THE CALCITONIN GENE PEPTIDE FAMILY

IN HEALTH AND MALIGNANT DISEASE

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to the UNIVERSITY OF LONDON

for the degree of

DOCTOR OF PHILOSOPHY

1988

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London

ABSTRACT

The α-calcitonin gene encodes the precursors of three circulating peptides: calcitonin, katacalcin and calcitonin gene-related peptide (α-CGRP), whilst the β-calcitonin/CGRP gene is known to encode β-CGRP. This thesis describes a study of the expression of calcitonin and its related peptides in healthy volunteers and cancer patients.

To investigate the secretion of the calcitonin gene peptides in both normal and neoplastic conditions, sensitive and specific immunoassays were required. Improvements were made to the established radioimmunoassays (RIAs) for calcitonin and katacalcin. A calcitonin amplified enzyme immunoassay was developed as an alternative to the RIA and a sensitive RIA for human CGRP was also developed. The RIAs were then used to study diurnal changes in calcitonin and CGRP as well as the effects of age and sex on plasma levels of these peptides.

Using the information on calcitonin gene peptides in healthy individuals as a baseline the immunoassays were used to investigate patients with either familial or sporadic medullary thyroid carcinoma, MTC (a tumour of the calcitonin secreting thyroidal C-cells). Calcitonin and katacalcin were observed to circulate in approximately equimolar amounts confirming both these peptides to be useful tumour markers for MTC. CGRP was elevated in only 50% of patients with MTC however CGRP levels were significantly different between the familial and sporadic forms of this disease.

Circulating calcitonin gene peptides were elevated in patients with non thyroid malignancies, including carcinoids and phaeochromocytoma, but were most frequently observed in breast and lung cancer. Studies on patients with haematological disorders revealed that abnormal levels of calcitonin gene peptides were frequently present in myeloid leukaemia and myeloproliferative disorders. However calcitonin was only occasionally present in the plasma of patients with lymphoma and lymphoid leukaemia, whereas CGRP levels were not elevated in patients with lymphoid malignancies.

To investigate the cellular expression of the calcitonin/CGRP genes RNA extraction and northern blotting were used to study messenger RNA (mRNA) in familial and sporadic MTC. The ratios of calcitonin to CGRP mRNA varied between tumours but no difference was apparent between the familial and sporadic MTC tumours.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor Ian MacIntyre and Dr Carrie Hillyard for their guidance during my studies. I am also grateful to several other members of the Endocrine Unit for their contributions including David MacDonald, Colin Self and John Stevenson, as well as my colleagues Peter Bevis, Tricia Hill and Rashmi Seth. I would particularly like to thank Samia Girgis and Steve Legon for their invaluable help and advice.

I am additionally grateful to David Bates (IQ(Bio)) for preparation of the calcitonin-alkaline phosphatase conjugate, Peter Bevis for the conjugation of CGRP and immunisation of rabbits, Professor Howard Morris and colleagues for CGRP fragments and Maria Alevezaki for calcitonin gene probes. I would also like to thank Drs Goldman and McKenzie (Hammersmith Hospital) and Dr McCarthy (Westminster Hospital) for patient samples.

My thanks are also due to Cathy Jones for the expert preparation of this manuscript and to Anthony Lynch for much of the artwork. Finally, I would like to thank Richard and my parents for their continued encouragement.

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ABBREVIATIONS

AEIA	Amplified enzyme immunoassay
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
MTC	Medullary thyroid carcinoma
RIA	Radio immunossay
mRNA	Messenger ribonucleic acid
HPLC	High performance liquid chromatography
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
ADH	Alcohol dehydrogenase
CT	Calcitonin
KC	Katacalcin
PTH	Parathyroid hormone
SEM	Standard error of the mean
BSA	Bovine serum albumin
HSA	Human serum albumin

CHAPTER 1 LITERATURE REVIEW OF THE CALCITONIN GENE PEPTIDES

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1.1 INTRODUCTION

The α-calcitonin gene has been found to encode the peptides calcitonin, katacalcin and calcitonin gene-related peptide (CGRP), whilst a second gene has recently been discovered which also encodes CGRP. This is known as the β-calcitonin/CGRP gene. This thesis describes a study of the expression of calcitonin and its related gene peptides in health and malignant disease.

This study had three main objectives. The first was to develop sensitive and specific immunoassays capable of measuring peptide levels in normal individuals. The second was to apply these assays to the measurement of circulating calcitonin, katacalcin and CGRP in healthyvolunteers and cancer patients. The third was to investigate the expression of calcitonin and CGRP mRNA in tumour tissue using RNA extraction and Northern blotting techniques.

The thesis starts with a review of the discovery and known biological actions of calcitonin and the related gene peptides, katacalcin and CGRP. Chapter 2 describes how an amplified enzyme immunoassay was developed for calcitonin and compared with an improved calcitonin radioimmunoassay (RIA). Improvements were also made to the katacalcin RIA and a new assay was developed for human CGRP.

These immunoassays were subsequently applied to the direct plasma measurement of calcitonin, katacalcin and CGRP in healthy volunteers and cancer patients in order to discern the extend of calcitonin gene peptide production in malignant disease and to discover useful tumour markers. This work is described in Chapters 3 to 6. The diseases studied include medullary thyroid carcinoma (a tumour of the calcitonin producing thyroidal c-cells) and non-thyroid tumours of both endocrine and non-endocrine tissues.

Molecular biology techniques were used to investigate the expression of calcitonin and CGRP mRNA in medullary thyroid carcinoma. Tissue from patients with both familial and sporadic forms of this disease was studied together with cells from the squamous bronchial carcinoma cell line 'BEN'. This last phase of the study is described in Chapter 7 and an overall discussion and conclusions are presented in Chapter 8.

1.2 CALCITONIN

1.2.1 Discovery

In the early 1960s the existence of a second calcium regulating hormone was suggested by Copp and his colleagues (1962). They observed a rapid fall in plasma calcium when perfusing the isolated thyro-parathyroid glands of dogs with high calcium blood. This fall was greater than that produced by total thyroparathyroidectomy and led Copp to recommend that the factor responsible for the hypocalcaemic effects seen in his experiments be called "calcitonin". He also postulated the parathyroid gland to be the site of calcitonin release.

Support for the existence of calcitonin came from further perfusion experiments on dogs at the Hammersmith Hospital (Kumar et al 1963), whilst Hirsch et al (1963) described a thyroid hypocalcaemic factor (thyrocalcitonin) present in rat thyroid extracts. In the following year experiments conducted by the Hammersmith Group on goats (Foster, Baghdiantz et al 1964) showed that calcitonin was indeed of thyroid rather than parathyroid orgin and that thyrocalcitonin and calcitonin were probably identical.

Work by Foster, MacIntyre and Pearse (1964) strongly indicated that the mitochondrial rich cells of the thyroid (parafollicular or c-cells), described by Nonidez in 1932, were responsible for calcitonin secretion. Immunofluorescent antibody techniques were used by Bussolati and Pearse (1967) to confirm the production of calcitonin by these cells in both pig and dog.

Drawing together several cytological observations on endocrine cells, Pearse (1968) proposed his theory that cells producing polypeptide hormones were a related series, all possessing similar properties. The most common cytochemical properties of these cells were their content of endogenous amines, the uptake of amino acid precursors of dopamine and 5-hydroxy tryptamine (5HT) and their decarboxylation. The acronym APUD (amine precursor uptake and decarboxylation) is now used to describe this series of cells which includes c-cells, adrenal medulla cells and pituitary cells.

APUD cells were initially thought to be of neuroectodermal origin and although this has been proven for c-cells (LeDouarin and LeLievre (1972)) many of the other APUD cells may not have a similar origin. Human calcitonin was first isolated from a medullary thyroid carcinoma (MTC), a tumour of the thyroidal c-cells. Sequencing of the pure calcitonin (Byfield et al 1968, Neher et al 1968) has shown the hormone to be a 32 amino acid peptide with a carboxyl terminal prolinamide and a 1-7 disulphide bridge between the cysteine residues in these positions.

1.2.2 Calcitonin Secretion

The stimulatory effect of high plasma calcium levels on calcitonin secretion facilitated its discovery (MacIntyre 1968). Various other cations also stimulate calcitonin secretion, but not at physiological doses (Cooper 1975). Gut hormones, including gastrin and the analogue pentagastrin, have been noted to stimulate calcitonin secretion (Heath and Sizemore 1977, Heynen et al 1981). Also several steroids may induce calcitonin secretion such as Vitamin D (Raue et al 1983), estradiol and progesterone (Greenberg et al 1986). In addition a negative feedback control on calcitonin secretion has been suggested (Orme and Pento 1976).

1.2.3 Actions of Calcitonin

Calcitonin produces its hypocalcaemic actions on bone by diminishing bone resorption (Friedman and Raisz 1965) probably by inhibiting osteoclast activity (Chambers and Moore 1983). The overall effect of this action is to reduce the circulating levels of the products of bone resorption, ie. calcium, phosphate and hydroxyproline containing peptides (Stevenson and Evans 1981). Calcitonin produces marked lowering of plasma calcium in conditions where the rate of bone turnover is high such as that found in Paget's disease, children and young animals. In adults, where bone turnover is slow, even a complete inhibition of bone resorption will produce little change in plasma calcium. However an escape phenomenon exists in healthy bone cells exposed to chronic calcitonin (Ziegler et al 1984).

In addition to its actions on bone calcitonin has several pharmacological actions of unknown physiological significance. Calcitonin has been shown to act on the kidney to enhance conversion of 25 hydroxy vitamin D to 1,25 hydroxy vitamin D (Galante et al 1972). Increased excretion of calcium, sodium, potassium and phosphate by the kidney has also been noted in response to calcitonin (Haas et al 1971, Emmertsen 1985). The actions of calcitonin on both bone and kidney are probably mediated by cAMP (Marx et al 1972).

The secretion of gastric acid, insulin, gastrin, pepsin and pancreatic enzymes are all inhibited by calcitonin (Ziegler et al 1984, Stevenson 1980). Furthermore calcitonin has been found to inhibit feeding (Freed et al 1979, Twery et al 1982) and calcium absorption in the intestine (Swaminathan et al 1974). Calcitonin may also exert an effect on pituitary hormone secretion, reducing the secretion of thyroid stimulating hormone, luteinising hormone and growth hormone but increasing prolactin secretion (Stevenson and Evans 1981, Lengyel and Tannenbaum 1987). Analgesic properties have also been attributed to calcitonin (Fraioli et al 1982).

The major function of calcitonin however, is probably in the maintenance of the skeleton, particularly in times of physiological need, e.g. in the foetus, neonate and growing child (Klein et al 1984) and in pregnant and lactating mothers (Stevenson et al 1979).

1.2.4 Evolution of Calcitonin

Calcitonin immunoreactivity has been detected in several representatives of the Phylum Chordata including the lower chordates; urochordata (tunicates) and cephalochordata (amphioxus), as well as vertebrates. Immunohistochemical and immunofluorescent techniques have shown human calcitonin immunoreactivity in the gut (Fritsch et al 1980) and the pharynx (Thorndyke and Probert 1979) of tunicates.

Specific radioimmunoassays for immunoreactive human and salmon calcitonin (ihCT and isCT) have been used to screen column fractions following HPLC and gel chromatography of various tissue extracts. Human calcitonin immunoreactivity (but not sCT) was observed in the nervous system of several tunicates, amphioxus and in the jawless

vertebrate, hagfish (Girgis et al 1980). Following the evolutionary development of the ultimobronchial gland (UBG) in cartilaginous fish (chondrichthyes) both hCT and sCT have been localised to this gland in many subsequently evolving vertebrates suggesting the existence of 2 calcitonin genes. Eel UBG has been shown to contain both ihCT and isCT whilst studies on amphibians have also shown isCT and/or ihCT in the UBGs (Perez Cano et al 1981, 1982a). In reptiles isCT has been localised to the UBG, brain and parathyroids (Galan-Galan et al 1981, Boudbid et al 1987) but in birds both ihCT and isCT are present (Perez Cano et al 1982b). The thyroid developed as the main source of calcitonin in mammals but ihCT and isCT have also been found in human brain (Fischer et al 1983).

These comparative studies suggest that calcitonin is a highly conserved peptide and that gene duplication probably took place early in the evolution of the vertebrates. An alignment of the amino acid sequences of a number of species is illustrated in Figure 1.1. The calcitonins appear to fit into 3 groups: primate-rodent, artiodactyl and teleost. Whilst there is considerable species variation in amino acid sequence, there are 9 invariant residues with the principle sequence homology being at the amino-terminal.

	Human α	Rat	Sa.2	Sa.3	Sa.1	Eel	Chick.	Porc.	Bov.	Ov.	Human β*
	H										
1	Cys										Tyr
2	Gly		Ser	Ser	Ser	Ser	Ala	Ser	Ser	Ser	Ser
3	Asn						Ser				
4	Leu										
5	Ser										
6	Thr										
7	Cys										
8	Met			Val	Val	Val	Val	Val	Val	Val	Leu
9	Leu										Gln
10	Gly							Ser	Ser	Ser	
11	Thr		Lys	Lys	Lys	Lys	Lys	Ala	Ala	Ala	
12	Tyr		Leu	Leu	Leu	Leu	Leu				
13	Thr		Ser	Ser	Ser	Ser	Ser	Trp	Trp	Trp	Leu
14	Gln							Arg	Lys	Lys	
15	Asp				Glu	Glu	Glu	Asn			Tyr
16	Phe	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
17	Asn		His	His	His	His	His				Lys
18	Lys							Asn	Asn	Asn	Asn
19	Phe		Leu	Leu	Leu	Leu	Leu		Tyr	Tyr	
20	His		Gln	Gln	Gln	Gln	Gln				
21	Thr							Arg	Arg	Arg	Met
22	Phe				Tyr	Tyr	Tyr			Tyr	
23	Pro						Ser	Ser	Ser		
24	Gln		Arg	Arg	Arg	Arg	Arg	Gly	Gly	Gly	Gly
25	Thr							Met	Met	Met	Ile
26	Ala	Ser	Asn	Asn	Asn	Asp	Asp	Gly	Gly	Gly	Asn
27	Ile		Thr	Thr	Thr	Val	Val	Phe	Phe	Phe	Phe
28	Gly										
29	Val		Ala	Ala	Ser	Ala	Ala	Pro	Pro	Pro	Pro
30	Gly						Glu	Glu	Glu	Glu	Gln
31	Ala		Val	Val	Thr	Thr	Thr	Thr	Thr	Thr	Ile
32	Pro										
	NH_2										

*Predicted from β EXON 4

Figure 1.1 Amino acid sequences of the calcitonins
(α=from the α-calcitonin gene, β=from the β-calcitonin/CGRP gene, Sa.=salmon)

1.3 KATACALCIN

1.3.1 Discovery

Investigations into the biosynthesis and secretion of calcitonin by c-cells suggested the existence of a high molecular weight calcitonin precursor (Roos et al 1974). Recombinant DNA techniques were then applied to the analysis of rat calcitonin messenger RNA (mRNA). In addition to calcitonin, the precursor mRNA was found to encode a 16 amino acid peptide at the carboxyl terminal of the precursor and an amino terminal peptide (Amara et al 1982a). Birnbaum and colleagues (1982) went on to report the presence of c-terminal peptide immunoreactivity in extracts of normal rat thyroid and MTC tissue.

