduction of corticotroph cell number in the anterior pituitary (Baker and Drummond, 1972). Dexamethasone, however, has been found to reduce (Sakakura et al, 1981) or exert no effect (McNicol et al, 1988) upon corticotrophs indicating that the effect obtained with this compound is dependent upon the treatment regime employed. Our results show that dexamethazone treatment significantly decreased the anterior lobe wet weight in keeping with reports of its inhibitory effects upon pituitary growth.

Glucocorticoids exert wide ranging effects in many tissues. In the pituitary, they also suppress TSH secretion in hypothyroid animals and influence TSH gene expression (Ahlquist et al, 1987). In addition, they inhibit prolactin secretion (Oosterom et al, 1985), increase the synthesis and content of growth

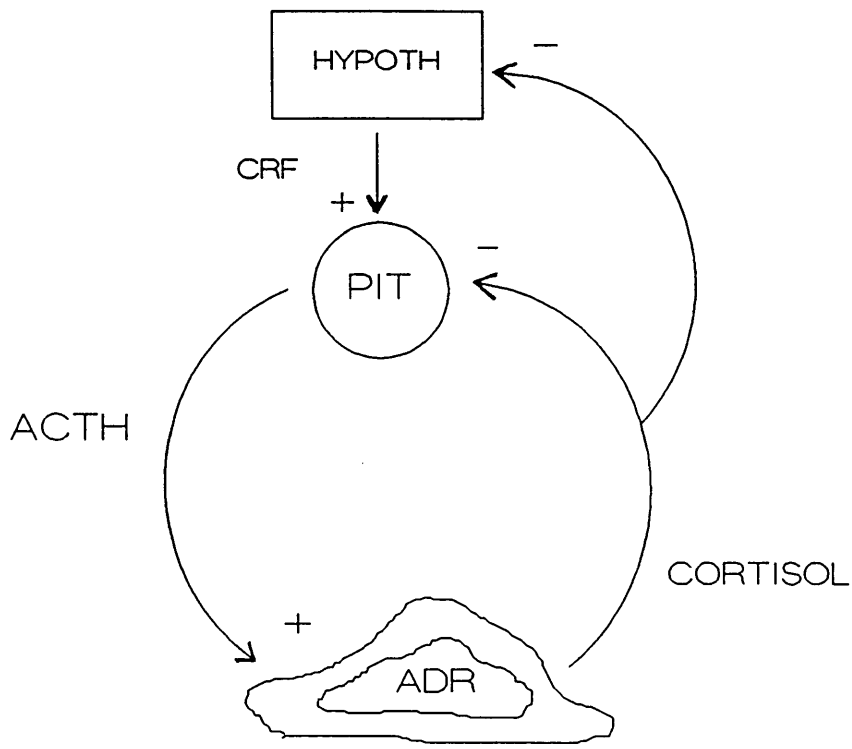


Figure 6: 7 A simplified diagrammatic representation of the hypothalamic-pituitary-adrenal axis.

hormone in pituitary cells in vitro and sensitise the response of somatotrophs to GRF (Wehrenburgh et al, 1989).

Recent studies have demonstrated that adrenal status can also affect enzymic activity. A Leu-Phe cleaving endopeptidase has been found which is sensitive to glucocorticoids thereby regulating γ - and β- endorphin levels in tissues containing proopiomelanocorticotropin producing cells (Lebouille et al, 1988). This finding has obvious repercussions upon the processing of other peptide prohormones if such enzymic sensitivity is widespread.

3. Dopamine Agonists And Antagonists

Physiologically relevant hypothalamic releasing factors have been characterized for five of the six anterior pituitary hormones. To date, however, the releasing factor regulating prolactin secretion remains elusive. Prolactin release is generally thought to be controlled through inhibition by a prolactin release inhibiting factor (McLeod, 1976), while acute stimulation of prolactin release is believed to be caused by a prolactin releasing factor (Shin, 1980). It is the general concensus that the inhibitory factor is dopamine whilst candidates for the role of releasing factor include TRH, VIP and vasopressin (Shin, 1987).

In our study, treatment of animals with the ergot derivative bromocriptine and antagonist haloperidol did not change the anterior pituitary neuromedin U content, although both plasma and anterior lobe prolactin levels were clearly altered. The results of these experiments cannot however exclude a more subtle modulatory role for dopamine upon changes in neuromedin U content as has been demonstrated for TRH induced stimulation of prolactin secretion in lactating rats (Plotsky and Neill, 1982).

Conclusion

The results of these experiments reveal that anterior pituitary neuromedin U is directly responsive to alterations in the circulating levels of the gonadal steroids estrogen and testosterone. An increase in anterior lobe neuromedin U content is observed following adrenalectomy and is suppressed following treatment with dexamethazone, suggesting the co-localization of this peptide with ACTH in this tissue and thereby strengthening previous histochemical data. Interestingly, the presence of intact gonads is a requirement for the adrenalectomy induced increase in male anterior lobe neuromedin U content since this effect is abolished and reversed when animals are simultaneously adrenalectomized and gonadectomized. The facilitatory factor is unlikely to be testosterone since

treatment of both gonadectomized and intact animals with this steroid led to a decrease in gland content. Alterations in dopamine status were without effect upon neuromedin U content suggesting that neuromedin U and prolactin levels are unlikely to be directly influenced by each other and that the estrogen effects are unlikely to be mediated via prolactin secretion.

Quantitation of anterior pituitary neuromedin U content following endocrine manipulations is continued in the next chapter which examines the effect of thyroid hormone status.

CHAPTER 7

NEUROMEDIN U AND THE PITUITARY-THYROID AXIS

Introduction

The secretion of thyroid stimulating hormone (TSH or thyrotropin) from the anterior pituitary is controlled by two mechanisms. The first is neuronal, regulated by the hypothalamus. The second is hormonal, operating through the secretions of the thyroid gland (Reichlin et al, 1978). Both elements act to maintain blood thyroid hormone concentrations at the physiological level. The hypothalamus exerts a dominant stimulatory action over TSH synthesis and secretion since circulating levels of TSH decrease and hypothyroidism ensues following hypothalamic-pituitary dissociation (Brolin, 1947). Furthermore, TSH secretion can be increased following the electrical stimulation of specific hypothalamic areas (Martin and Reichlin, 1972).

The existence of a hypothalamic thyrotropin releasing hormone (TRH) was predicted in the early 1950's by physiological studies (Greer, 1951). The tripeptide was

finally purified and characterized from hundreds of thousands of hypothalami in 1969 (Boler et al, 1969). Intravenous injection of TRH induces a prompt increase in circulating TSH levels within 2 minutes in rats and within 5 minutes in man. In the circulation, TRH has a half life of about 4 minutes (Redding and Schally, 1969). Elevated levels of circulating TSH stimulate the release of thyroid hormones, which in turn, inhibit further TSH secretion by acting directly upon the pituitary and the hypothalamus where TRH synthesis and secretion is inhibited (Segerson et al, 1987) (Figure 7:1). Although TRH was purified on the basis of its action upon TSH secretion, it is also a prolactin releasing factor and regulator of growth hormone secretion under certain circumstances (Shin et al, 1987).

Manipulation of thyroid status has previously identified changes in the anterior pituitary content of several peptides including substance P (Jonassen et al, 1987), neurotensin (Goedert et al, 1982) and neuromedin B (Steel et al, 1988). Due to the complexity of the resultant response however, full interpretation of such findings is difficult. Nevertheless, once a change has been identified, futher physiological and pharmacological studies can then lead to a better understanding of the role these peptides play in the pituitary. The aim of this study was to identify changes

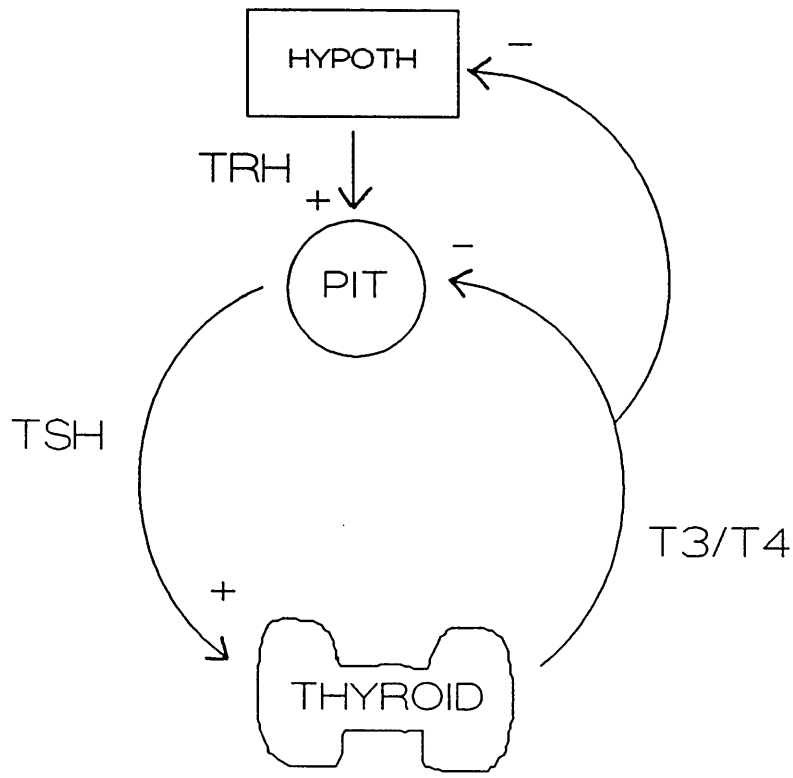


Figure 7:1 A simplified diagrammatic representation of the hypothalamic-pituitary-thyroid axis

* Animals were supplied by either Olac Laboratories, Bicester, Oxon, UK or Banfin and Kingman Ltd, Aldbrough, Hull, UK. Surgical thyroidectomy was performed by the supplier at source.

in anterior pituitary neuromedin U immunoreactivity, and, if possible, explain the findings in terms of the changes known to occur following TRH treatment and alteration in thyroid status.