The isolation of mRNA from human MTC tissue and the subsequent production of calcitonin precursor complimentary DNA (cDNA) allowed partial nucleotide sequencing of the precursor mRNA and identification of the encoded peptides (Craig et al 1982). This work indicated that in humans the c-terminal peptide, PDN-21, consisted of 21 rather than 16 amino acids. The divergence in amino acid sequences between species suggests that the c-terminal peptide has little biological importance. PDN-21 immunoreactivity has been demonstrated in extracted MTC and non-neoplastic thyroid (Roos et al 1983) as well as in the plasma of MTC patients (Hillyard et al 1983).

1.3.2 Action of Katacalcin

The hypocalcaemic actions produced by calcitonin in young animals have been used as the basis for the calcitonin bioassay described by Kumar and colleagues (1965). Using an adaptation of this rat bioassay the presence of calcitonin was demonstrated in plasma and tumour from an MTC patient (Cunliffe et al 1968) and later calcium lowering activity was observed in extracted normal plasma (Gudmundsson et al 1969). Following the discovery of PDN-21 the calcitonin bioassay was used to determine whether any hypocalcaemic bioactivity could be ascribed to this peptide. Initial results suggested that synthetic PDN-21 had hypocalcaemic actions in 50 g rats as well as inhibiting

calcium release from cultured mouse calvariae (MacIntyre et al 1982). The hormone was renamed katacalcin (KC) following these observations, however subsequent studies were unable to confirm these findings (MacIntyre et al 1984; Roos et al 1986) and no further actions of katacalcin have yet been described.

Figure 1.2 shows the position of katacalcin (KC, PDN-21) attached to the carboxyl end of calcitonin within the initial translation product of calcitonin mRNA, preprocalcitonin.

1.4 CALCITONIN GENE RELATED PEPTIDE

1.4.1 Discovery

Studies into the nature of the calcitonin gene progressed further following observations on a serially transplanted rat MTC. Calcitonin production by this tumour switched from high to low calcitonin output, associated with the production of a different cytoplasmic mRNA which is chromosomally linked to calcitonin sequences (Rosenfeld et al 1982). Sequencing of cDNA to the variant mRNA indicated the existence of a primary translation product of 128 amino acids (Amara et al 1982b). This alternative precursor could be processed further to give an N-terminal peptide identical to that of the calcitonin precursor as well as a putative 37 amino acid peptide, designated calcitonin gene-related peptide (CGRP).

Investigations by Rosenfeld et al (1983) indicated that expression of the calcitonin gene was tissue specific, with calcitonin being the main product in thyroidal c-cells and CGRP the major product in neural tissue. Amara and colleagues (1984) determined that although the same transcription initiation site was common to both mRNAs they had individual polyadenylation sites. The choice of a specific polyadenylation site, associated with differential splicing to produce a particular mRNA, may require a regulatory factor (Leff et al 1987).

The existence of CGRP in man was confirmed by the isolation and sequencing of CGRP from human MTC tissue (Morris et al 1984). Human CGRP (hCGRP) was also found to be a 37 amino acid peptide with a disulphide bridge between cysteine residues at positions 2 and 7, but with 4 amino acid substitutions compared with rat CGRP.

Calcitonin RNA processing events in man are similar to those in the rat (Nelkin et al 1984). Primary transcription of the calcitonin gene is followed by RNA processing to remove intervening sequences and generate one of two possible mature RNAs. Transcripts of exons 1, 2, 3 and 4 are found in the mature calcitonin mRNA whereas exons 1, 2, 3, 5 and 6 are represented in CGRP mRNA. Translation and proteolytic cleavage of the precursor peptides gives rise to an N-terminal peptide and either calcitonin and PDN-21 or CGRP and a tetrapeptide (Figure 1.2).

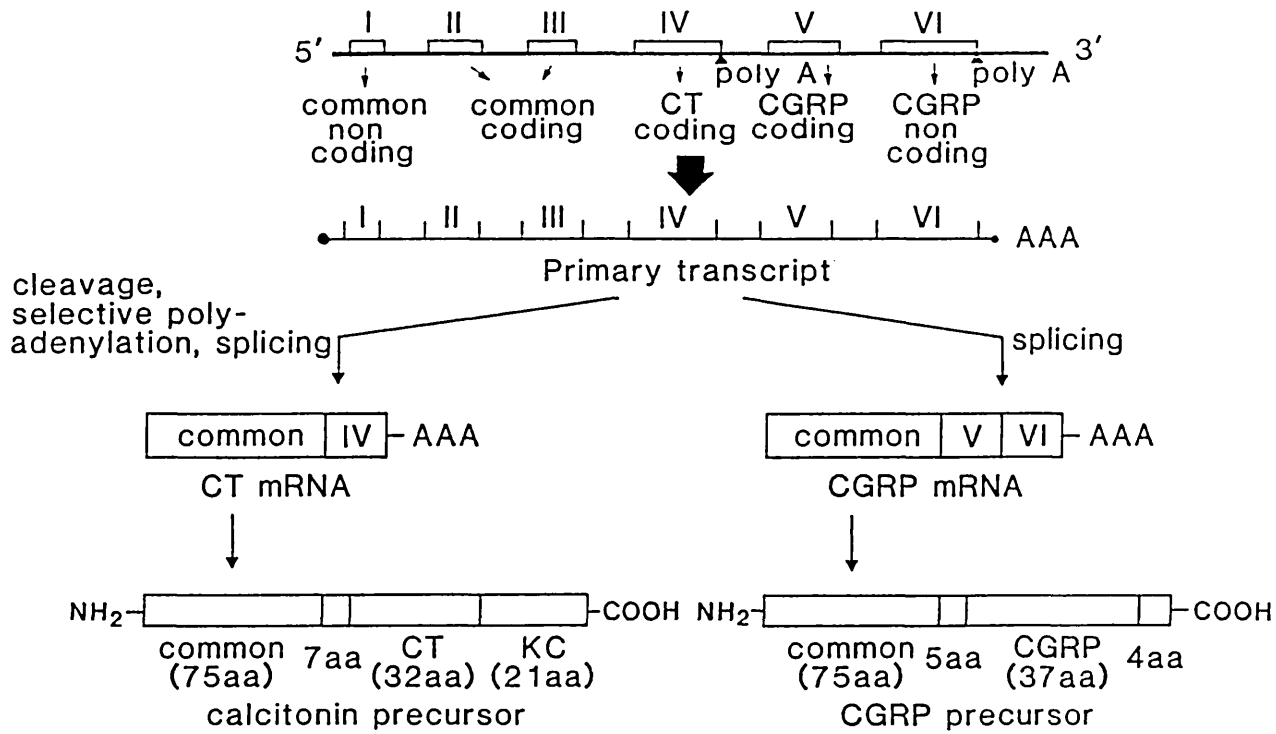

Figure 1.2 Processing of the α-calcitonin/CGRP gene (human)

Screening of cDNA libraries from both human (Steenbergh et al 1985) and rat MTC tissue (Amara et al 1985) with CGRP specific probes resulted in the discovery of a gene encoding a second CGRP (β-CGRP). A difference of only one amino acid was found in the β-CGRP sequence compared to CGRP from the rat calcitonin gene (α-CGRP). Human β-CGRP differed from α-CGRP by 3 amino acid substitutions. Alevizaki and colleagues (1986) in this laboratory have recently isolated and sequenced a region of the β-CGRP gene which has 67% homology to the exon 4 region of the alpha calcitonin gene. Whilst a beta preprocalcitonin is unlikely to be expressed there is a calcitonin like region with considerable homology to mammalian calcitonins.

1.4.2 Distribution of CGRP

In keeping with the theory of tissue specific processing of the calcitonin gene (Rosenfeld et al 1983) several studies have localised CGRP to the nervous system. Immunoreactive CGRP fibres have been found in the trigeminal ganglion, amygdala, parabrachial area and various other brain regions (Kawai 1985, Shimada et al 1985 and McCulloch et al 1986). CGRP immunoreactivity has also been observed in the spinal cord (Tschopp et al 1985) and is widely distributed throughout the peripheral nervous system (Rosenfeld et al 1983, Terenghi et al 1985, Saito et al 1986) including nerve fibres associated with blood vessels, heart, lung, gastrointestinal tract and eyes. CGRP has also been located in several endocrine tissues including adrenal medulla, thyroid, pituitary and pancreas (Rosenfeld et al 1983, Tschopp et al 1985, Sternini and Brecha 1986).

1.4.3 Actions of CGRP

A complete picture of the physiological role of CGRP has not yet been obtained. In addition to neuromodulator activities CGRP is also a potent vasodilator when administered locally to both cerebrovascular and peripheral blood vessels (Brain et al 1985, 1986; McCulloch et al 1986). CGRP also appears to be involved in inflammatory oedema (Brain and Williams 1985, Piotrowski and Foreman 1986) and to produce cardiovascular effects of decreased blood pressure and increased heart rate (Fisher et al 1983, Saito et al 1986). Certain calcitonin like actions have also been attributed to CGRP such as inhibition of gastric acid secretion (Tache et al 1984, Lenz et al 1986) and a minor hypocalcaemic action in the rat bioassay (Zaidi, Bevis, Lynch et al unpublished).

1.4.4 Evolution of CGRP

The existence of CGRP in the calcitonin gene and the discovery of β-CGRP have posed further questions about the evolution of the calcitonin gene. Bearing in mind the localisation of calcitonin in

protochordate neural tissue and of CGRP in the CNS it is possible that a partial duplication of an ancestral gene occurred. The resulting gene contains CGRP, which maintains the original neuromodulator function, and calcitonin which has developed calcium regulating abilities. The β-CGRP gene is probably a duplication of the α-gene with loss of expression of the β exon 4 region. The known amino acid sequences of CGRP from various species are shown in Figure 1.3 together with the salmon calcitonin sequence for comparison. The finding of CGRP immunoreactivity in the neural ganglion of the tunicates slyela and ciona (Dr S I Girgis, personal communication) and the sequencing of porcine and avian CGRP (Kimura et al 1987, Minvielle et al 1986) supports the idea that CGRP is a highly conserved molecule.

	Humanα CGRP	Humanβ CGRP	Porc. CGRP	Ratα CGRP	Ratβ CGRP	Chick. CGRP	Salmon Calcitonin	Human Calcitonin
	H							
1	Ala		Ser	Ser	Ser		Cys	Cys
2	Cys						Ser	Gly
3	Asp	Asn	Asn	Asn	Asn	Asn	Asn	Asn
4	Thr						Leu	Leu
5	Ala						Ser	Ser
6	Thr							
7	Cys							
8	Val						Val	Met
9	Thr						Leu	Leu
10	His						Gly	Gly
11	Arg						Lys	Thr
12	Leu							Tyr
13	Ala						Ser	Thr
14	Gly					Asp	Gln	Gln
15	Leu					Phe	Glu	Asp
16	Leu							Phe
17	Ser						His	Asn
18	Arg						Lys	Lys
19	Ser						*	*
20	Gly						*	*
21	Gly						*	*
22	Val	Met	Met				*	*
23	Val						*	*
24	Lys						Leu	Phe
25	Asn	Ser	Ser	Asp	Asp		Gln	His
26	Asn						Thr	Thr
27	Phe						Tyr	
28	Val						Pro	Pro
29	Pro						Arg	Gln
30	Thr							
31	Asn		Asp					Ala
32	Val						Thr	Ile
33	Gly							
34	Ser							Val
35	Lys		Glu	Glu			Gly	Gly
36	Ala						Thr	Ala
37	Phe						Pro	Pro
	NH_2							

* Spaces introduced to maximise homology

Figure 1.3 Amino acid sequences of known CGRPs

CHAPTER 2 IMMUNOASSAY METHODS

2.1 INTRODUCTION

The principles of radio and enzyme immunoassays are described in this chapter as well as the work undertaken to improve immunoassays for the three circulating peptides from the calcitonin gene. To obtain assays of sufficient sensitivity to be able to detect normal levels of these peptides, a calcitonin amplified enzyme immunoassay and a human CGRP radioimmunoassay were developed.

2.1.1 Radioimmunoassays

2.1.1.1 Basic Principles

The development of radioimmunoassay (RIA) in the early 1960s was of great significance to endocrinologists. The first assays of this type were described by Yalow and Berson (1960), who used specific antibodies in an immunoassay for insulin, and Ekins (1960) whose use of a naturally occurring binding protein enabled him to measure thyroxine.

The principle of radioimmunoassay depends on the competition for binding to a specific antibody between the hormone present in the sample and hormone labelled with a radioactive marker. The amount of labelled hormone bound at the end of the reaction is inversely proportional to the amount of unlabelled hormone. Radioimmunoassays are applicable to a wide variety of biological compounds and have several advantages over traditional bioassays. Radioimmunoassays are more sensitive than bioassays allowing hormone determinations on small sample volumes. The RIA has the potential to be extremely specific, particularly if region specific antisera are employed, and the simplicity of the procedure allows large numbers of samples to be assayed simultaneously. However, care should be taken to distinguish between immunoreactivity, which can include reactivity with fragments and precursors of a hormone, and the biological activity indicated by a bioassay.

Further advances in RIA were made following the work of Greenwood and colleagues in 1963. The Chloramine-T oxidation method used by this group to label peptides with ^{131}I was simpler than previous methods and produced tracers with a higher specific activity. The availability and longer half-life of ^{125}I has now made it the principle isotope used for peptide labelling by this method. In addition assays have been developed in which the antibody is labelled. The antigen is usually reacted with a first antibody attached to a solid phase. A second labelled antibody is then added resulting in a sandwich complex. Such assays are known as immunoradiometric assays (IRMA) (Woodhead *et al* 1974).

The principal components of an RIA system are: an antiserum, specific to the antigen to be investigated; labelled antigen; and unlabelled antigen. Following the reaction a separation step is employed to distinguish between bound and unbound label.

2.1.1.2 Production of Antisera

There is considerable debate over the best means of producing antisera and several factors can influence antibody production including the animal to be immunised, the immunogen, the adjuvant and the immunisation procedure.

- The Animal

The animal chosen for immunisation should be a species of suitable difference from the source of the antigen. The rabbit is the most commonly selected animal being relatively cheap, easy to handle and giving 10-30 ml of serum per bleed (Szelke 1983).

- The Immunogen

Low molecular weight compounds may not be immunogenic in themselves but can be rendered so by coupling to a high molecular weight carrier protein. Such a carrier protein should be immunogenic and therefore from a species different to the chosen animal. Additionally the carrier protein should have adequate binding sites for the low molecular weight compounds (hapten). One of the most popular carrier proteins is bovine serum albumin (BSA). The specificity of an antiserum is dependent on the orientation of the hapten to the carrier protein and is usually directed against that part of the hapten which is furthest from the carrier.

Coupling of hapten and carrier protein can be achieved with various coupling reagents including bis-diazonium compounds, diimido esters and halogenated triazines but glutaraldehyde and carbodiimides are the most popular. Glutaraldehyde is a bifunctional reagent which forms a bridge between primary amine groups, either amino terminal or side chain, of the hapten and carrier molecule. Glutaraldehyde is thus a very effective reagent for coupling peptides and proteins containing lysine (O'Shaughnessy 1982).

$$R'NH_2 \quad + \quad CHO\ (CH_2)_3\ CHO \quad + \quad R^2NH_2$$

Hapten — Glutaraldehyde — Carrier

$$\downarrow \text{ pH } 7.5$$

$$R'NCH\ (CH_2)_3\ CHNR^2 \quad + \quad 2H_2O$$

Conjugate

A linkage of this nature could allow rotation of the hapten possibly reducing steric hinderance by the larger carrier molecule giving freer access to the host immune system. The disadvantage of this simultaneous reaction is that the reaction mixture will contain dimers and polymers of hapten and carrier protein as well as the desired conjugate.

The most commonly used carbodiimide is the water soluble 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide. The two step reaction of the carbodiimide with hapten and carrier protein involves condensation of any free carboxyl and primary amino groups to form CO-NH bonds. The linkage between hapten and carrier can therefore be formed with amino or carboxyl terminals as well as the side chains of lysyl, aspartate or glutamate residues. A variety of antigenic responses may then be produced as coupling can occur at several alternative points on the peptide.

$$R'COOH \quad + \quad CH_3CH_2\ NCN(CH_2)_3\ N(CH_3)_2$$

Hapten/Carrier — Carbodiimide

$$\downarrow$$

$$CH_3CH_2\ NHCN(CH_2)_3\ N(CH_3)_2$$

$$|$$

$$COOR'$$

$$\downarrow + \ R^2NH_2 \quad \text{Hapten/Carrier}$$

$$R'CONHR^2 \quad + \quad CH_3CH_2\ HNCONH(CH_2)_3\ N(CH_3)_2$$

Conjugate

Carbodiimides are the reagent of choice when the only available functional group is carboxyl; however the peptide bond is such that no rotation can occur and hence steric hinderance is possible.

- The Adjuvant

When using antigens which are poorly immunogenic the antibody response can be enhanced by using an emulsion of conjugate and Freund's adjuvant (a mixture of the emulsifying agent Arlacel A, n-hexadecane and killed mycobacteria (O'Shaughnessy 1982). The adjuvant allows slow release of the immunogen into the host circulation and may protect the conjugate from destruction. The effect of mycobacteria is to increase further the immune response by activating B-lymphocytes. To elicit a secondary immune response booster injections are usually carried out with incomplete adjuvant (lacking the mycobacteria to avoid a hypersensitivity reaction).

- Routes of Administration

There are several possible routes of administration including intradermal, footpad, intramuscular, subcutaneous, intranodal and intraperitoneal. Technical difficulties and ulceration in the animal can restrict the choice of injection site (Hurn 1974). The dose of immunogen is selected to avoid high and low dose tolerance and is usually about 100 nmol per rabbit for the primary injection. Peak antibody production occurs at approximately 18 days after the primary injection and to produce an antibody of high avidity and titre monthly booster injections are given after the primary response declines (Szelke 1983).

2.1.1.3 Radiolabelling of Antigen

- Radioiodination Methods

There are two types of radioiodination procedure; the direct method, where ^{125}I is incorporated into tyrosine or histidine residues of the peptide, or the conjugation method where a reactive group containing ^{125}I is conjugated to a side chain of the protein. Direct radioiodination methods frequently involve the oxidation of radioactive iodide to iodine in the presence of the peptide to be labelled.

Removal of the oxidising agent from the labelling mixture or addition of a reducing agent is then necessary to stop the reaction. There are several examples of direct radioiodination procedures involving oxidation including chloramine-T, iodine monochloride, sodium hypochlorite and Iodo-gen (1,3,4,6-tetrachloro 3a,6a diphenylglycoluril) (Bolton 1985). To avoid chemical damage to the peptide by oxidising and reducing agents a non-oxidising iodination technique has been developed using lactoperoxidase to catalyse the reaction (Bryant 1982). Conjugation labelling with reagents such as Bolton-Hunter reagent (N-succinimidyl 3-(4-hydroxyphenyl) propionate) offers a further alternative to oxidation methods (Bolton and Hunter 1973).

The chloramine-T method is the most popular direct labelling method because, although this method exposes the peptide to chemical damage, it is a quick and simple procedure which usually provides labels with a high incorporation of radioiodine. In aqueous solution (pH 7.5) chloramine-T acts as an oxidising agent promoting the conversion of sodium iodide to iodine. The iodine can then react with the phenolic side chain of tyrosine. Mono- and di-iodinations of the tyrosine side chain are possible but following monosubstitution the tyrosine residue becomes less reactive. Excess chloramine-T and free iodine are reduced by the addition of sodium metabisulphite at the end of the reaction. The pH optimum for the iodiation of tyrosine is 7.5, however above pH 8.0-8.5 the substitution of iodine into the imidazole ring of histidine is enhanced.

- Label Purification

Following the completion of the radioiodination reaction purification of the labelled antigen from the reaction components, unlabelled antigen and damaged label is necessary. Various techniques have been employed to attain this separation including several column chromatography methods such as affinity columns, ion exchange columns, gel permeation chromatography and HPLC (Wood and Sokolowski 1981). The inability of many of these methods to distinguish adequately between labelled and unlabelled peptide and the time involved in applying gel chromatography (up to 24 hours) can be major disadvantages.

High performance liquid chromatography (HPLC) has several advantages as a separation technique, frequently being able to distinguish between unlabelled, damaged, monoiodinated and multi-iodinated peptide. Resolution of the peptide by HPLC is achieved on the basis of hydrophobicity when using reverse phase columns whereas differences in peptide charge lead to separation with ion exchange HPLC. Stable, high specific activity labels can be obtained using HPLC. However restricted finance may prevent the allocation of HPLC equipment solely for label purification.

2.1.1.4 Separation of Bound from Free Antigen

A suitable incubation time for the RIA will allow a state of equilibrium to be achieved in the reaction between antibody and antigen. Subsequently separation of bound and free antigen is required prior to counting the assay. The ideal separation system should completely separate bound and free fractions whilst being simple, quick and unaffected by plasma and serum components.

The principal separation methods can be classified according to their mode of action i.e. adsorption, precipitation or solid phase. Adsorption methods using charcoal, silicates or ion exchange resins depend upon attachment of the free fraction to the adsorbant followed by centrifugation. Chemical precipitation of the antigen-antibody complex can be achieved by the addition of polyethylene glycol, ammonium sulphate or ethanol. However immunological precipitation requires a second antibody which is specific to immunoglobin from the animal which produced the first antibody. Binding of the second antibody to the antigen-antibody complex renders it insoluble or alternatively the second antibody can be bound to solid phase e.g. cellulose particles. Antibodies involved in the primary antigen reaction can also be adsorbed to solid phases such as plastic surfaces, however a washing step is then necessary to remove unwanted material which has non-specifically adhered.

2.1.2 Enzyme Immunoassays

2.1.2.1 Non-Isotopic Labels

The principal disadvantages of traditional radioimmunoassays are largely due to the radiolabel. As well as the health risks encountered in handling radioactive material, most iodine labels have a short shelf-life, require complex measuring equipment, long counting times and present difficulties in disposal. Consequently several alternative immunoassays have been developed and the resulting assays named according to the nature of the label, e.g. enzyme immunoassays (EIA), fluoroimmunoassays and chemiluminescence immunoasays. Enzyme labels are frequently chosen as substitutes for radiolabels because of the relative cheapness of spectrophotometers for measuring coloured end products. The visual reading of coloured substrates means that EIAs are invaluable as simple field tests. As with radiolabels the enzyme can be conjugated to either the antigen (competition assays) or to an antibody (immunometric/sandwich assays).

2.1.2.2 Enzyme Conjugation

Popular enzyme labels include horseradish peroxidase, β-galactosidase and alkaline phosphatase which have various coloured and fluorescent substrates (Trivers et al 1983).

There are several methods available for the enzyme conjugation step (Ishikawa et al 1983) including glutaraldehyde and periodate oxidation coupling techniques. Unfortunately both these coupling agents can lead to polymerisation with consequent loss of enzyme activity. More specific conjugation can be achieved using various maleimide reagents such as the analogue N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) which has been developed to be stable at pH 7.0. Maleimides react with thiol groups on both hapten and enzyme and the resulting conjugate usually shows minimal loss of enzyme activity.

2.1.2.3 Separation

EIAs can be either competitive or non-competitive e.g. double antibody sandwich assays (Monroe 1984). The competitive immunoassay follows the same principles as conventional RIA except that the antiserum is usually adsorbed to a solid phase such as microtitre plates. The advantage of microtitre plates is that separation of bound and free fractions can be achieved simply by washing which eliminates a centrifugation step.

Antibody is thought to adsorb to plastic surfaces tenaciously by hydrophobic interaction (Parsons 1981); however the solid phase chosen should be a consistent product to minimise adsorption variability.

2.1.2.4 Detection

Following the wash step the enzyme substrate is added to the wells of the microtitre plate. The substrate is usually chosen to give a coloured product following interaction with the enyzme, the rate of colour development being proportional to the amount of bound enzyme conjugate.

The catalytic action of the enzyme in producing the coloured product may be further amplified by the use of an enzyme cycling system. One such enzyme amplification system is that described by Self (1985) in which the enzyme label alkaline phosphatase is exposed to the substrate NADP, dephosphorylating it to NAD. The proportion of NAD present is then determined by means of a highly NAD-specific redox cycle in which NAD is reduced to NADH by the action of alcohol dehydrogenase. The oxidation of NADH back to NAD is catalysed by diaphorase which simultaneously reduces colourless p-iodonitrotetrazolium violet (INT) to a coloured product (Figure 2.1). The cycling of NAD in the secondary system means that each molecule of NAD from the primary reaction results in several molecules of coloured product and hence the initial enzyme signal is considerably amplified. Also, because the redox cycle is specific to NAD there is no need to remove the NADP remaining from the primary reaction.

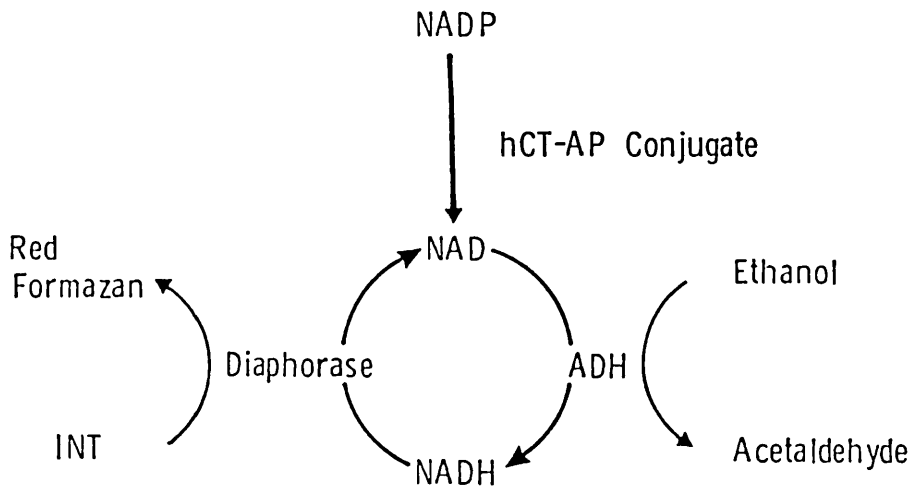

Figure 2.1 Enzyme amplified cycle triggered by alkaline phosphatase - calcitonin conjugate (hCT-AP conjugate)

2.2 CALCITONIN IMMUNOASSAY METHODS

The calcitonin RIA established within the department has been used routinely in the detection and follow up of MTC patients. For research studies on normal and ectopic secretion of calcitonin a more sensitive assay was required to measure plasma calcitonin directly (without prior extraction). To solve this problem a sensitive amplified enzyme immunoassay (AEIA) was developed and its attributes compared with those of an improved RIA.

2.2.1 Calcitonin Amplified Enzyme Immunoassay

2.2.1.1 Antiserum

The anti-calcitonin antiserum (OC31) used in both RIA and AEIA was raised in rabbits by repeated foot-pad immunisation with a synthetic calcitonin-ovalbumin conjugate. The antiserum was tested against a wide range of peptides and found to be specific for CT.

2.2.1.2 Enzyme Conjugate

A calcitonin alkaline phosphatase conjugate was prepared by reducing the N-terminal disulphide bridge of calcitonin using dithiothreitol. Calf-intestinal alkaline phosphatase was activated by reaction with SMCC (Ishikawa *et al* 1983) and then coupled to the thiols resulting from reduction of the hCT disulphide bridge. The calcitonin-alkaline phosphatase conjugate was then purified on HPLC.

2.2.1.3 Plate Coating

Microtitre plates were coated with antiserum OC31 in 20 mmol/ℓ carbonate buffer (pH 9.6). Diluted antiserum (120 μl) was added to each well and the plates incubated overnight at room temperature. Each plate was sealed with polythene plate sealers to reduce uneven evaporation and improve plate coating. The antibody solution was then shaken out and the wells washed 4 times with a protein buffer at pH 7.4 (5% lactose, 0.5% bovine serum albumin and 0.2% Tween 20). Plates were then dried at room temperature and covered with plate sealers for storage.

2.2.1.4 Assay Procedure

Calcitonin standards were prepared by serial dilution of synthetic calcitonin in plasma protein fraction (PPF). The alkaline phosphatase conjugated calcitonin was diluted in TRIS buffered saline (appendix) containing 'Trasylol' (aprotinin proteinase inhibitor) to prevent protein degradation. Sample or standard (80 μl) and 20 μl of enzyme conjugate were mixed in the wells and incubated overnight (20°C). The samples were then shaken out and the wells washed 4 times with TRIS buffered saline containing 0.02% Tween 20.

A substrate solution was added to each well: 100 μl of 0.2 mmol/ℓ NADP in 50 mmol/ℓ diethanolamine, pH 9.5. Following a 20 minutes incubation period 200 μl of amplifier solution was mixed into each well (60 units/ml alcohol dehydrogenase EC1.1.1.1, 0.1 units/ml diaphorase EC1.6.4.3, 0.55 mmol/ℓ INT and 4% ethanol in 25 mmol/ℓ phosphate buffer, pH 7.0). The reaction proceeded until the colour of the zero standard wells reached approximately 2.0 absorbance units at 492 nm. To stop the reaction sulphuric acid (50 μl of 0.2 mmol/ℓ) was added and the absorbance of the wells read at 492 nm in a Titertek Multiskan MCC.

2.2.1.5 Working Dilutions

To determine the optimal dilutions of both antiserum and conjugate, plates were coated with antiserum at dilutions of 1:20,000, 1:40,000, 1:80,000, 1:160,000 and 1:320,000. Standard curves were tested at each antiserum dilution with varying conjugate dilutions between 1:10,000 and 1:30,000. The choice of conjugate and antiserum dilution was dependent on practical as well as performance criteria. Low concentrations of antiserum required unsatisfactory periods of time to reach the desired colour density whereas at high conjugate concentration the rate of colour development was too rapid for convenience. Figure 2.2 shows standard curves at 4 antiserum dilutions using a conjugate dilution of 1:25,000. Compromising between assay sensitivity and practical considerations an antiserum dilution of 1:40,000 was selected using a conjugation dilution of 1:25,000. The resulting amplifier colour development was then approximately 20 minutes giving a typical detection limit of 6 pmol/ℓ (20 ng/ℓ).