Materials And Methods

Animals

Male rats of Sprague Dawley or Wistar strains (150-200g) were maintained in a constant temperature (21°C) and humidity environment with a 13h light: 11 h darkness cycle. All animals were allowed water ad libitum. *

Effect Of Exogenous TRH And Manipulation Of Thyroid Status

Sprague Dawley rats (n>10) of body weight 200-250g were treated with either methimazole (Sigma Chemical Company Ltd. Poole, U.K) (5 mg/100g per day), thyroxine (T4) (Eltroxin®, Glaxo Pharmaceuticals Ltd, Greenford, U.K) (5 µg/100g per day) or TRH (Roche Products Ltd, Welwyn Garden City, U.K) (20mg/100g per day) by daily administration to their food. Treatment with each drug was continued for 12 days. A further two groups of control and surgically thyroidectomized animals received food without additives.

Treatment Of Thyroidectomized Animals With TRH And Tri-iodothyronine (T3)

Male Wistar rats of body weight 130-170 g were surgically thyroidectomized 3 weeks before study. Groups of animals (n=8) were injected s.c (1ml) with either TRH (250 µg/100g per day), tri-iodothyronine (T3) (Tertroxin® Glaxo Laboratories Ltd; 15 µg/100g per day), TRH (250µg/100g per day) and T3 (2.5 µg/100g per day) or TRH (250µg/100g per day) and T3 (15 µg/100g per day) for 14 days. Control animals received injections of vehicle alone (0.9% w/v NaCl).

Dose-Effect Relationship Between Exogenous TRH And Anterior Lobe Neuromedin U Content

Groups of adult Wistar rats (200-250g) (n=10) received daily subcutaneous injections of either saline or TRH (20ng-2mg/100g body weight) over a 14 day period. All rats were fed on standard chow and were allowed water ad libitum.

Time Course Of The TRH Induced Changes In Anterior Lobe Neuromedin U Content

Two groups of adult Wistar rats (200-250g) received daily s.c injections of either saline or TRH (0.2mg/100g body weight). Animals from each group (n=10) were

sacrificed following 1, 3, 5, 7, 10 or 14 days of treatment. All animals were killed 24 hours ± 15 minutes following their last injection.

Tissue Extraction

At the end of each study period, rats were lightly anaesthetized with ether and killed immediately by cardiac puncture. Blood was collected into heparinized tubes and the plasma separated after centrifugation. Anterior pituitary glands were dissected with the aid of a microscope and plunged into boiling 0.5 mol/l acetic acid for 10-15 min. Both tissue extracts and plasma samples were frozen immediately on dry ice and stored at -20^{0}C until assay.

Chromatography

Extracts of the anterior pituitary lobe from control and TRH treated animals (2mg/100g/day for 14 days) were subjected to gel permeation and reversed phase HPLC analysis as outlined in chapter 3.

Radioimmunoassays

Neuromedin U-like immunoreactivity was measured as described in chapter 2. TSH, T3 and T4 were measured

using conventional double antibody radioimmunoassays. Reagents for the rat TSH assay were obtained from Dr A F Parlow, UCLA Medical Centre USA. Radioimmunoassays for T3 and T4 were performed as described previously (Brajkovich et al, 1983). All results are expressed as mean ± SEM. Statistical significance was assessed by analysis of variance and Students t-test.

Results

TRH Treatment And Modulation Of Thyroid Status

Oral administration of TRH (20mg/100g per day) for 12 days produced a five-fold increase in the anterior pituitary content of neuromedin U immunoreactivity when compared with animals receiving vehicle (Figure 7: 2). Oral T4 treatment (5µg/100g per day) did not change the neuromedin U content of this gland. Thyroidectomy, both surgical and chemical produced a significant fall in neuromedin U content. The success of each treatment was verified by measurement of serum T4 and TSH concentrations (Table 7: 1).

Treatment Of Thyroidectomized Animals With TRH And Tri-iodothyronine (T3)

Administration of TRH (250 µg/100g per day) s.c to thyroidectomized rats for 14 days did not alter anterior

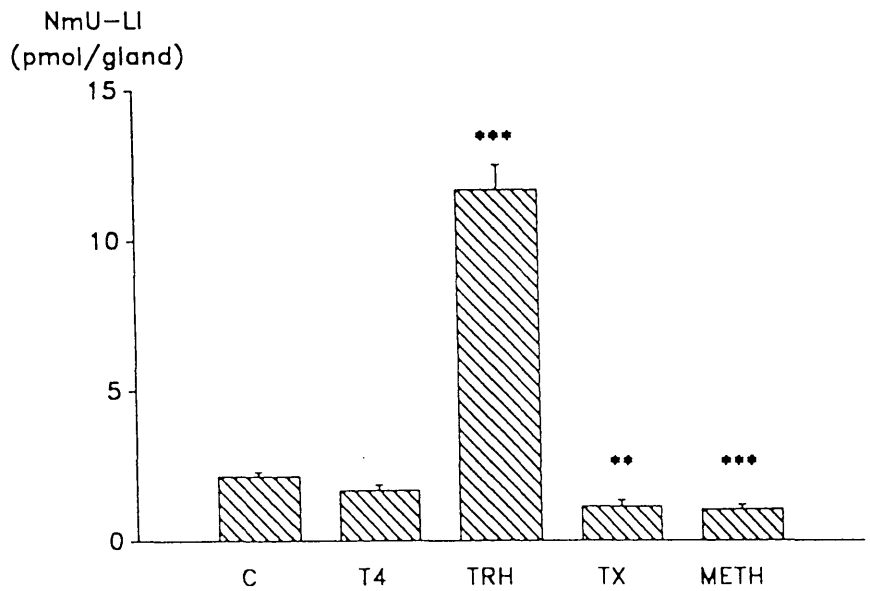


Figure 7.2 Euthyroid rat anterior pituitary content of neuromedin U-like immunoreactivity (NmU-LI) following oral administration of thyroxine (T4) (5 μg/100g per day), TRH (20mg/100g per day) and methimazole (METH) (5mg/100g per day). Treatments were continued for 12 days. Control (C) and surgically thyroidectomized rats (TX) received normal rat chow. Results are expressed as mean ± SEM n>10. ** $p<0.01$, *** $p<0.001$ compared with control (analysis of varience and Students t test). **(n=>10)**

Table 7:1 Serum TSH and thyroxine (T4) concentrations in euthyroid rats orally treated with T4, TRH and methimazole for 14 days. Control and surgically thyroidectomized rats received normal rat chow. Results are expressed as mean ± SEM. **(n=>10)**

Treatment	TSH (µg/l)	T4 (nmol/l)
Control	297±16	39±3
T4	<78	240±14
TRH	925±43	90±3
TX	5292±311	<10
METH	1528±82	<10

lobe neuromedin U content whilst high dose T3 treatment (15 μg/100g per day) produced a small but significant increase in anterior pituitary neuromedin U levels (Figure 7: 3). The greatest changes in gland content were observed when both TRH (250 μg/100g per day) and low dose T3 (2.5 μg/100g per day) were given simultaneously. When the same dose of TRH was given together with a higher dose of T3 (15 μg/100g per day) a smaller increase in anterior lobe neuromedin U content was observed. All doses of T3 suppressed the TSH hypersecretion associated with thyroidectomy (Table 7: 2).

Dose-Effect Relationship Of Exogenous TRH And Anterior Pituitary Neuromedin U Content

The effect of 14 days treatment with daily s.c injections of various doses of TRH are shown in Figure 7: 4. Treatment with 2μg/100g/day produced a statistically significant increase in the anterior pituitary neuromedin U levels. Above this dose, the neuromedin U content increased linearly up to the maximum dose of 2mg/100g/day. Over this range the correlation coefficient between the change in anterior lobe neuromedin U content and TRH dose was Rval=0.75.

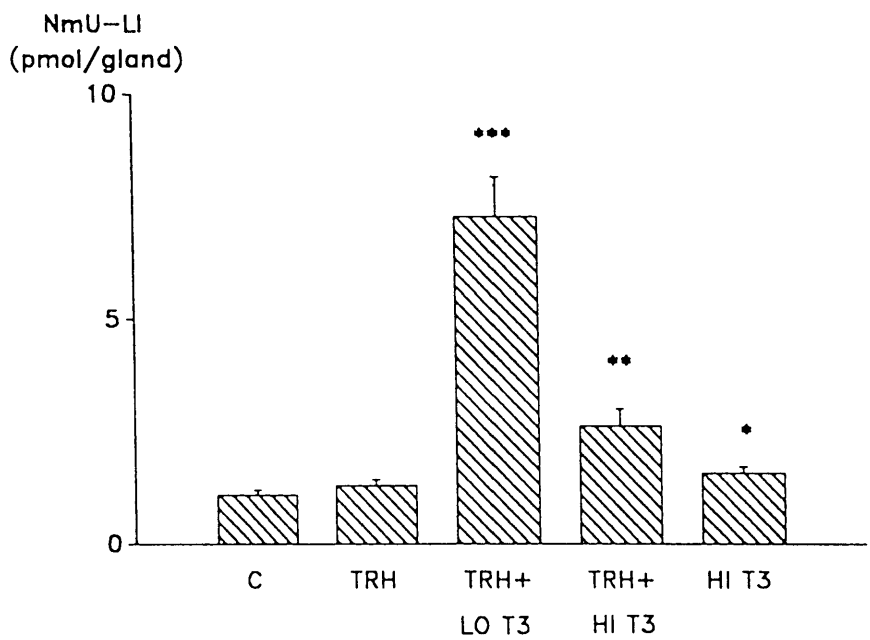


Figure 7:3 Anterior pituitary lobe content of neuromedin U-like immunoreactivity in surgically thyroidectomized rats injected s.c with TRH (250 μg/100g per day), TRH (250 μg/100g per day) and tri-iodothyronine (T3) (2.5 μg/100g per day)(TRH+LO T3), TRH (250 μg/100g per day) and T3 (15 μg/100g per day)(TRH+HI T3) and T3 alone (15 μg/100g/day). Treatments were continued for 14 days. Results are expressed as mean ± SEM. **(n=8)**

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with control (analysis of variance and Students t test).

Table 7:2 Serum TSH and tri-iodothyronine (T3) concentrations in thyroidectomized rats s.c treated for 14 days with TRH, TRH and low dose T3 replacement (TRH+ LO T3), TRH and high dose T3 replacement (TRH+ HI T3) and high dose T3 replacement alone (T3). Results are expressed as mean ± SEM (n=8).

* $p<0.001$ comparing high dose and low dose T3 treatment (one way analysis of variance and Students t test).

Treatment	TSH (µg/l)	T3 (nmol/l)
Control	2052±334	0.46±0.07
TRH	2189±214	0.59±0.05
TRH+LO T3	65±9	1.00±0.06
TRH+HI T3	72±9	3.03±0.37*
HI T3	62±8	2.16±0.24*

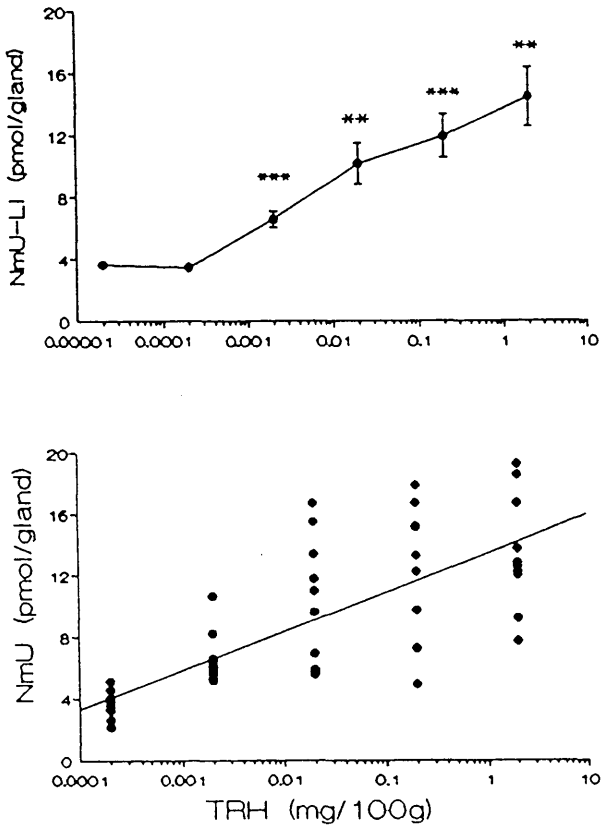


Figure 7:4 The effect of TRH upon the anterior lobe neuromedin U content following 14 days treatment.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with control (analysis of variance and Students t test).(n=10)

Time Course Of The TRH Induced Change In Anterior Pituitary Neuromedin U Content

The increase in anterior lobe neuromedin U content induced by daily injections of TRH (0.2mg/100g/day) is shown in Figure 7:5. All animals were killed 24 hours ± 15 minutes after their final injection. The first statistically significant change in neuromedin U content was observed after 3 days of treatment. This increase became more pronounced with time, giving a hyperbolic relationship up to 14 days treatment.

Chromatography

Gel permeation profiles of anterior pituitary gland extracts confirmed the single peak of neuromedin U-like immunoreactivity originally described in chapter 4 (Figure 7:6). Following 14 days of treatment with TRH (0.2mg/100g/day), the increase in content was almost solely due to an increase in this molecular form. An additional molecular form, with a smaller apparent molecular weight, was also observed which eluted ahead of synthetic neuromedin U8. Fractionation of gland extracts from treated animals by reversed phase HPLC also revealed a predominant molecular form which coeluted with both synthetic rat neurmedin U and the immunoreactivity present in the pituitary of untreated animals (fraction 43). A small amount of a second

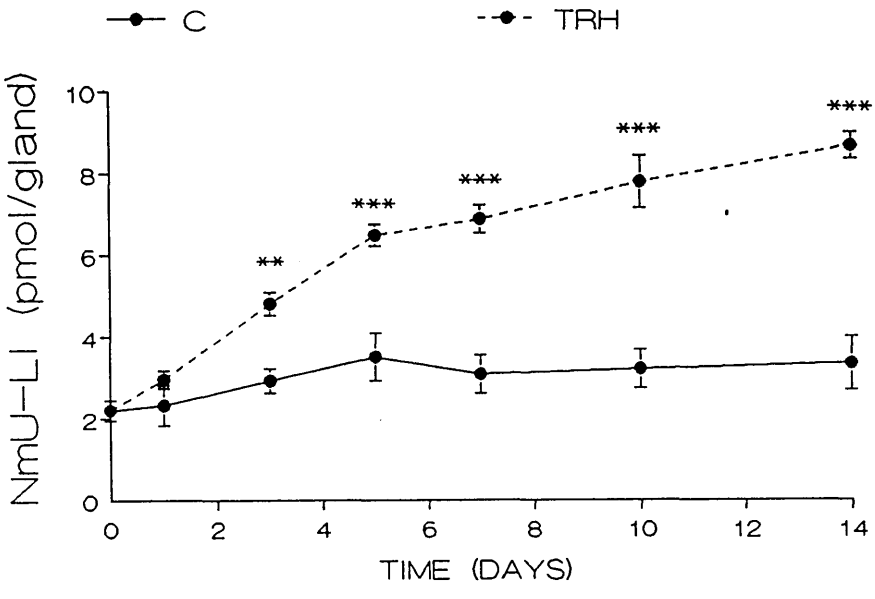


Figure 7:5 The effect of time upon the change in anterior lobe neuromedin U content following daily s.c. injection of TRH (0.2mg/100g/day). (n=10)

* p<0.05, ** p<0.01, *** p<0.001 compared with control (analysis of variance and Students t test).