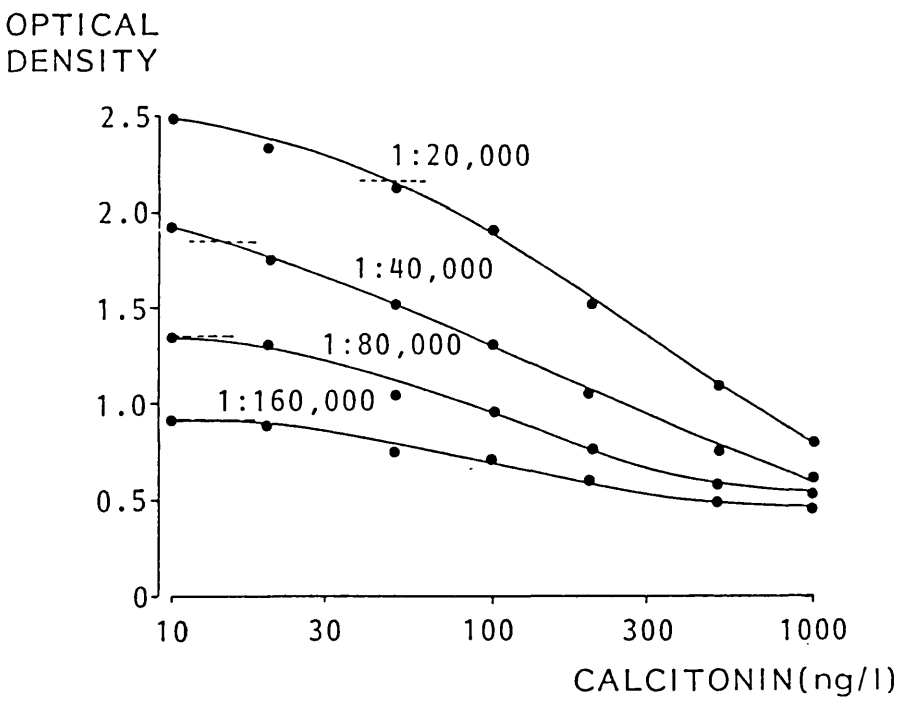

Figure 2.2 Calcitonin standard curves at 4 dilutions of antiserum OC31 with 1:25,000 conjugate dilution (dotted line indicates 10% of zero standard).

Intra-assay coefficient of variation (CV) was calculated as 12% for a quality control pool of 73 pmol/ℓ and 10% for a pool of 293 pmol/ℓ. For the same pools inter-assay CVs were found to be 11.8% for the low pool and 11.2% for the high pool.

2.2.2 Calcitonin Radioimmunoassay

The RIA method employed was a modification of the previously published method (Coombes et al 1974) using antiserum OC31 (described earlier). Standards were prepared in PPF as for AEIA and 100 μl of phosphate buffer (appendix) was added to 100 μl of sample or standard in Luckam LP3 tubes. Diluted antiserum (50 μl) was then added to each tube to give a final dilution of 1:180,000. The assay was then incubated for 2 days at 4°C, 50 μl of ^{125}I-calcitonin added and then incubated for a further day. Cellulose bound second antibody was used as the separation method and the bound fraction counted on an LKB Wallac 1261 multigamma counter.

2.2.2.1 Radioiodination

Initially the radioiodination of synthetic hCT was performed using an adaptation of the Chloramine-T method of Greenwood and colleagues which is detailed in Table 2.1a. Purification of the label was then carried out on a CG400 "Amberlit" ion exchange column (eluting with acetate buffer, pH 4.8). The radiolabel obtained by this procedure had a relatively short shelf-life of 3-4 weeks and the usual assay sensitivity was about 29 pmol/ℓ using a three day assay with label addition after 48 hours. Frequent visits to the radioiodination laboratory and inadequate sensitivity indicated that a better quality radiolabel was required. To carry out such improvements alterations were made to the radioiodination protocol and an HPLC system was constructed for label purification.

Several minor changes were made to the iodination procedure and are summarised in Table 2.1b. The overall amount of peptide to be iodinated was increased to combat product loss on the HPLC column. As radioactive iodine is delivered in sodium hydroxide, phosphate buffer was added at a concentration suitable to maintain the pH at 7.4. The amount of Chloramine-T was reduced to slow the reaction in compensation for the elevated peptide concentration.

Table 2.1 Radioiodination of synthetic human calcitonin:
(a) Established method
(b) Alternative method

	(a)	(b)
Peptide	2 μg freeze-dried	10 μg freeze-dried
Phosphate buffer	50 μl 0.5 mol/ℓ pH 7.4	50 μl 0.25 mol/ℓ pH 7.4
^{125}I 100 μCi/μl	10 μl	10 μl
Chloramine-T	25 μl 1.6 mg/ml	25 μl 1.0 mg/ml
Na Metabisulphite	25 μl 3.2 mg/ml	100 μl 0.75 mg/ml

2.2.2.2 HPLC Purification

An HPLC system was constructed and housed within a lead box kept permanently in the radioiodination laboratory. A gradient maker and pump were attached to a 10 cm reverse phase C18 ODS (octadecasilyl) column. Fractions eluting from the column were collected in an automatic fraction collector.

A calcitonin radiolabel was prepared as detailed in Table 2.1b and applied to the HPLC column. A wide linear gradient of acetonitrile/water/0.1% TFA was used initially to purify the label i.e. 25 to 45% acetonitrile. The major peak of radioactivity also exhibited the greatest binding to antiserum OC31. Subsequent labels were purified on a linear gradient of 27 to 37% acetonitrile. The radioactive profile of the eluted fractions for one such purification is shown in Figure 2.3.

2.2.2.3 Improved Radioimmunoassay

Labels prepared according to this protocol showed an extended shelf-life of up to 3 months (although longer counting times were necessary by 3 months) and enhanced the performance of the assay. The detection limit of an overnight assay improved from 88 pmol/ℓ to 6-12 pmol/ℓ, and from 29 pmol/ℓ to 0.71 pmol/ℓ for a 3 day disequilibrium assay. Inter assay coefficient of variation was calculated as 12.9% for a quality control pool of 293 pmol/ℓ and 10.9% for a pool of 85 pmol/ℓ. Intra assay coefficient of variation was 10.4% for the high pool and 10.4% for the low pool.

2.2.3 Comparison of Calcitonin AEIA and RIA

A selection of samples from patients with MTC and non-thyroid malignancies were assayed using both RIA and AEIA. A significant correlation was found between the results obtained by the two methods ($P<0.005$) shown in Figure 2.4.

A comparison of the performance of the two assays indicates that AEIA shows slightly greater sensitivity (6 pmol/ℓ) than RIA (12 pmol/ℓ) with similar variability. Additionally the AEIA offers the advantage of

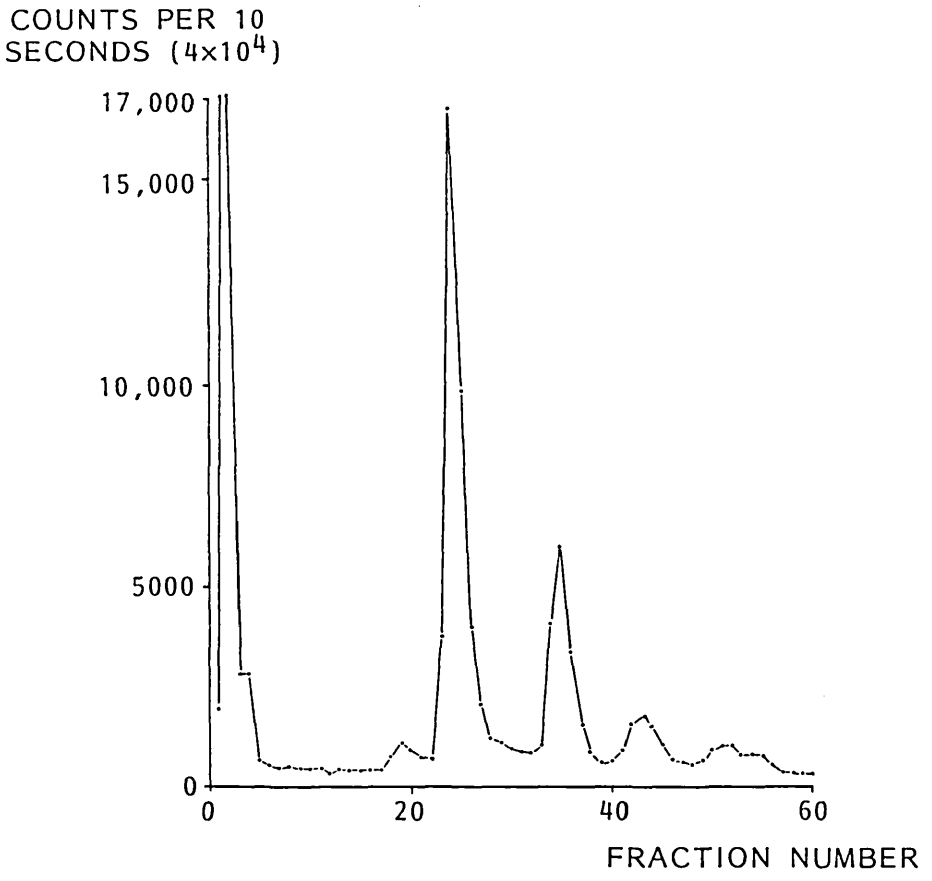

Figure 2.3 HPLC purification of calcitonin label
(Gradient: 27-37% acetonitrile)

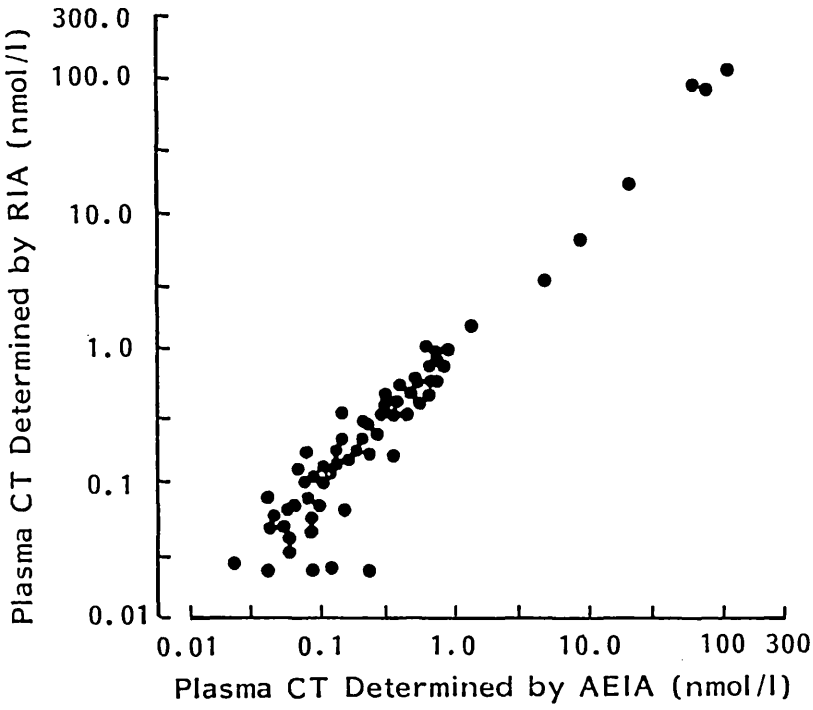

Figure 2.4 Correlation of calcitonin levels in patient samples measured by RIA and AEIA

a label of indefinite shelf-life without the health hazards of ^{125}I labels. The use of microtitre plates allows convenient loading of reagents using multichannel pipettes. Furthermore the developed plates can be read quickly using plate readers whereas long counting times are necessary for RIA, particularly with ageing radiolabels.

Unfortunately there were two disadvantages with the AEIA. Firstly, uneven plate coating was occasionally observed at the edges of the plates. Strategically placed zero standards at the periphery of the plates allowed this problem to be monitored but reduced the number of wells available for samples. RIA, therefore, enabled a greater throughput of samples than EIA. Secondly, the cost of amplifier reagents was greater than for in-house radioiodination.

Within the Endocrine Unit (Hammersmith Hospital) further work is underway to produce a better AEIA for calcitonin. A two-site monoclonal assay is currently being developed to produce a sandwich assay of increased sensitivity and specificity.

Whilst the improved calcitonin RIA was chosen for both routine and research work for this study monoclonal AEIA will probably offer a worthy alternative for the future. The amplification system has the potential to enhance assay sensitivity considerably and the coloured end product offers possibilities for qualitative visual field tests. Where funds for equipment are limited the relatively low cost of spectrophotometers means that AEIA will be increasingly chosen when new laboratories are set up. However existing laboratories with gamma-counters and established radioimmunoassays will continue ^{125}I radiolabelling for some time.

2.3 KATACALCIN RADIOIMMUNOASSAY

Plasma katacalcin was measured directly using an established RIA (Hillyard et al 1983) and was performed according to the same protocol as the 3 day calcitonin assay using synthetic katacalcin as standard. The anti-katacalcin antiserum (CP23) was raised in rabbits by subcutaneous immunisation with a katacalcin ovalbumin conjugate. The antiserum was used at a final dilution of 1:60,000 in the assay. Radioiodination of tyrosinated katacalcin ([Tyr]°katacalcin) was performed using the same iodination procedure as described for calcitonin (Table 2.1b). The katacalcin radiolabel was then purified on HPLC using a linear gradient of 10-30% acetonitrile and the principle peak eluted at approximately 25% acetonitrile (Figure 2.5). The assay sensitivity for a 3 day disequilibrium assay was 15 pmol/ℓ with inter assay CVs of 11.6%, 12% and 8.7% for pools of approximately 35, 175 and 850 pmol/ℓ respectively.

Plasma samples were assayed by duplicate determinations with further duplicate measurements on 50% dilution of the plasma in PPF.