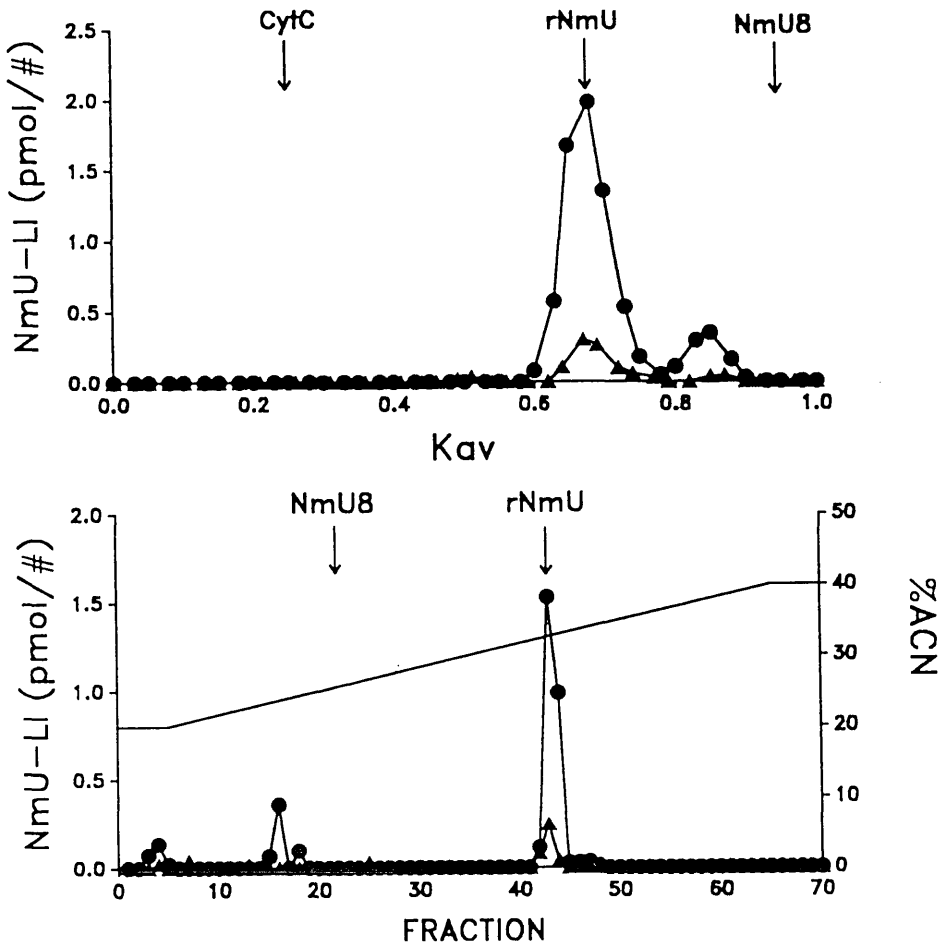


Figure 7:6 Fractionation of rat anterior lobe extract following 14 days treatment with saline (▲) or TRH (0.2mg/100g/day) (●). The upper panel shows the results of Sephadex G50 chromatrography (0.9x100cm). The lower panel, the results obtained by reversed phase HPLC using a 5μm Techsil C_{18} column. Arrows indicate the elution position of horse heart cytochrome C(Cyt C), synthetic rat neuromedin U (rNmU) and neuromedin U8 (NmU8).

molecular form was also detected, eluting earlier at fraction 16 ahead of synthetic neuromedin U8 (fraction 22).

Discussion

The results of this study demonstrate that chronic treatment with TRH significantly increases the rat anterior pituitary content of neuromedin U in euthyroid animals (Figure 7: 2). Since exogenous TRH administration would induce a compensatory thyroid hormone response, we investigated whether this increase in content was the result of an increased exposure to thyroid hormones. Treatment of euthyroid rats with exogenous T4, at a dose which elevated circulating T4 concentrations above those found following TRH treatment, failed to elicit similar effects (Table 7: 1).

We also examined if the increased neuromedin U content was related to increased TSH secretion. Both chemical and surgical thyroidectomy however produced a significant decrease in anterior lobe neuromedin U despite raising circulating TSH levels above those found in the TRH treated group (Table 7: 1). The mechanism of the observed TRH effect therefore appeared to be dependent upon TRH stimulation in the presence of circulating thyroid hormones.

To test this hypothesis, surgically thyroidectomized male rats were treated with TRH in the presence and absence of exogenous T3, the bioactive form of thyroid hormone (Larsen, 1982). The results from this study confirmed our hypothesis. TRH administration to these animals failed to alter anterior lobe neuromedin U content without simultaneous T3 replacement (Figure 7:3). The reason for this dependence is unknown, although it may be related to the general decrease in gene expression accompanying the hypothyroid condition (Oppenheimer et al, 1987). Interestingly, when the dose of T3 was raised still further, the neuromedin U response to TRH decreased, with no further fall in circulating TSH (Table 7:2).

The suppressive effect of high dose T3 cannot be explained in terms of thyrotrope toxicity (De Fesi et al, 1979) since the same dose of T3 slightly increased anterior pituitary neuromedin U concentrations when given alone (Figure 7:3). The effect may however be the result of a thyroid hormone induced down regulation of TRH receptor number (Hinkle et al, 1981). A similar biphasic relationship of TRH-induced effects and thyroid hormone concentration was recently reported for the growth hormone secretory response of urethane anaesthetized rats (Szabo et al, 1985).

The effect of exogenous TRH upon the pituitary

neuromedin U content was dependent upon the dose administered over the range 0.2 µg/100g/day to 2 mg/100g/day (Figure 7:4). At a dose of 0.2mg/100g/day TRH produced a significant increase in neuromedin U content after 3 days treatment (Figure 7:5). The neuromedin U response is therefore the result of the chronic effects of TRH treatment.

Chromatographic analysis confirmed that the increase in anterior lobe neuromedin U-like immunoreactivity following TRH treatment was primarily due to an increase in the single molecular form of rat neuromedin U present in the normal animal (chapter 4). A second, as yet uncharacterized molecular form was also evident after TRH treatment. Further work is now needed to determine its identity although it possibly represents a truncated form of the rat neuromedin U sequence.

Recent immunocytochemical studies have reported a co-localization of neuromedin U immunoreactivity with ACTH in the rat anterior lobe (Steel et al, 1988; Ballesta et al, 1988). Using the antiserum employed in those reports an increase in the neuromedin U/ACTH immunoreactive cell number was observed following TRH treatment, but, no corresponding increase in the cell content was evident (Domin et al, 1989). The explanation for this finding is, at present, also unknown since TRH is not recognised to exert effects on corticotrope activity.

It was possible that the effect of TRH treatment upon neuromedin U levels was the result of alterations in prolactin secretion and synthesis. Treatment of euthyroid animals with the dopamine agonist bromocriptine and antagonist haloperidol was, however, without effect upon the anterior pituitary levels of neuromedin U-like immunoreactivity (chapter 6).

The physiological significance of the observed neuromedin U response to TRH is unclear since the doses administered in our studies were pharmacological rather than physiological. The presence of circulating thyroid hormones near the normal range do, however, appear to provide a condition in which the effects of exogenously administered TRH become maximal. In addition, it is possible that the observed alterations in anterior lobe neuromedin U content in vivo may be secondary to the indirect effects of TRH and thyroid hormones upon other regulatory factors.

In conclusion, we have demonstrated that exogenous TRH treatment increases the anterior lobe content of the peptide neuromedin U. This effect is dependent upon circulating thyroid hormone concentrations. Although the physiological significance of these changes is unclear, our results indicate an association between the control of the pituitary-thyroid axis and the anterior lobe concentrations of this novel regulatory peptide.

CHAPTER 8

SOME PHARMACOLOGICAL ASPECTS OF NEUROMEDIN U

A. Comparative Bioactivity Of Pig, Rat And Frog Neuromedin U

Introduction

The ability of peptides such as substance P and bombesin to contract rat uterine smooth muscle *in vitro* is well established (Von Euler and Gaddum, 1931; Melchiorri, 1980). Using this bioassay system, Naoto Minamino and his collegues purified a series of "neuromedins" from extracts of porcine spinal cord (Minamino et al, 1985a). This method of monitoring peptide purification is however, non specific, as exemplified by the diversity of amino acid sequence found amongst these peptides (Chapter 1). Nevertheless, the approach has allowed his group to monitor their purifications rapidly without specialized pharmacological equipment or specific radioimmunoassays.

Each "neuromedin" exerts either potent pressor or vasodepressor effects. They thus join a number of established vasoactive peptides including calcitonin gene related peptide (CGRP), substance P, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), angiotensin II, atrial naturetic peptide (ANP) (Polak and Bloom, 1986) and the recently discovered endothelin (Yanagisawa et al, 1988). Both forms of porcine neuromedin U, like the bombesin peptides, NPY and endothelin, exert pressor effects when administered to rats in vivo. Sumi et al (1987) and Gardiner et al (in press) later extended these original observations and showed that, in addition to the pressor effect, neuromedin U specifically reduced blood flow in the canine and rat superior mesenteric artery and portal vein respectively.

In this study, the effects of the recently characterized rat and frog neuromedin U (see chapter 5) are examined upon rat uterine smooth muscle and systemic blood pressure. The responses observed are also compared to those of the porcine forms.

Materials And Methods

(i) Rat Uterus Bioassay

Female Wistar rats (200-250g) were killed and both horns

of the uterus removed and mounted in 10ml organ baths containing modified Locke solution (NaCl 154 mmol/l, KCl 5.6 mmol/l, $CaCl_2$ 0.54 mmol/l, $NaHCO_3$ 5.95 mmol/l) and glucose 2.78 mmol/l. Tissues were maintained at 30°C and bubbled with 100% O_2. Recordings were made using a T3 isotonic transducer (Palmer Bioscience, Kent UK) and transferred to a Washington 400MD4C four-channel recorder (Palmer Bioscience). Pure synthetic porcine neuromedin U8 (CRB Ltd, Cambridge, UK), neuromedin U25 (Bachem, Torrance USA), rat neuromedin U (IAF BioChem, Quebec, Canada) and C-terminally amidated amphibian neuromedin U (custom synthesis, IAF BioChem) were diluted in Locke solution and added to the bath in volumes of up to 0.3ml. Dose response curves were constructed for each peptide on tissue from each rat.

(ii) Rat Blood Pressure Bioassay

Male Wistar rats (220-300g) were anaesthetized with Hypnorm® (0.7 ml/kg) (Janssen Pharmaceuticals Ltd, Oxford UK) s/c and (0.7 ml/kg) Hypnovel® (Roche Products Ltd, Welwyn Garden City, UK) i.p. A heated pad was used to maintain the temperature of the rat at 37-38°C. Blood pressure was measured via a PT 400 blood pressure transducer (Palmer Bioscience) connected to the left femoral artery and recorded on a Washington 400MD4C four channel recorder. Peptides were dissolved in saline and administered via the left femoral vein as a bolus dose

of 0.1-0.2ml, flushed in with 0.3ml saline at 10 minute intervals. Dose response curves were constructed for each peptide in each rat. Dose ratios were calculated using one way analysis of variance and 95% confidence limits were derived from Fieller's theorem.

Results

In the rat uterus bioassay, amphibian neuromedin U was slightly less potent than rat and porcine neuromedin U25 although this difference was not statistically significant (Figure 8:1). Amphibian neuromedin U was however, 5.3 times more potent than porcine neuromedin U 8 with 95% confidence limits of 3.6 to 7.3 times as potent.

The results of the blood pressure assay for each peptide were qualitatively similar. Amphibian neuromedin U was slightly, but not significantly, more potent than porcine neuromedin U 8 and slightly less potent than neuromedin U25 (Figure 8:2). Amphibian neuromedin U was only 0.5 times as potent as rat neuromedin U with 95% confidence limits of 0.3 to 0.7 times as potent.

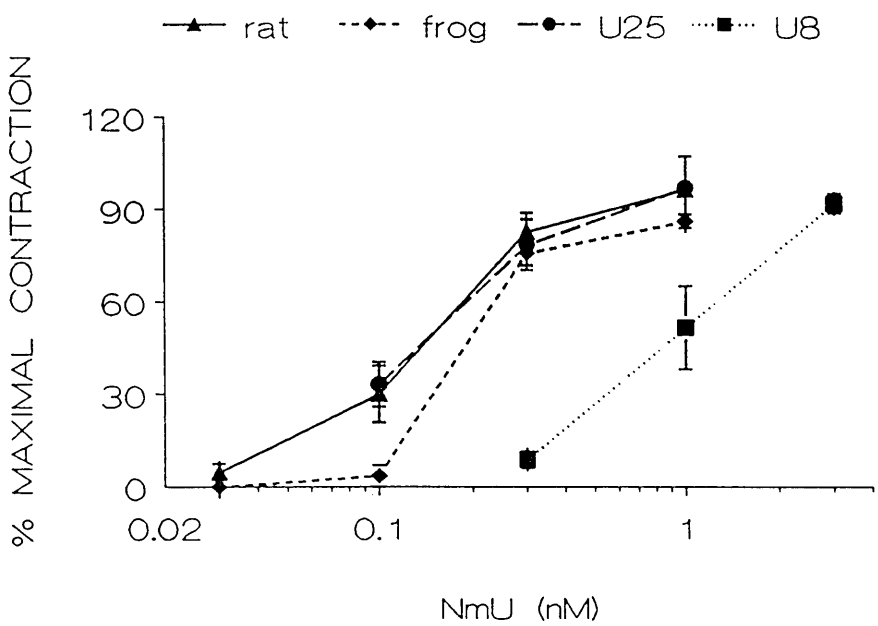


Figure 8: 1 The effect of porcine neuromedin U 8 and U25, rat and frog neuromedin U on rat uterine contraction. Symbols represent the means from at least 5 preparations and vertical bars the corresponding standard error of the mean.

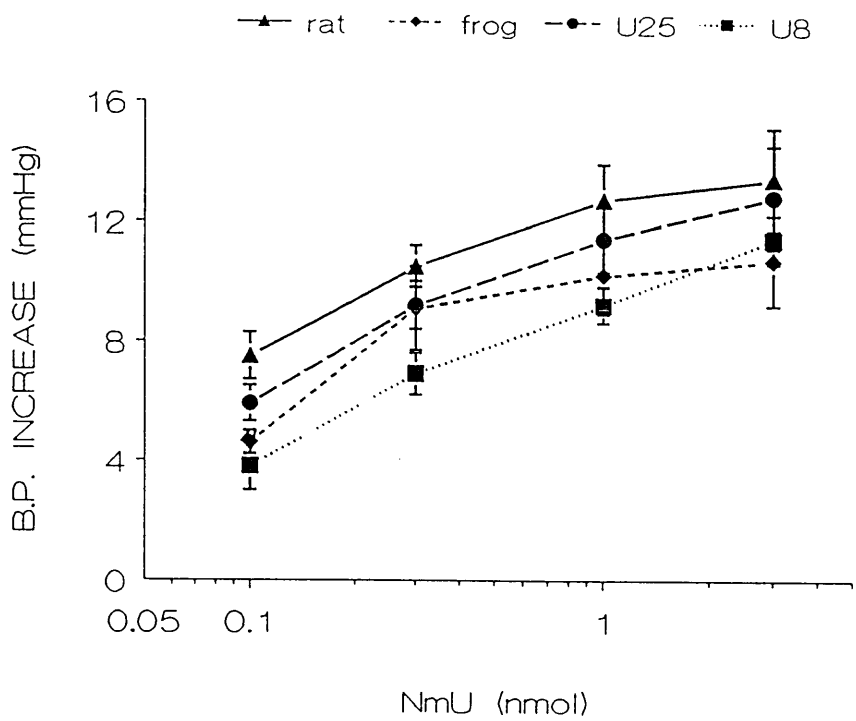


Figure 8: 2 The effect of porcine neuromedin U8 and U25, rat and frog neuromedin U on rat systemic blood pressure. Symbols represent the means from at least 5 preparations and vertical bars indicate the corresponding standard error of the mean.

Discussion

The results of this study demonstrate that rat and frog neuromedin U both show bioactivity similar to the porcine forms of this peptide in the models of uterine smooth muscle contraction and systemic blood pressure. In each preparation, frog and rat neuromedin U had a potency similar to that of neuromedin U25 and slightly greater than that of neuromedin U8. Thus, although the C-terminal region of the peptide confers biological activity, the mid to N-terminal region appears to facilitate receptor affinity.

The observed effect of rat neuromedin U upon rat uterine contraction was recently confirmed by Minamino et al (1988). His group concluded, however, that the C-terminal heptapeptide is essential for eliciting biological activity. Comparison of primary amino acid sequence reveals that frog neuromedin U differs at its C-terminal region in position 20 from the pig and rat forms (valine replacing leucine)(Figure 8:3). This structural alteration, clearly, does not preclude biological activity. The exact amino acid sequence required for biological activity is therefore, currently, unknown. An amidated form of the strictly conserved, C-terminal pentapeptide has however, consistantly failed to show any biological activity in our assay systems (data not shown). It appears that only

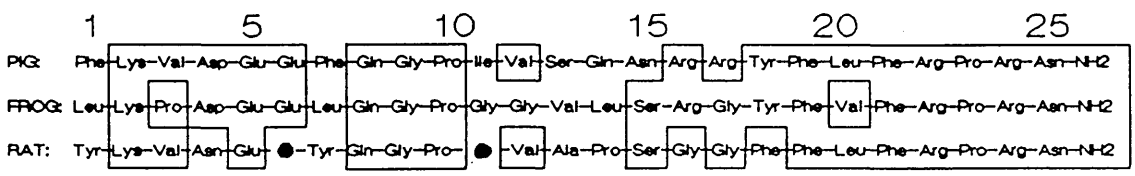


Figure 8: 3 The primary amino acid sequence of pig, rat and frog neuromedin U.

a comprehensive structure-function study will answer this question.

The cardiovascular effects of neuromedin U are particularly interesting because of the reports detailing its specificity in decreasing mesenteric blood flow (Sumi et al, 1987 and Gardiner et al, in press). Another peptide which displays marked superior mesenteric vasoconstriction is vasopressin (Bennett et al, 1988). Vasopressin however, also causes a marked reduction in hind limb blood flow and a substantial bradycardia. Despite such additional effects, vasopressin has been used in the treatment of the complications of cirrhosis (Soderlund, 1987). Thus, neuromedin U administration in man may prove useful in a clinical context since its effect is more specific, longer lasting and appears to be without side effects, at least in animal models.

B. In Vitro Studies On Neuromedin U And The Anterior Pituitary Gland

Introduction

In chapters 6 and 7 the effects of a number of endocrine manipulations upon the anterior pituitary content of immunoreactive neuromedin U were investigated. This work revealed that the anterior lobe content of neuromedin U

was altered by exogenous TRH, gonadal steroids and adrenal manipulation. Quantitation of tissue content alone is nevertheless, limiting. It is not known whether the observed changes reflect alterations in the rate of synthesis, the rate of secretion or both processes. Currently, a cDNA probe for neuromedin U is unavailable, making the study of its synthetic rate difficult. The secretion of neuromedin U from the anterior pituitary is not, however, inherently difficult to examine.