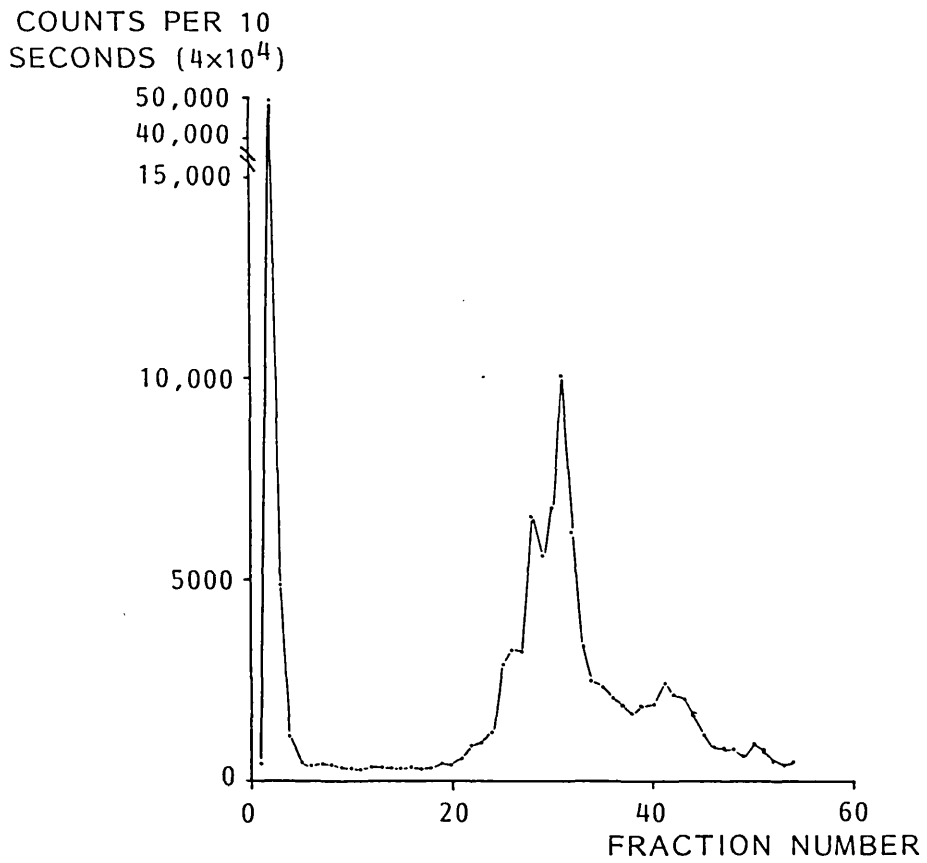

Figure 2.5 HPLC purification of katacalcin label
(Gradient: 10-30% acetonitrile)

2.4 CALCITONIN GENE-RELATED PEPTIDE RADIOIMMUNOASSAYS

2.4.1 Assay with Antiserum CG39

An anti-CGRP antiserum (CG39) was raised in rabbits by immunisation with a conjugate of the C-terminal decapeptide of rat CGRP and ovalbumin. The assay was performed according to the calcitonin assay procedure using synthetic human CGRP as standard and with a final antiserum dilution of 1:60,000. A five day disequilibrium assay was used with addition of the label after 3 days. The radiolabeled human CGRP (2-[^{125}I]-iodohistidyl^{10}CGRP) was obtained from Amersham. A standard curve for CGRP is shown in Figure 2.6.

Whilst allowing the measurement of high levels of CGRP in the plasma of MTC patients the assay was not sufficiently sensitive for studies on normal circulating CGRP. Consequently the need arose for a new anti-human CGRP antiserum to develop a sensitive assay.

2.4.2 Assay with Antiserum CC2/1

2.4.2.1 Raising Anti-Human CGRP Antisera

Bovine serum albumin was selected as the carrier protein for conjugation to hCGRP. Two BSA-CGRP conjugates were prepared for immunisation, one conjugated with glutaraldehyde and one conjugated with carbodiimide.

Dutch rabbits were used to raise the anti-CGRP antiserum and two groups of 3 rabbits each were used because of individual differences in immune response between animals. The first groups were injected with conjugate coupled by glutaraldehyde (GC1, 2 and 3) and the second with carbodiimide coupled conjugate (CC1, 2 and 3). The immunisation procedure is detailed in Table 2.2.

2.4.2.2 Testing Antibodies

Serum from each bleed was tested for binding with radioactive CGRP (Amersham). Doubling dilutions of the sera in phosphate buffer were incubated overnight with radioactive CGRP and free label was

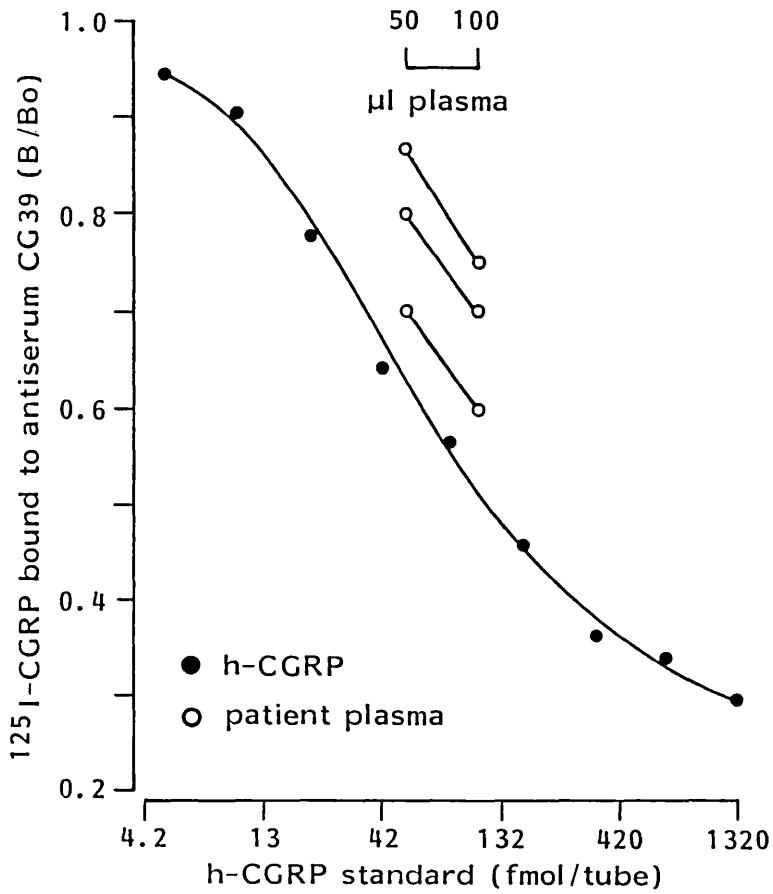

Figure 2.6 CGRP standard curve using antiserum CG39

Table 2.2 The immunisation procedure for raising CGRP antisera

	GC1, 2 & 3	CC1, 2 & 3
Primary Injection		
CGRP-BSA Conjugate	100 nmol CGRP/rabbit	50 nmol CGRP/rabbit
Adjuvant	Freund's complete	Freund's complete
Site	4 subcutaneous sites	4 subcutaneous sites
First Booster (December)		
Time	7 weeks after primary	7 weeks after primary
CGRP Conjugate	30 nmol/rabbit	15 nmol/rabbit
Adjuvant	Freund's incomplete	Freund's incomplete
Site	6 subcutaneous sites	6 subcutaneous sites
Bleed (12)	9 days after boost	9 days after boost
Second Booster (January)		
Time	5 weeks after first boost	5 weeks after first boost
Injection	As first boost	As first boost
Bleed (1)	9 days after boost	9 days after boost

separated from bound using activated charcoal (0.5% charcoal with 0.05% dextran). The percentage of radiolabel binding for each serum is shown in Figure 2.7 (January bleed GC/1 and CC/1). Antiserum CC2/1 showed the greatest binding and was used to develop an RIA.

2.4.2.3 Working Dilutions

The following experiments were carried out using plasma protein fraction (PPF) as additional protein in the incubation mixtures.

The two separation methods available within the laboratory were activated charcoal and cellulose bound sheep anti-rabbit second antibody (SAR 2nd Ab). Both methods were tested on a range of antiserum dilutions between 1:6,000 and 1:60,000 (final dilution). SAR second antibody proved to be a better separation method than charcoal, having a similar percentage binding of labelled CGRP but showing less non-specific binding. However, all the CC2/1 antiserum dilutions gave binding of greater than 50%. A further range of antiserum dilutions between 1:60,000 and 1:360,000 were then tested for incubation times between 1 to 7 days. A dilution of 1:240,000 showed the optimal binding of 40-50% at about 5 days. To achieve the greatest sensitivity with this assay standard curves were incubated in both five day equilibrium and 3+2 disequilibrium reactions. Standard curves for these different incubation times were approximately 10 pmol/ℓ more sensitive in disequilibrium than equilibrium reactions. A standard curve for the CC2/1 assay is shown in Figure 2.8 (5 day disequilibrium reaction). As the assay was intended for the direct measuremnet of CGRP in plasma samples, a suitable plasma substitute for standard curves was required. A series of protein substitutes were tested including BSA, HSA, charcoal stripped plasma and PPF. PPF and stripped plasma exhibited similar standard curves and subsequently PPF was used as a plasma substitute because of its greater availability.

The assay was established with a final antiserum dilution of 1:240,000 using (2-[^{125}I]-iodohistidyl^{10}) CGRP as the radiolabel and PPF as the plasma substitute. The typical assay sensitivity was 10 pmol/ℓ.

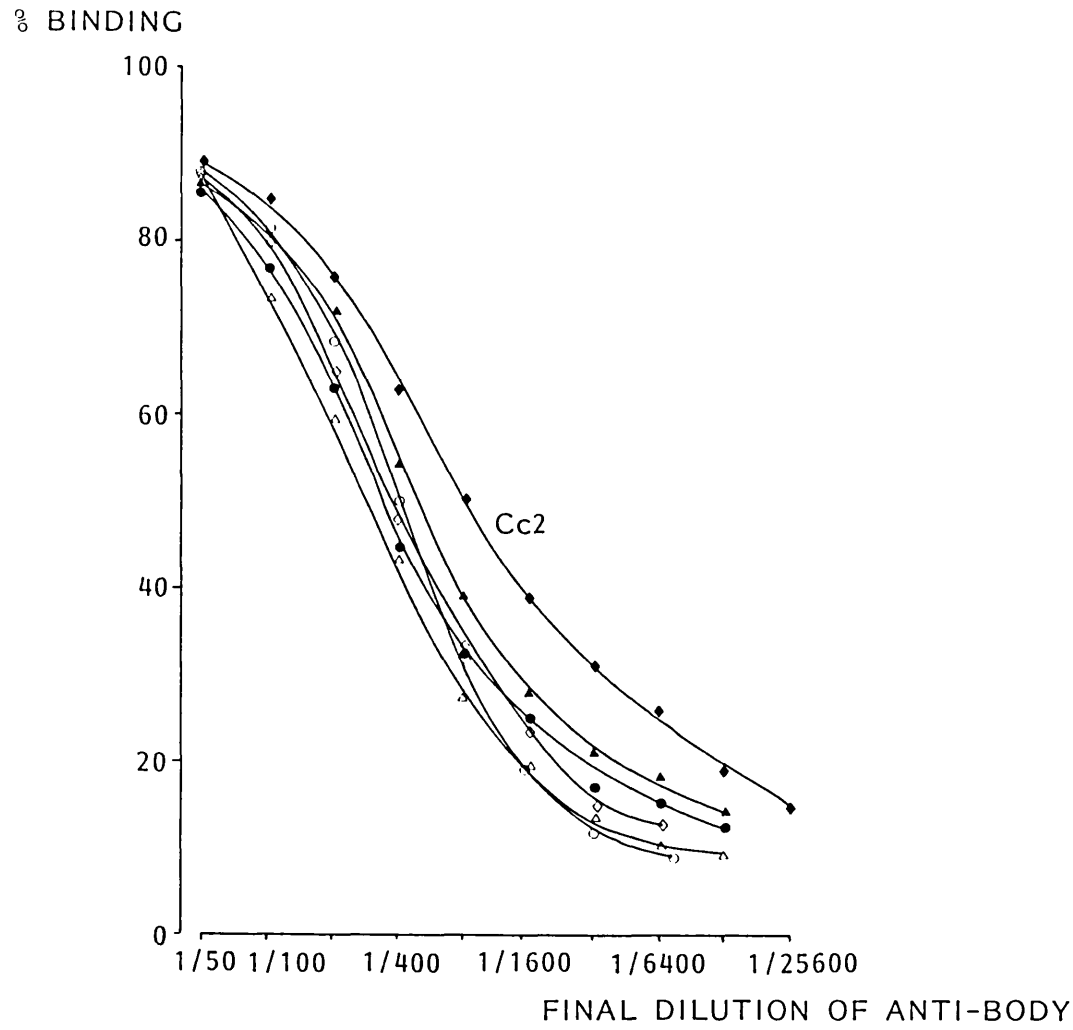

Figure 2.7 Antiserum dilution curves following second boost
(CC1 ● , CC2 ◆ , CC3 ▲ ,
GC1 ○ , GC2 ◇ , GC3 △)

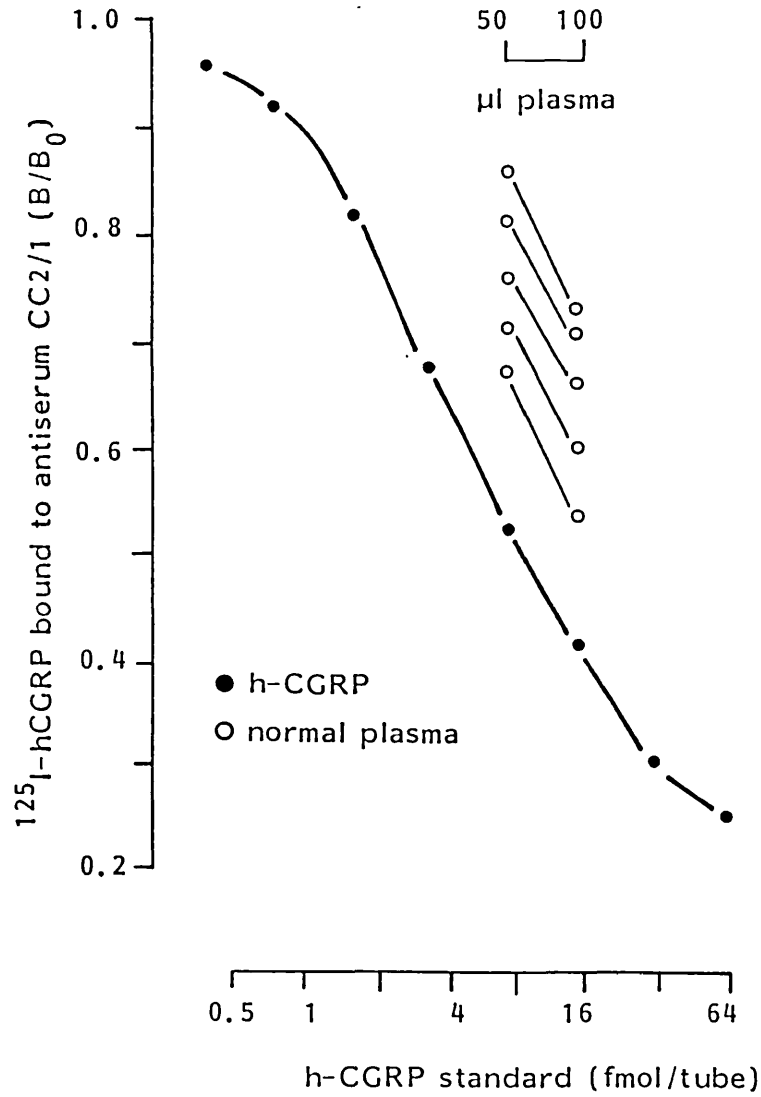

Figure 2.8 CGRP standard curve using antiserum CC2/1

2.4.2.4 Assay Specificity

The antiserum CC2/1 was tested for cross reactivity with a wide range of peptides as listed in Table 2.3 and typical anticoagulants. No cross reactivity was observed with up to 1 μg of the peptides or with anticoagulants for the amounts used in blood collecting tubes. However the antiserum cross reacted with heparin at a concentation of 25,000 units/ml.