Analysis of anterior pituitary hormone secretion lends itself readily to in vitro experimentation (Levine and Ramirez, 1986). The isolation of the gland from the multitude of extrapituitary trophic substances allows manipulation of the tissue under more precisely controlled conditions than can ever be attained in vivo. Intact pituitary glands, hemipitutaries, tissue fragments and enzymically dispersed cells can all been used for such work.

In this study, two techniques are employed. The first involves the static incubation of dispersed pituitary cells together with test substances. The medium containing pituitary secretions is then harvested after a period of time. The second uses a perifusion system where test substances are introduced into a continous flow of medium around pituitary pieces. Secreted moieties are then removed and replaced by fresh medium

at a rate governed by the flow rate of the system.

The aim of this study was to examine the effect of pig and rat neuromedin U upon rat anterior pituitary hormone secretion. With such data, a possible biological role for neuromedin U could be postulated. Any effect would complement both its localization in this tissue and possibly explain the significance of specific changes in tissue content observed following endocrine manipulations. The secretion of immunoreactive neuromedin U from the anterior pituitary *in vitro* is also studied to examine the effect of membrane depolarization and acute TRH and T3 challenge.

(i) The Effect of Neuromedin U On Pituitary Hormone Secretion In Static Culture.

Materials And Methods

Pituitary Cultures

Male Wistar rats (180-220g) were killed by decapitation and the pituitary gland removed within 30 seconds. The anterior lobe was parted from the posterior and neurointermediate lobe and placed into phosphate buffered saline (PBS; 7.4 mmol/l Na_2HPO_4, 0.7 mmol/l KH_2PO_4, 0.15 mmol/l NaCl pH 7.3) and diced into approximately $1mm^3$ cubes. The tissue was incubated at

37°C with 0.125% (w/v) trypsin and 0.02% (w/v) EDTA in PBS. After 15 minutes, the tissue was centrifuged (2000g, 4min) and resuspended in fresh trypsin solution. This procedure was repeated three times, followed by two 20 minute incubations with 0.15% (w/v) collagenase in PBS. The dispersed cells were washed twice in PBS and resuspended in culture medium consisting of Earle's minimal essential medium containing 10% (v/v) heat inactivated fetal calf serum (Gibco, Paisley UK), 100 U/ml penicillin, 100 µg/ml streptomycin (Glaxo, Greenford UK) and 2.5 mg/ml fungizone (Gibco), buffered with Hepes.

Dispersed pituitary cells were suspended in culture medium and divided into multiwells (Gibco), each well containing 1ml of culture medium and 0.3-0.4 million cells. Cultures were initially incubated at 37°C for 2 days to allow for cell attachment to the well and recovery. Experiments were carried out at 37°C with randomization of cultures for each study. Triplicate cultures were used for both control and variable. The cells were washed with fresh culture medium before each experiment. The cultures were incubated for 4 hours in culture medium (1ml)(basal period), then washed and incubated for the same time period (experimental period) in fresh medium together with 100 nM neuromedin U8 (IAF Biochem), neuromedin U25 (Bachem), rat neuromedin U (IAF Biochem) or without any additive (control). Media were

stored at -20^{0}C before radioimmunoassay.

Radioimmunoassays

Concentrations of rat growth hormone (GH), thyroid stimulating hormone (TSH), adenocorticotropin (ACTH), lutenising hormone (LH) and prolactin (PRL) in the media were measured by direct antibody techniques provided by the NIAMMD, Bethesda, MD, USA. Results are expressed as the mean±SEM of the percentage of hormone secreted by cells in the experimental period compared with that produced by the same cells in the preceding basal period. The significance of the data was determined by one way ANOVA and Student's t test.

Table 8:1 The effect of porcine neuromedin U8, neuromedin U25 and rat neuromedin U upon anterior pituitary hormone secretion from dispersed cells. All results are expressed as percentage change from a period of basal secretion immediately preceeding the test period ± SEM (n=3).

	% BASAL SECRETION				
	ACTH	GH	LH	PRL	TSH
CONTROL	54±7	67±4	74±3	81±3	64±1
NmU 8	54±5	78±5	84±0.5*	78±4	76±0.5**
NmU 25	53±0.3	71±3	122±10**	72±2	92±2**
Rat NmU	56±0.5	71±6	84±0.5*	78±4	62±6

* $p<0.05$

** $p<0.001$ compared to control (Student's t-test)

Results

At a concentration of 100 nM, neuromedin U8, U25 and rat neuromedin U all stimulated LH secretion from dispersed pituitary cells in vitro (Table 8:1). At the same concentration, both forms of porcine but not rat neuromedin U stimulated the secretion of TSH from this preparation. No effects of neuromedin U were observed upon ACTH, GH or PRL secretion.

(ii) The Effect Of Hypothalamic And Pituitary Hormones Upon Neuromedin U Secretion

Materials And Methods

Male Wistar rats (150-250g)(n=8) were killed by decapitation and the pituitary glands placed into ice cooled Krebs bicarbonate buffer (NaCl 118 mmol/l, KCl 4.6 mmol/l, $MgSO_4$ 1.17 mmol/l, $CaCl_2$ 2.5 mmol/l, KH_2PO_4 1.17 mmol/l, $NaHCO_3$ 25mmol/l) containing 10 mmol/l glucose gassed with 95% O_2-5% CO_2 . Each gland was halved and the tissue from eight animals placed into a perifusion chamber as shown in Figure 8:4. The equipment consists essentially of a buffer reservoir, a perifusion chamber, a peristaltic pump and a fraction collector. The perifusion chamber was constructed from the barrel of a 2ml disposable

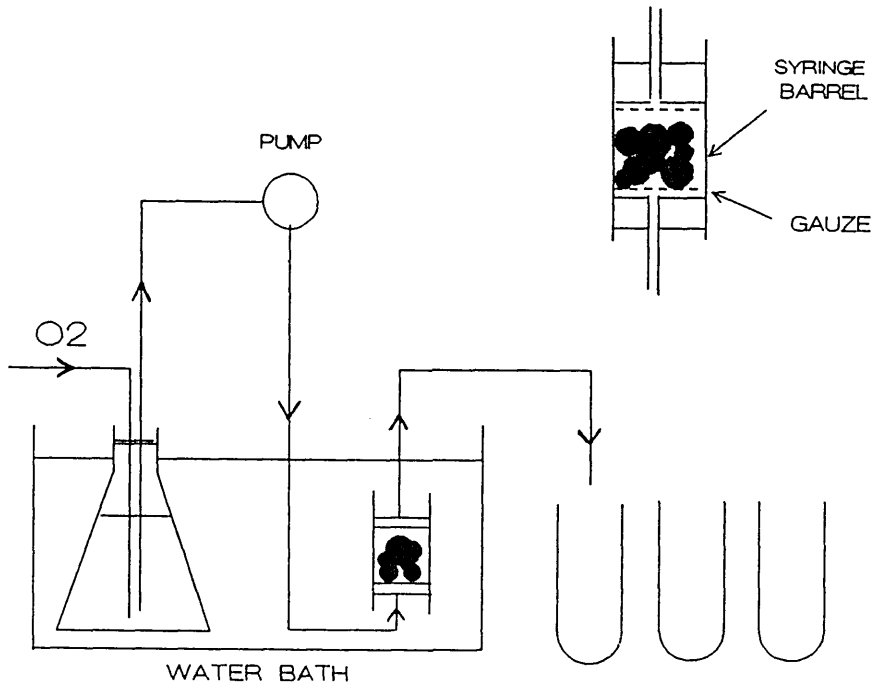


Figure 8: 4 A schematic representation of the perifusion system used in this study.

syringe. Two rubber rings made from the plungers were covered with a film of fine nylon mesh to support the tissue pieces and avoid exit of tissue fragments. A connector was then inserted in each end to allow coupling to the incoming and outgoing lines. The volume of the cell was between 0.5-1ml. The perifusion medium, Krebs bicarbonate buffer was gassed as above, and maintained at 37^{0}C as was each chamber for the duration of the experiment. The tissue was perifused in an upward manner at a flow rate of 0.3ml/min. The effluent during the first 90 minutes of the perifusion was discarded. Fractions were then collected at 3 minute intervals. Test substances were introduced for periods of 15 minutes with a recovery period of at least 60 minutes between successive challenges. When investigating the combined effect of T3 and TRH the tissue was perifused with a solution of T3 for 6 minutes prior to perifusion with a mixture of T3 and TRH for 15 minutes. Fractions (0.9ml) were collected and assayed immediately.

Where appropriate, responses were quantitated by taking seven samples immediately prior to the response and obtaining a mean basal secretion of neuromedin U. This value was then subtracted from each of seven samples obtained after the start of the TRH stimulus. The sum of these values was used as the net neuromedin U response to the respective stimulus.

Radioimmunoassay

Neuromedin U-like immunoreactivity was measured as described in chapter 2.

Results

At the start of each experimental period, the tissues produced a stable baseline secretion of neuromedin U like immunoreactivity. When the concentration of potassium ions in the perifusion medium was elevated to 56 mmol/l for a 15 minute period, a marked secretion of neuromedin U-like immunoreactivity was observed (Figure 8:5). Repeated stimulation produced an ever decreasing response. When the tissues were challenged with pulses of perifusion medium, no release of neuromedin U immunoreactivity was observed. The same preparations were however responsive to a potassium challenge (Figure 8:6).

The effect of T3 (1 μmol/l) was initially investigated but it produced no effect upon basal neuromedin U secretion (Figure 8:7). The effect of TRH (1 μmol/l) was then examined in the absence and presence of T3 (1 μmol/l). At this concentration, TRH produced an increased release of neuromedin U-like immunoreactivity

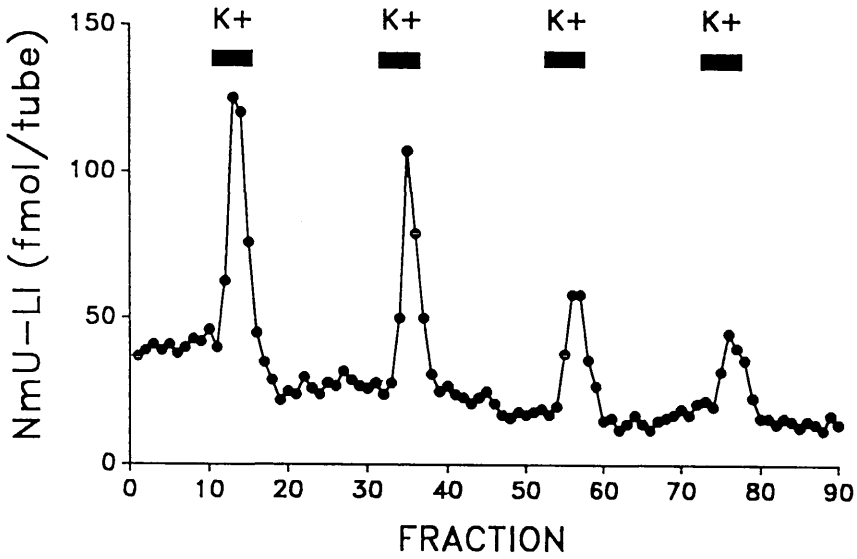


Figure 8:5 The effect of sequential pulses of potassium chloride (56 mmol/l) upon the release of neuromedin U-like immunoreactivity from perifused anterior pituitary fragments.

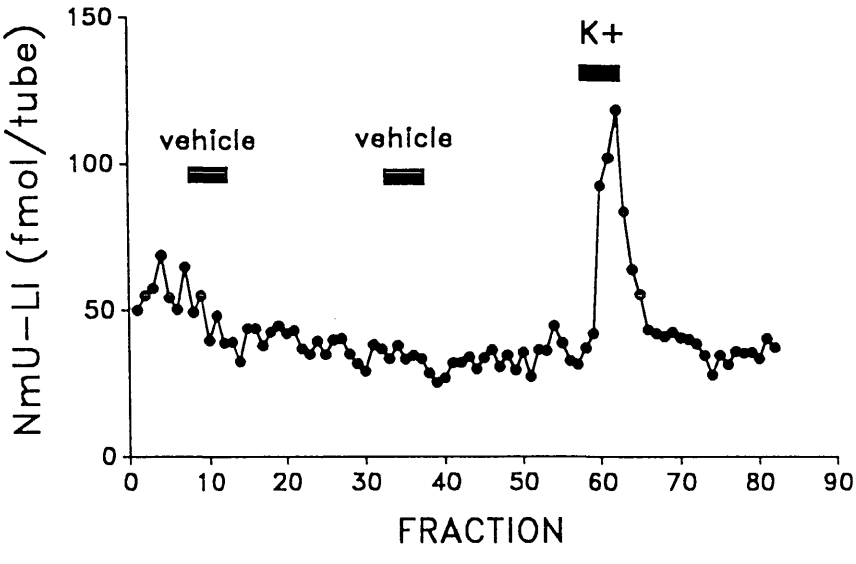


Figure 8: 6 The effect of vehicle alone and a single pulse of potassium chloride (56 mmol/l) upon the release of neuromedin U-like immunoreactivity from perifused anterior pituitary fragments.

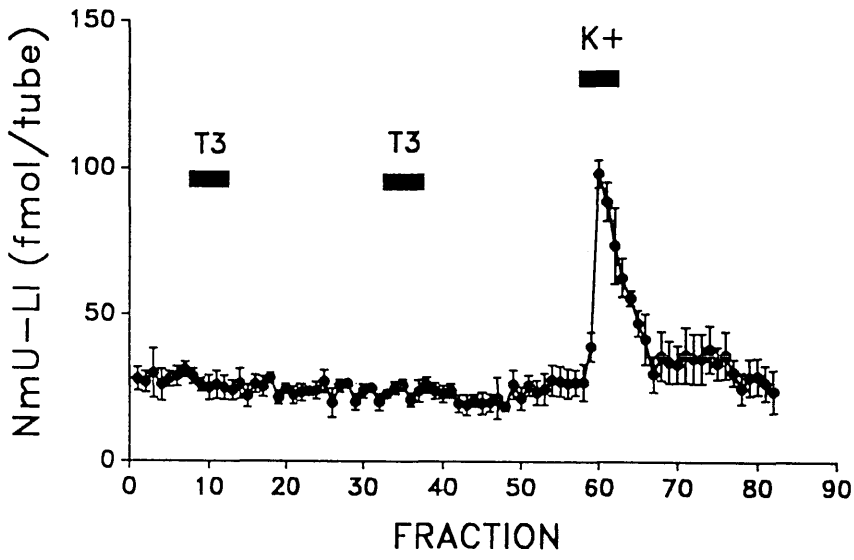


Figure 8: 7 The effect of T3 (1 μmol/l) and a single pulse of potassium chloride (56 mmol/l) upon the release of neuromedin U-like immunoreactivity from perifused anterior pituitary fragments (n=4).

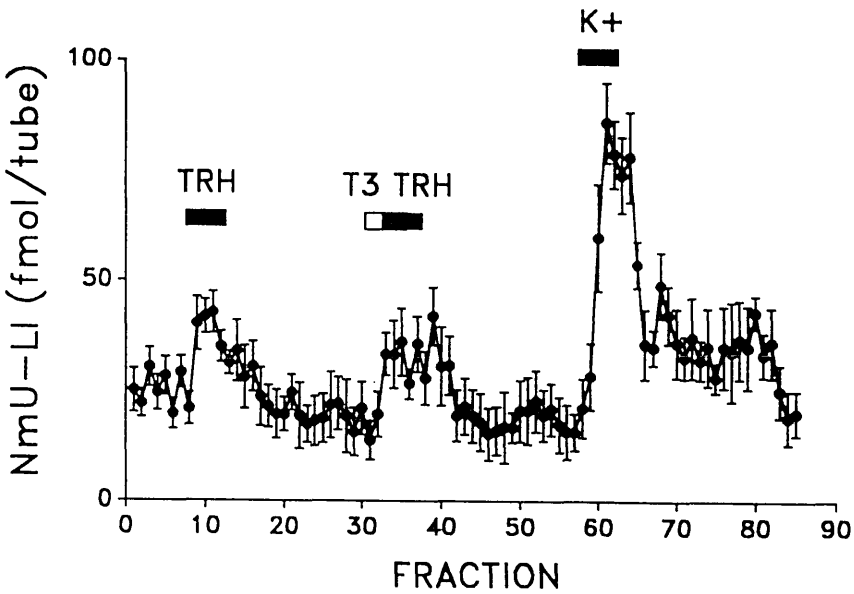


Figure 8:8 The effect of TRH (1 μmol/l), TRH (1 μmol/l) together with T3 (1 μmol/l) and a single pulse of potassium chloride (56 mmol/l) upon the release of neuromedin U-like immunoreactivity from perifused anterior pituitary fragments (n=6).

Table 8: 2 A list of substances examined for potential effects upon the secretion of neuromedin U like immunoreactivity from perifused pituitary pieces *in vitro*. All were used at a final concentration of 1μmol/l.

ACTH
Angiotensin II
Corticotropin releasing factor
Dopamine
FSH/LH
Growth hormone releasing factor
Growth hormone
LHRH
Met enkephalin
Oxytocin
TSH
Vasopressin

from this tissue. This release was independent of the presence of T3 (Figure 8:8). When the net neuromedin U secretion following each stimulus was calculated, no significant difference between the two responses was observed using a Students t test (TRH 75±5 fmol, TRH+T3 91±22 fmol mean ± SEM).