2.4.2.5 Characterisation of Antisera

CGRP(α) from both rat and human as well as hCGRP(β) were tested for immunoreactivity with the antisera CC2/1 and CG39. In addition various fragments of CGRP were also tested for cross reactivity. The fragments were prepared by Howard Morris and colleagues (Imperial College) by proteolytic digestion of the hCGRP(α) with HPLC purification.

The cross reactivity of the antisera CG39 and CC2/1 with CGRP ($\alpha+\beta$) and fragments are shown in Figure 2.9 and 2.10 respectively. As well as CGRP(α), CG39 also cross reacted with CGRP(β) and the fragments Ala^{1}-Arg^{18} CGRP, Ser^{19}-lys^{37} CGRP. CC2/1 cross reacted with CGRP(β) and the fragment Ala^{1}-Arg^{18} CGRP but neither CG39 nor CC2/1 showed any cross reactivity with fragments Ala^{1}-Arg^{11}, Leu^{12}-Arg^{18}, Asn^{25}-Lys^{35} and Ser^{19}-Lys^{35} CGRP. Whilst CG39 was expected to bind the fragment Ser^{19}-lys^{37} CGRP because it was raised against rat C-terminal decapeptide, the observation of cross reactivity with the fragment Ala^{1}-Arg^{18} CGRP is probably indicative of contamination of this fragment with whole molecule CGRP. Consequently the N-terminal reactivity of CC2/1 is ambiguous. Antiserum CC2/1 cross-reacted with both human and rat α-CGRP to the same extent.

2.4.2.6 Precision

Three quality control pools were prepared and used to assess the precision of the assay. The pools were prepared to give approximate CGRP concentrations of 528, 132 and 33 pmol/ℓ. The intra assay coefficients of variation (CVs) wre found to be 10.5, 7 and 9% respectively for the high, medium and low pools, whilst inter assay CVs were 9.2, 8.7 and 13.1% respectively.

Table 2.3 Human CGRP immunoassay: specificity

No cross reactivity with 1 μg amounts of these peptides

CALCITONIN	BOMBESIN	PROLACTIN
KATACALCIN	GLUCAGON	CRF
SUBSTANCE P	VASOPRESSIN	GROWTH HORMONE
SOMATOSTATIN	OXYTOCIN	ACTH
PROOPIOMELANOCORTIN	GASTRIN RELEASING PEPTIDE	met-ENKEPHALIN
ATRIOPEPTIN III	GASTRIN RELEASING FACTOR	VIP
SYNACTHEN	PARATHYROID HORMONE	
ANGIOTENSIN II	INSULIN	

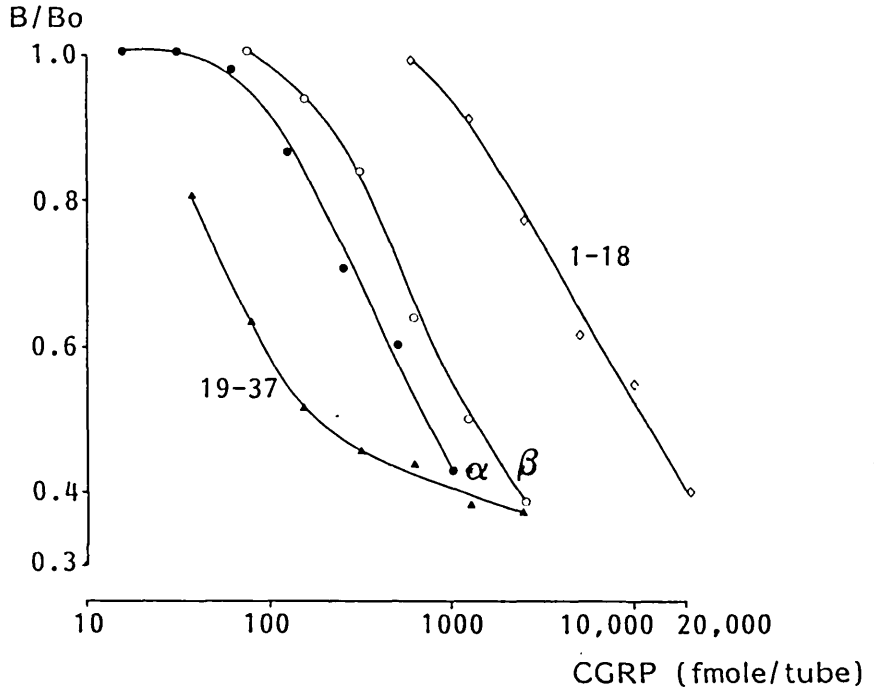

Figure 2.9 Cross reactivity of antiserum CG39 with CGRP(α) (●), CGRP(β) (○) and CGRP fragments Ala^{1}-Arg^{18} CGRP (◇) and Ser^{19}-lys^{37} CGRP (▲)

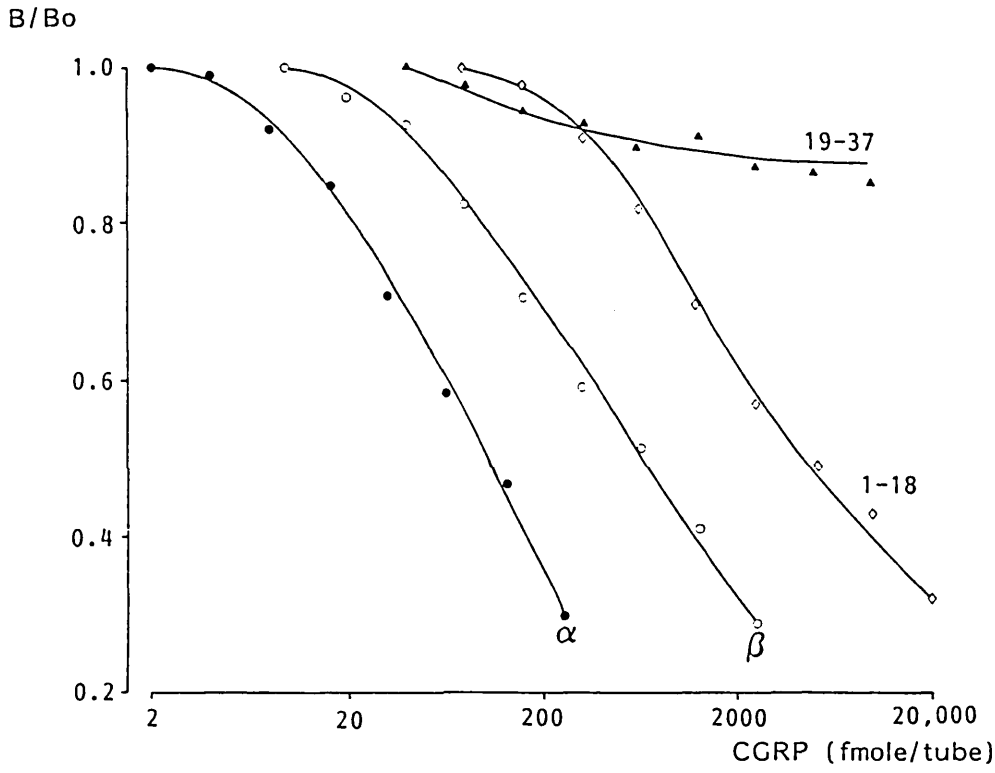

Figure 2.10 Cross reactivity of antiserum CC2/1 with CGRP(α) (●), CGRP(β) (○) and CGRP fragments Ala^{1}-Arg^{18} CGRP (◇) and Ser^{19}-lys^{37} CGRP (▲)

2.4.3 Discussion

The raising of a new antiserum (CC2/1) has successfully enabled the development of a sensitive RIA capable of measuring much lower levels of CGRP (10 pmol/ℓ) than the previous assay (CG39). Both assays are incapable of discriminating between the two types of hCGRP (α and β). Polyclonal antisera are complex, heterogenous mixtures of antibodies. Hence it is unlikely that CC2/1 is composed entirely of antibodies directed against the whole CGRP molecule, despite the apparent sole immunoreactivity with the complete molecule. More probable is the possibility that the antibodies within CC2/1 have been diluted to a point where the immunoreactivity of one particular clone of antibodies predominates within the mixture. Alternatively recent hypotheses concerning antibody binding enhancement (Thompson and Jackson 1984) may be relevant to CC2/1 antibodies. In one theory binding of a first antibody and antigen produces a conformational change in the antigen such that binding of a second antibody is greatly enhanced. Binding enhancement may also result if the second antibody recognises an epitope on the first antibody induced by antigen binding or an epitope created at the junction between the primary antibody and antigen because of their coming together.

The second hypothesis requires that an antigen has two distinct epitopes each of which are recognised by different antibodies. This allows the formation of a stable complex of two antigens with one of each antibody. Some supporting evidence for this model has been provided by studies on monoclonal antibodies. Ehrlich and Moyle (1983) found that certain combined pairs of anti-human chorionic gonadotrophin (hCG) monoclonals had a ten-fold greater avidity for hCG than either antibody alone. Using size exclusion chromatography Holmes and Parham (1983) discerned that a co-operating pair of monoclonals directed against HLA-A2 formed a complex of molecular size consistent with two antigens and two antibodies. Monoclonal enhancement effects may explain the low affinity of single monoclonals compared with often high avidity of polyclonal antisera to the same antigen. Additionally such enhancing effects will allow the future development of assays which employ carefully selected and defined oligoclonal antiserum.

The antiserum CC2/1 was raised against a carbodiimide conjugate of CGRP to BSA and therefore CGRP may have been coupled to BSA at different points. A variety of sites on the CGRP molecule would then be presented to the rabbit immune system resulting in more than one clone of antibodies. The high avidity of antiserum CC2/1 for whole molecule CGRP may thus result from the combined effects of suitably enhancing sub-populations of antibodies. Whilst the individual affinities of the participating antibody binding sites may be unchanged, the overall avidity of the antibody mixture for the antigen will be greatly enhanced.

2.5 SUMMARY

Improvements in the calcitonin RIA have allowed the direct measurement of plasma hCT down to 0.71 pmol/ℓ using a long incubation. The calcitonin AEIA compared favourably with the RIA but RIA offered a greater sample capacity for each assay.

Development of a sensitive hCGRP assay has facilitated the detection of pmol levels of this peptide. These two assays, together with the katacalcin RIA, have enabled studies on the circulation of the three calcitonin gene peptides (hCT, KC and hCGRP) in normals and in various malignant diseases.

CHAPTER 3 NORMAL CIRCULATING CALCITONIN GENE PEPTIDES

3.1 INTRODUCTION

Calcitonin was first measured in normal plasma by extraction and bioassay (Gudmundsson et al 1969). Greater calcitonin bioactivity was observed in plasma samples taken in the afternoon than at 9 am although the samples were not obtained from the same individuals. Following development of calcitonin radioimmunoassays at various centres more extensive investigations of normal calcitonin levels have been possible. Several centres quote an hCT normal range of around 100 ng/ℓ or less (Heath and Sizemore 1977, Parthemore and Deftos 1978, Emmertsen et al 1982).

There have been conflicting reports concerning circadian fluctuations in hCT. Hillyard and colleagues (1977) reported a diurnal rhythm in hCT with a midday peak but other workers have not observed a similar variation (Robinson et al 1982, Emmertsen et al 1983A). A study on calcium regulating hormones during the menstrual cycle (Pitkin et al 1978) indicated a mid cycle peak of hCT but was unconfirmed in a smaller study by Baran et al (1980).

Greater consistency is apparent in the findings by various groups of sex and age difference in normal circulating levels of calcitonin. Higher levels of plasma calcitonin have been observed in normal men than in women (Heath and Sizemore 1977, Hillyard et al 1978) although there was no significant difference between men and women using oral contraceptives. A progressive decrease in circulating calcitonin levels has been reported from birth to 6 years of age (Klein et al 1984) continuing between childhood and adulthood (Samaan et al 1975) with further decreases through adult life (Deftos et al 1980). Stevenson (1985) was also able to demonstrate a sharp fall in calcitonin levels at the menopause, which lead to speculation that the relative deficiency of calcitonin in women, together with low oestrogen, may contribute to the onset of post-menopausal osteoporosis. Additionally the observation that circulating calcitonin was higher than normal throughout pregnancy and during lactation suggests that protection of the maternal skeleton during calcium stress may be one of the main physiological roles of calcitonin (Stevenson et al 1979).

Studies on normal katacalcin show a similar sex difference in circulating levels to those of calcitonin (Hillyard et al 1983) whilst women on oral contraceptives had higher circulating katacalcin levels than normal women.

Clearly it is important to take into account the various factors which may affect normal secretion of calcitonin and katacalcin when interpreting clinical results. The studies described in this chapter were undertaken with reference to previous observations on normal circulating calcitonin and katacalcin to establish the normal plasma levels of CGRP.

3.2 SUBJECTS AND METHODS

3.2.1 Normal Range of CGRP

Fifty healthy volunteers were studied, 15 female and 35 male, with an age range of 20-65 years. None of the individuals were on medication. Fasting blood samples were taken into heparinised tubes, rapidly separated and stored at -20°C until assayed.

- CGRP Immunoassays

Plasma samples were assayed for hCGRP using antisera CG39 and CC2/1. The assays were performed according to the protocols detailed in Chapter 2 using a five-day disequilibrium incubation.

3.2.2 Diurnal Variation

The diurnal variation in CGRP was studied in five healthy volunteers who were ambulant throughout the period of study. At the commencement of observations an indwelling catheter was inserted into the forearm of each individual and the line flushed with "Hepsal" (heparinised saline, 200 units/ml) after each blood sample to prevent blockage. Blood samples were taken at 09.00, 10.30, 12.00, 13.30, 15.00, 18.00 and 21.00 hours. The initial sample was taken after an overnight fast following which breakfast was consumed. Subsequent meals were taken after the 12.00 hours sample and the 18.00 hours sample.

In a second experiment two individuals were maintained in a recumbent condition during the day. Samples were taken at hourly intervals between 09.00 and 21.00 hours. Meals were consumed following the 13.00 and 18.00 hours samples.

CGRP was assayed using antiserum CC2/1 according to the protocol in Chapter 2.

3.2.3 Interrelationships between Circulating Calcitonin Gene Peptides

A further group of healthy volunteers was studied to investigate interrelationships between the circulating calcitonin gene peptides. CGRP, calcitonin and katacalcin measurements were performed on fasting 9 am plasma samples from 52 individuals (17 male, 38 female; age range 21-85). Calcitonin was assayed using a 3-day disequilibrium assay, otherwise the assays were carried out according to the protocols in Chapter 2.