The effects of a range of other hypothalamic releasing hormones and pituitary hormones were also examined (Table 8:2). None were found to elicit a detectable effect upon the secretion of neuromedin U-like immunoreactivity from the anterior pituitary.

General Discussion

The results of this study show that neuromedin U stimulates LH and TSH secretion from dispersed anterior pituitary cells *in vitro*. Furthermore, its own secretion from this tissue is stimulated by membrane depolarization and TRH.

The experiments shown above are however incomplete and the results should therefore be treated with certain caution. Complementary dose-response data and the effects of neuromedin U in combination with the appropriate hypothalamic releasing factors ie LHRH must also be studied. The data is nevertheless representative. The results of this work are however, of

great importance to the findings presented in chapters 4,6 and 7. It was previously unknown what the effects of neuromedin U upon pituitary hormone secretion were, if indeed there were any. These studies reveal that neuromedin U can be secreted from the anterior pituitary thereby supporting the hypothesis that neuromedin U has an autocrine/paracrine role within the pituitary.

Despite the conceptual advantages such in vitro studies have, removal of the pitutary gland from its normal vascular perfusion, incurs practical limitations in maintaining a viable preparation. Although tissue fragments maintain a degree of morphological integrity, necrosis becomes a problem with time. Enzymatic dispersion avoids this problem, but cellular function and morphology is then altered. In addition, damage of the cell surface and most importantly, cell surface receptors may also occur. To exemplify this point, no neuromedin U immunoreactivity could be detected in a trypsinised preparation of anterior pituitary cells even after a 24hr refractory period (data not shown).

In perifusion experiments, consistency of response is also a problem. Thus pre-equilibration of the tissue is usually extended until a stable basal release has been attained. Basal release can however be the subject of experimental artefacts since unavoidable leakage of stored content may occur as a consequence of tissue

erosion caused by the circulation of perifusion medium. Peak resolution may also be limited by dead spaces in the perifusion system, diffusion in the tubing and mixing in the pump. Although such problems can be minimised, they are rarely eradicated.

In conclusion, these studies have revealed that neuromedin U stimulates the secretion of LH and TSH from dispersed anterior pituitary cells in vitro. Furthermore, its own secretion from the pituitary can be stimulated by tissue depolarization and exogenous TRH. These studies are consistent with the hypothesis that neuromedin U is a putative paracrine/autocrine regulator of pituitary hormone secretion. Further studies are now required to determine the exact physiological role of neuromedin U in this tissue and explain the significance of the changes in its content following specific endocrine manipulations.

CHAPTER 9

CONCLUSIONS

When porcine neuromedin U was characterized in 1985, the regional distribution of this peptide was unknown. Knowledge of its biological activity was restricted to the contraction of rat uterine smooth muscle and its ability to exert pressor effects when administered *in vivo*. The simultaneous purification of two molecular forms indicated that both occurred naturally.

Some other recently isolated peptides have been shown to have sequence similarity with known peptides eg. PHI/PHM with VIP, neuromedin B with bombesin and neuromedins K and L with substance P. Such similarity has provided obvious avenues of research into the comparative distribution and physiological function of these novel peptides. Neuromedin U, however, despite its misleading name, does not appear to belong to any established peptide family.

The development of a radioimmunoassay for neuromedin U provided no problems. A specific antiserum for this purpose was raised within the course of only a few

months. The production of antisera towards neuromedin U should therefore not prove to be a problem for other investigators hoping to develop their own assay system. The extraction of neuromedin U from rat gut was found to be optimal when the samples were boiled in aqueous acetic or hydrochloric acid. Thereafter, all tissues were routinely extracted in aqueous acetic acid.

Neuromedin U-like immunoreactivity was detected in several species ranging from man to frog. Chromatographic characterization of tissue extracts indicated that the peptide was predominantly present in a molecular form similar to porcine neuromedin U25 (with the exception of guinea pig). In porcine tissue, no immunoreactivity corresponding to neuromedin U8 could be detected. This implies that neuromedin U8, dispite being biologically active, does not exist in vivo and that it represents an artefactual metabolite of neuromedin U25.

Although inter-species molecular heterogeneity was evident, the amino acid sequence corresponding to neuromedin U8 was known to be conserved to some degree. Without this, antibody cross-reaction and subsequent detection could not occur across species.

The distribution of neuromedin U-like peptides was found to extend beyond the spinal cord and central nervous system. High concentrations of immunoreactivity were

found in the gastrointestinal tract and in the pituitary gland. Lower levels of were also found in the rat thyroid gland and urogenital tract. The brain-gut distribution of this peptide was subsequently confirmed by other groups using both radioimmunoassay and immunohistochemistry.

Recent studies reveal that neuromedin U may play an important role in the regulation of gastrointestinal function since it has been found to selectively reduce mesenteric blood flow in both dog and rat. If this effect can be reproduced in man, neuromedin U or its analogues, may find a use in a clinical setting. One immediate application would be in the treatment of gastrointestinal haemorrage. Furthermore, neuromedin U8 has been found to alter ion flux across the gut wall.

Since the majority of studies investigating the biological acitivity of this peptide were being performed on rat tissue or in the rat, the characterization of rat neuromedin U was a priority. This peptide was purified from extracts of gastrointestinal tract and found to be 23 amino acid residues long, 15 of which were identical to neuromedin U25. High concentrations of neuromedin U immunoreactivity in tissues from the common frog, Rana temporaria also allowed the purification of neuromedin U from this species. Frog neuromedin U is 25 residues in length, and, somewhat suprisingly, has a slightly higher degree of sequence similarity with porcine neuromedin U25

than rat neuromedin U. Both rat and frog neuromedin U contract uterine smooth muscle and exert pressor effects in the rat in a manner similar to neuromedin U25. The sequence of human neuromedin U however, remains elusive.

Immunocytochemical studies confirmed the presence of neuromedin U immunoreactivity in the pituitary gland and showed it to be colocalized with ACTH. The results of rat endocrine manipulations presented in chapters 6 and 7 show that the anterior lobe content of neuromedin U is decreased by gonadal steroids and dexamethasone and increased by TRH and adrenalectomy. Whilst the changes following manipulation of adrenal state could have been anticipated on the basis of the proposed co-localization with ACTH, those evoked by TRH, estrogen and testosterone could not. Furthermore, the effect of chronic TRH treatment has been clearly shown to be dependent upon the circulating levels of thyroid hormone and the effect of adrenalectomy upon intact gonads in male rats.

In chapter 8, the effect of neuromedin U upon pituitary hormone secretion was consequently investigated. The results, although preliminary, indicate that this peptide may be a novel modulator of both LH and TSH secretion. Studies involving the perifusion of pituitary pieces reveal that membrane depolarization with potassium chloride (56 mmol/l) leads to a transient secretion of neuromedin U immunoreactivity from this tissue.

Furthermore, acute challenge with TRH also stimulated its secretion. This effect was independent of the presence of T3. Such results complement those of endocrine manipulation and because of the observed specificity, indicate that neuromedin U is a novel candidate involved in the autocrine/paracrine control of anterior pituitary hormone secretion.

Currently, physiological studies involving neuromedin U lag behind biochemical ones. The future will, no doubt, reveal new biological roles for neuromedin U, determine the elusive sequence of human neuromedin U and perhaps identify the significance of this peptide in human health and disease.

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APPENDIX

All reagents used in this thesis were of Analar grade

A. BUFFERS

1. 50 mM Acetate Buffer (pH=5.0)

Dissolve 4.1g sodium acetate in 1l of distilled water.

2. 60 mM Phosphate Buffer (pH=7.4)

To make 5l:-

48.00g $Na_2HPO_4.2H_2O$

4.13g KH_2PO_4

18.61g $Na_2H_2(EDTA).2H_2O$ (Ethylenediaminetetraacetic acid disodium salt).

2.5g NaN_3

Preboil 5l of distilled water. If gelatine is to be added, dissolve 12.5g of gelatine in this just boiled water by leaving it to stir for 15 minutes. When cool, add and dissolve the other constituents. This buffer is 60 mM in phosphate, 136 mM in sodium, 6mM in potassium, 10 mM in EDTA and 77mM in azide with an approximate ionic strength of 0.23.

3. 50mM Veronal Buffer (pH=8.0)

To make 5l:-

51.55g Barbitone sodium

20 ml 5M HCl

2.5g NaN_3

Pre-boil 5l of distilled water. When cool, dissolve the barbitone sodium completely before adding the acid. Allow the acid to disperse well and then add the azide.

B. FORMULAE FOR CALCULATIONS

1. Scatchard Plot (Scatchard, 1949)

A typical standard curve is recalculated in terms of the total peptide bound and free. In this calculation, the total peptide bound is calculated by taking the percent bound and multiplying by the total amount of peptide added as standard plus the amount added as label. The percent bound/free is plotted on the Y-axis against the total peptide bound along the X-axis. The intercept on the Y-axis gives K x N, where K is the affinity constant of the antibody (l/mol) and N is the total number of binding sites (mol/l). The value of N is obtained from the intercept on the X-axis.