3.3 RESULTS

3.3.1 CGRP Normal Range

The distribution of CGRP levels, measured using antiserum CC2/1, is shown in Figure 3.1. The range of CGRP levels was from undetectable to 70 pmol/ℓ but no significant difference was found in those samples between male and female. The CGRP results determined using antiserum CG39 were largely undetectable (94%). The range of those which were detectable extended up to 0.17 nmol/ℓ.

3.3.2 Diurnal Variation

Figure 3.2 shows the CGRP and calcitonin levels for the ambulant volunteers during the period of study. No diurnal variation was clear in either calcitonin or CGRP levels. The CGRP and calcitonin results for the 2 recumbent volunteers are shown in Figure 3.3 but again no diurnal variation was apparent in the circulating levels of either of these peptides.

3.3.3 Interrelationships between Circulating Calcitonin Gene Peptides

Statistical analysis of the peptide results on the second group of normal individuals indicated a significant difference ($p<0.001$) in calcitonin levels between males and females (Figure 3.4) using a Student's t-test, but no such difference was apparent in either katacalcin or CGRP results. No significant correlation was observed between age and any of the three calcitonin gene peptides measured in this group of individuals. However, a weak correlation was observed between calcitonin and katacalcin ($p<0.05$), whilst calcitonin and CGRP levels showed a weak negative correlation ($p<0.05$). No correlation was observed between katacalcin and CGRP levels.

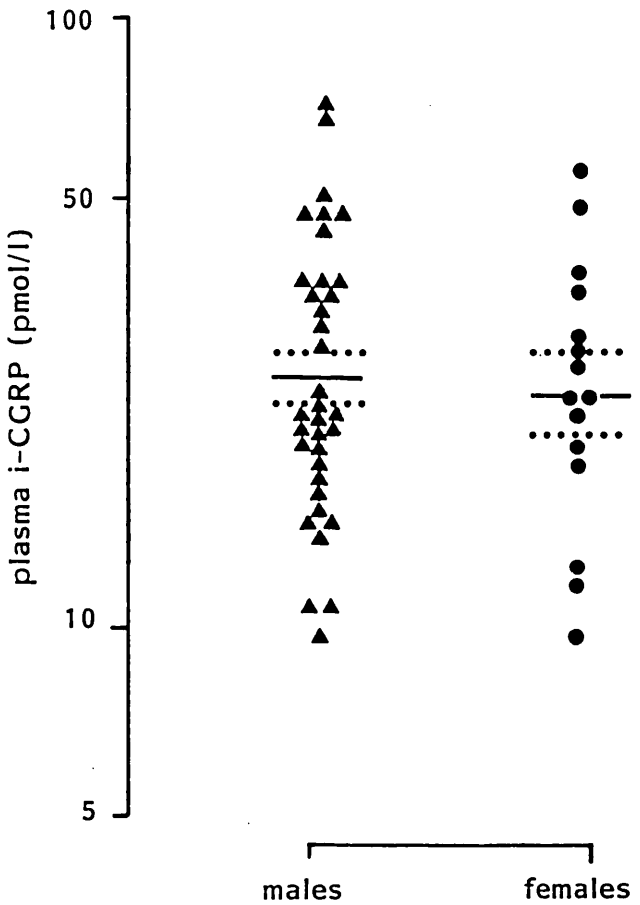

Figure 3.1 Plasma immunoreactive CGRP concentration in 15 normal females (●) and 35 males (▲)

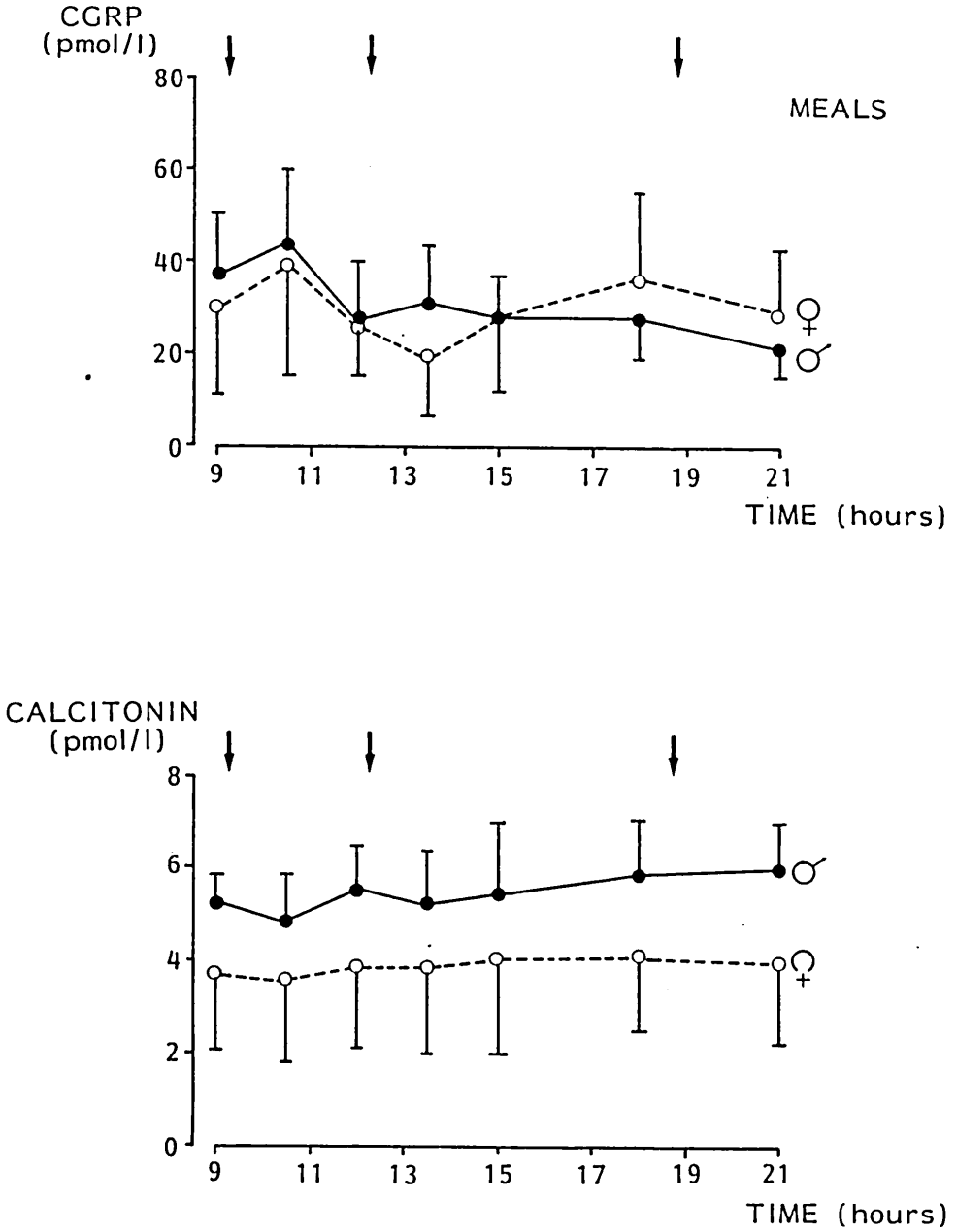

Figure 3.2 Mean plasma CGRP and calcitonin diurnal variation in six ambulant individuals (3 male (●), 3 female (○)) (Mean, SEM)

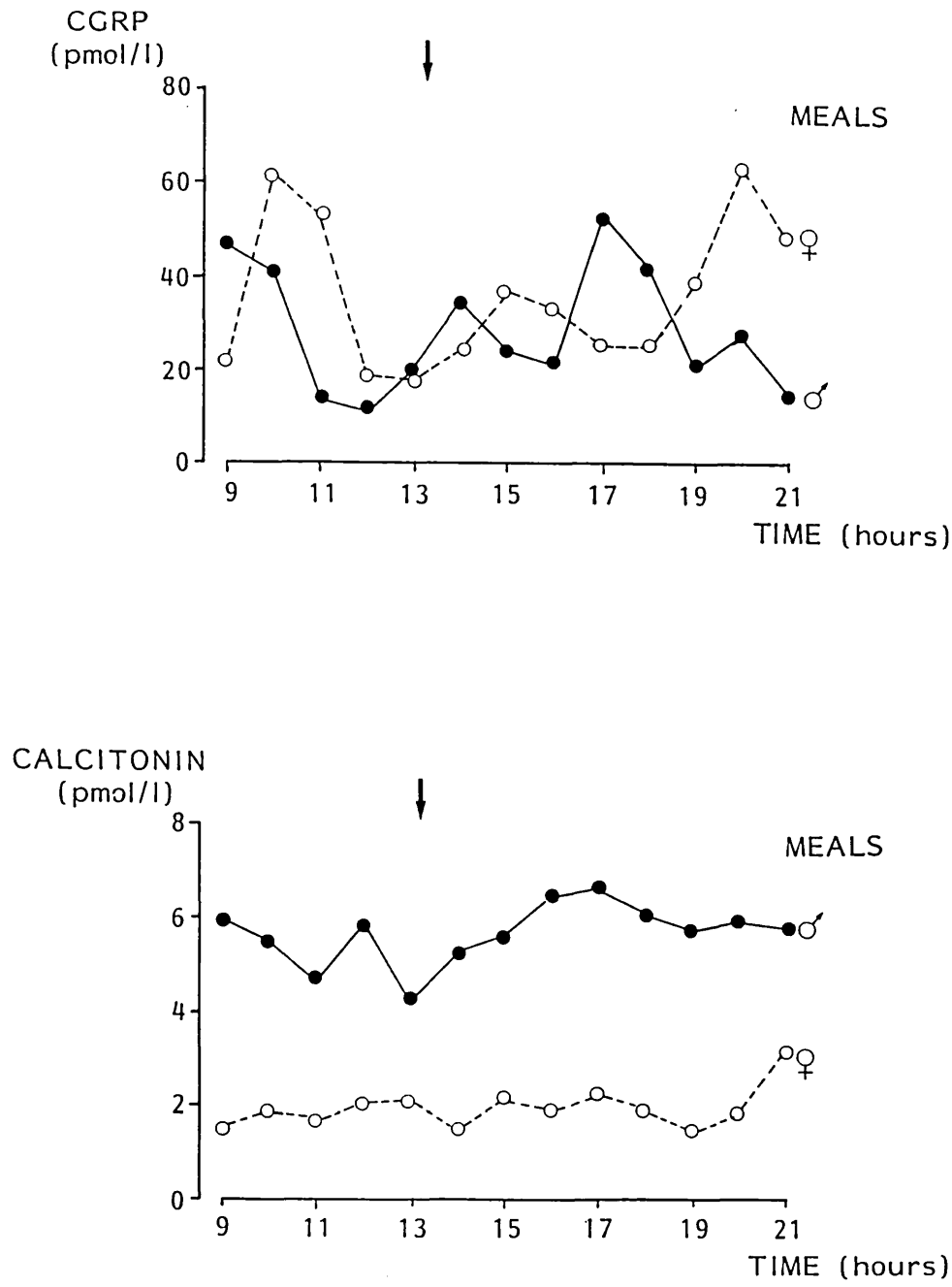

Figure 3.3 Plasma CGRP and calcitonin diurnal variation in two recumbent individuals

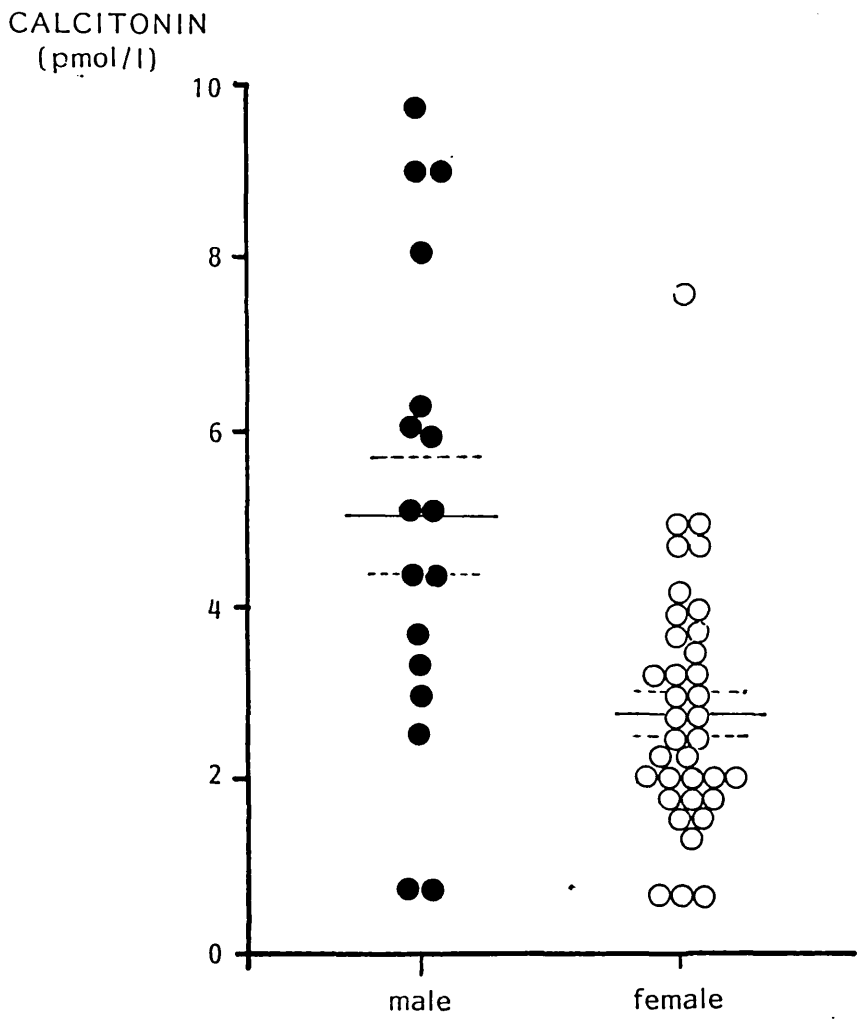

Figure 3.4 Plasma calcitonin levels in normal males and females (mean, SEM)

3.4 DISCUSSION

The development of a sensitive human CGRP RIA (Chapter 2) has facilitated the determination of a normal range for circulating CGRP. Measurement of a CGRP normal range would not have been possible using antiserum CG39 in the previous assay.

The new human CGRP assay was used in the study of diurnal variation in CGRP. The results from each person showed considerable fluctuation over the period of study but no overall pattern was apparent in either ambulant or recumbent investigations.

The calcitonin measurements on this group of individuals were unable to demonstrate a diurnal variation in circulating calcitonin. The contrast between the present findings and those of Hillyard et al (1977) may be a consequence of using different antisera. The real pattern of calcitonin or CGRP secretion may be obscured by the episodic or pulsatile release of these petpides from the c-cells or other sources.

The calcitonin and katacalcin measurements on the samples from the second group of normal individuals showed a weak positive correlation. However this was not an equimolar relationship since katacalcin levels were higher than calcitonin levels. Hillyard et al (1983) found an equimolar relationship in calcitonin and katacalcin levels over a group of normal individuals and MTC patients. The dis-similarity between such observations and the present findings is largely due to low calcitonin values. The calcitonin normal range for this assay has previously been set as up to 80 ng/ℓ (23 pmol/ℓ), whilst the present results suggest the normal range to be considerably lower (up to 10 pmol/ℓ). As the higher level has become established as a decision point for abnormal calcitonin in MTC this point will also be used for defining elevated calcitonin in non-thyroid disease. Motte et al (1986) have found a calcitonin normal range which is even lower using their two-site immunoradiometric assay (IRMA). The discrepancy in normal ranges is probably the result of differences in immunospecificity between different calcitonin antisera. This could mean that OC31 in the improved RIA, like the 2-site IRMA, was more specific for whole calcitonin molecules than other antisera which react with the fragments and precursors of calcitonin present in normal plasma.

Alternatively calcitonin may be cleared from the circulation more rapidly than katacalcin. As a physiological role for katacalcin has not yet been established, then katacalcin receptors may not exist. The binding of calcitonin to receptors as well as metabolic clearance would mean a lesser half life for calcitonin in the blood than katacalcin. The much greater quantities of calcitonin and katacalcin secreted by MTC patients would overcome such a differential effect and present an equimolar relationship in the plasma of such patients .

The application of a direct plasma assay to the measurement of normal circulating immunoreactive calcitonin has confirmed the previous findings using extracted plasma that CGRP circulates at higher concentrations than calcitonin (Girgis et al 1985). CGRP has frequently been localised to peptidergic nerves and therefore the CGRP measured in plasma may have leaked into the circulation from nerve terminals.

The weak inverse relationship between circulating calcitonin and CGRP levels in this group of individuals may have been coincidental. However, if future investigations confirm this observation, interesting questions will be raised concerning control of expression of the calcitonin gene or the secretion of calcitonin and related peptides in normal physiology.

CHAPTER 4 CALCITONIN GENE PEPTIDES IN MEDULLARY THYROID CARCINOMA

4.1 INTRODUCTION

Medullary thyroid carcinoma (MTC) was first identified as a distinct histopathological entity by Hazard in 1959. The tumour was described as a solid, non-follicular structure with a sheet-like growth of cells and stromal amyloid. In 1961 Sipple noted the high incidence of this tumour with phaeochromocytoma and in 1966 Williams suggested that MTC was a tumour of the parafollicular or c-cells of the thyroid.

Medullary thyroid carcinoma is now known to occur in either a sporadic form or as part of an inherited multiple endocrine neoplasia (MEN) syndrome. Patients with MTC are usually classified as sporadic in the absence of any lesions characteristically associated with familial MTC and after the exclusion of familial involvement. Little is known of the aetiology and pathogenesis of sporadic MTC.

The classification of the MEN syndrome has been made according to the pattern of the component disorders. MEN I (Wermer Syndrome), which has anterior pituitary, parathyroid and pancreatic involvement, has been classified as distinct from the MEN syndromes with phaeochromocytoma and MTC (Steiner et al 1968). The remaining MEN syndromes with MTC involvement have been subclassified as MEN II (IIa, classical Sipple's syndrome) and MEN III (IIb) because of the difference in expression of the disease (Khairi et al 1975, Norton et al 1979). Patients exhibiting MTC associated with phaeochromocytoma and hyperparathyroidism are classified as MEN type II whereas MEN III involves MTC, phaeochromocytoma and associated mucosal neuromas, skeletal abnormalities and marfanoid habitus, giving the patient a distinct appearance.

Following the observations that calcitonin was produced by the parafollicular or c-cells of the thyroid (Foster et al 1964) and that MTC was a tumour of the c-cells (Williams 1966), calcitonin was detected in MTC tissue and plasma using bioassay (Cunliffe et al 1968, Melvin and Tashjian 1968).

Radioimmunoassays for calcitonin were subsequently developed (Clark et al 1969, Tashjian et al 1970) which greatly enhanced calcitonin measurements in MTC. The value of calcitonin measurements for the detection and follow up of MTC is now well established. Elevated calcitonin is measurable in almost all patients with clinically detectable MTC and in some patients who show no clinical signs (Jackson et al 1973). Tumour mass generally correlates well with calcitonin levels (Wells et al 1978b) although some disparity has been reported (Trump et al 1979). Patients with metastases usually have higher calcitonin levels than those in which the disease is localised to the thyroid (Stepanas et al 1979).

Calcitonin RIA is particularly important in widespread screening of families at risk from MTC. Familial MTC is thought to develop from areas of c-cell hyperplasia often bilateral and multifocal (Wolfe et al 1973, Carney et al 1979). A proportion of patients with MTC or c-cell hyperplasia may have undetectable basal levels of calcitonin and consequently several provocative tests have been developed to stimulate calcitonin secretion in such cases of latent hypercalcitoninaemia.

- Calcium Infusion

A 4-hour calcium infusion of 15 mg/kg body weight was originally used to provoke calcitonin secretion (Tashjian et al 1970). Unfortunately this was a time-consuming test, often causing nausea and vomiting. Shorter infusions of 2 mg calcium per kg body weight (given intravenously over a 1 minute period) were later introduced (Rude and Singer 1977) offering a more rapid test with reduced side effects.

- Pentagastrin Test

A rapid infusion of 0.5 μg/kg body weight pentagastrin induced a marked increase in plasma calcitonin in MTC patients, reaching a peak between 2 and 10 minutes post-injection (Hennessy et al 1973). However the test is accompanied by unpleasant side effects and abdominal discomfort (Emmertsen et al 1980).

- Whisky Test

The whisky test involves oral administration of 50 ml whisky to stimulate hCT secretion (Dymling et al 1976), which peaks around 15 minutes after the ingestion of alcohol. Whilst this test is generally more acceptable to patients than pentagastrin and calcium, the effects of consuming alcohol after a fast may be disconcerting, particularly for children.

In a comparison of stimulation tests, Emmertsen et al (1980) observed pentagastrin to give the greatest calcitonin stimulation overall but recommended the additional use of short term calcium infusion in equivocal cases. A provocative test combining pentagastrin and short term calcium infusion has now been introduced (Wells et al 1978A).

Whilst calcitonin remains the principle tumour marker for MTC, several other substances have been variously associated with this disease including ACTH, alpha-subunit human chorionic gonadotrophin, gastrin releasing peptide, serotonin, insulin, glucagon and the enzymes histaminase and dopadecarboxylase (Goltzman et al 1979, Lippman et al 1982, Rasmusson 1984, Wurzel et al 1984, Ghatei et al 1985, Sikri et al 1985). Carcinoembryonic antigen (CEA) has also been found to be secreted in MTC (Ishikawa and Hamada 1976). The doubling time of CEA has been correlated with disease progression but CEA was not found to be diagnostic of MTC in more than about three-quarters of patients (Busnardo et al 1984, Saad et al 1984A).

New tumour markers and prognostic indicators are being sought to confirm the diagnosis of MTC and assist the clinician in monitoring progress of the disease. The measurement of katacalcin and CGRP may offer potentially useful information when investigating MTC patients and the present chapter describes studies on circulating calcitonin gene peptides in this disease.

4.2 PATIENTS AND METHODS

4.2.1 Circulating Peptides

- Patients

Forty-five patients with histologically proven MTC were studied of which 30 had sporadic disease (11 male, 19 female; age range 21-76 years). The remaining 15 patients had familial MTC; 3 had familial MTC alone (1 male, 2 female; age range 32-49), 8 had MTC as part of MEN II (2 male, 6 female; age range 17-55 years) and 4 as part of MEN III (all male, age range 22-29).

- Assays

Fasting venous blood samples were taken from each patient, the plasma separated and stored at -20°C until assayed. The samples were assayed for calcitonin, katacalcin and CGRP (using both antisera) as described in Chapter 2.

4.2.2 Provocative Testing

- Whisky Tests

Radioimmunoassays for calcitonin, katacalcin and CGRP were performed on plasma samples from 3 patients and 5 normal volunteers. Whisky (50 ml) was administered orally and venous samples taken before and at 3, 10, 15 and 30 minutes after ingestion.

- Pentagastrin Stimulation Tests

Plasma samples from 4 patients who had undergone pentagastrin tests were assayed for the calcitonin gene peptides. Normal volunteers were not available for comparison but the unaffected siblings of certain patients had also undergone pentagastrin tests. Samples from combined pentagastrin-calcium infusion tests on 2 patients were also investigated.

4.2.3 Localisation

Selective venous catheterisation was performed on a patient (H) to localise the calcitonin secreting tumour. Blood samples were collected from various sites and and plasma stored at -20°C. The samples were analysed for calcitonin and CGRP (using antiserum CC2/1).

4.3 RESULTS

4.3.1 Circulating Peptides

Circulating levels of both calcitonin and katacalcin were elevated in all but 3 patients, whose katacalcin levels were undetectable. In the group of patients studied these two peptides were found to circulate in approximately equimolar amounts (Table 4.1, Figure 4.1). CGRP levels were elevated above normal in approximately 50% of patients using both antisera. Levels of CGRP correlated with each other (Table 4.1) although antiserum CG39 gave higher measurements than CC2/1. CGRP levels correlated weakly with both calcitonin and katacalcin (Table 4.1).

Statistical analysis of the results using two tailed unpaired Student's t-tests indicated that patients with sporadic MTC were significantly older than those with familial disease ($p<0.001$). Calcitonin and katacalcin levels were not signifcantly different between the two groups (Figure 4.2); however CGRP levels were significantly higher ($p<0.03$) in patients with sporadic tumours than in the familial group, using both antisera (Figures 4.3 and 4.4).

4.3.2 Provocative Testing

The results of the whisky stimulation tests on 3 patients and 5 normal volunteers are shown in Figure 4.5. Calcitonin and katacalcin levels were elevated above normal in the three MTC patients. Calcitonin levels reached a mean peak of 3 times basal levels whilst katacalcin peaked at a mean of 1.8 times basal. CGRP levels in 2 of these pateints were within the normal range whilst the third showed no stimulation.

No stimulation of either calcitonin, katacalcin or CGRP was observed for the 5 normal volunteers following the administration of whisky.

Figures 4.6 and 4.7 show the results of the pentagastrin and combined pentagastrin-calcium tests. Whilst calcitonin and katacalcin both stimulated in response to provocation, CGRP was only stimulated in 3 of the patients with advanced MTC (katacalcin results not available on 3 patients). The results of pentagastrin tests on unaffected siblings of MTC patients were within normal ranges.

Table 4.1 Correlation coefficients for plasma calcitonin, katacalcin and CGRP levels in MTC

	calcitonin	katacalcin	CGRP CC2/1
katacalcin	0.90***		
CC2/1	0.55***	0.56***	
CGRP CG39	0.40**	0.35*	0.82***

* $p<0.02$

** $p<0.01$

*** $p<0.001$

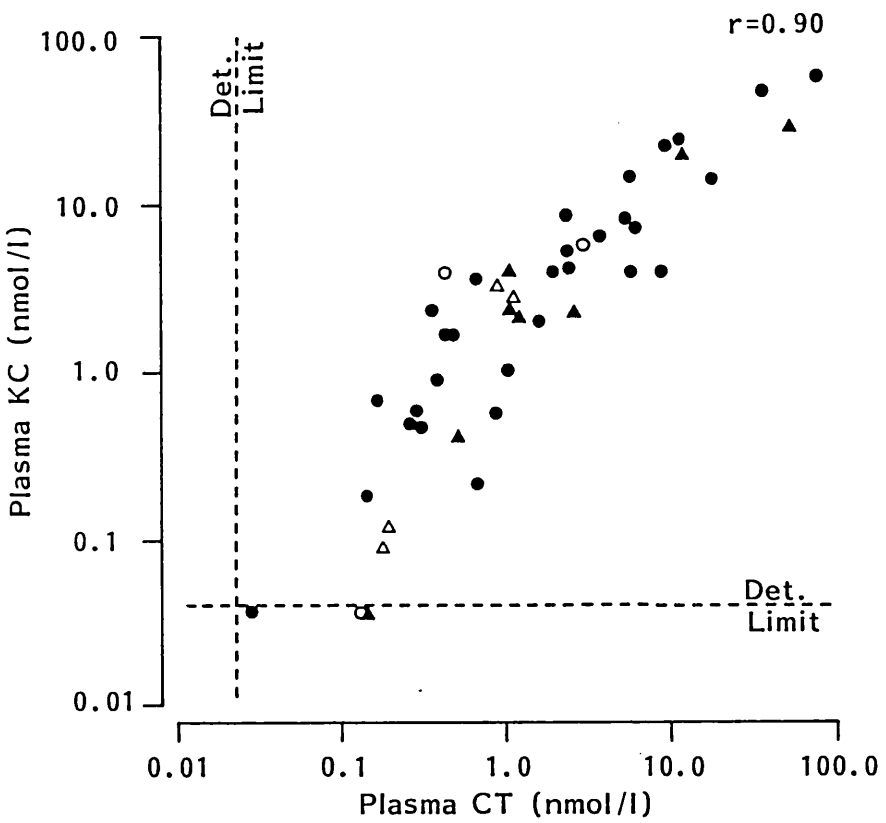

Figure 4.1 Correlation between plasma calcitonin and katacalcin levels in MTC (Sporadic MTC (●), Familial MTC alone (○), MEN II (▲) and MEN III (△))

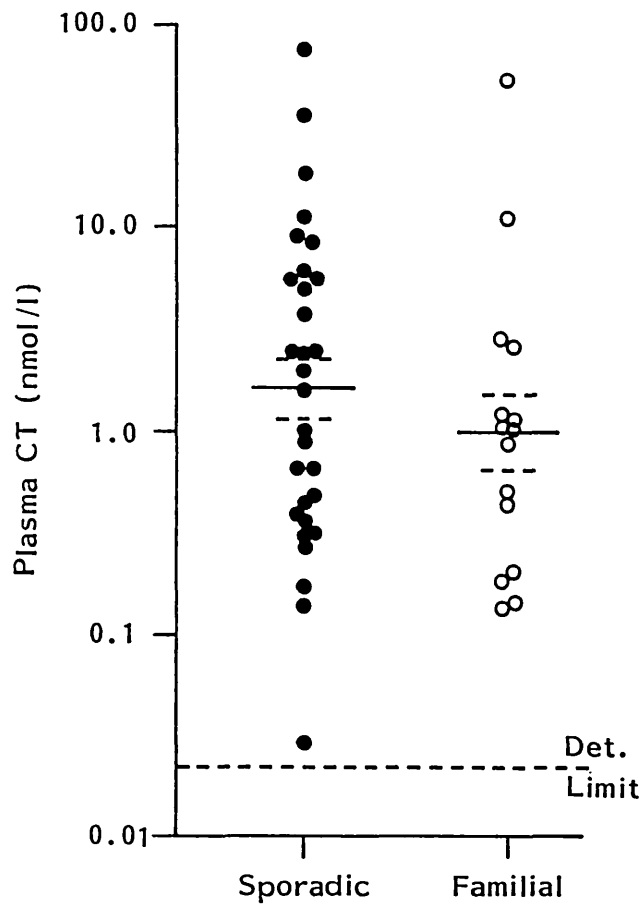

Figure 4.2 Plasma calcitonin levels in familial and sporadic MTC (mean, SEM)

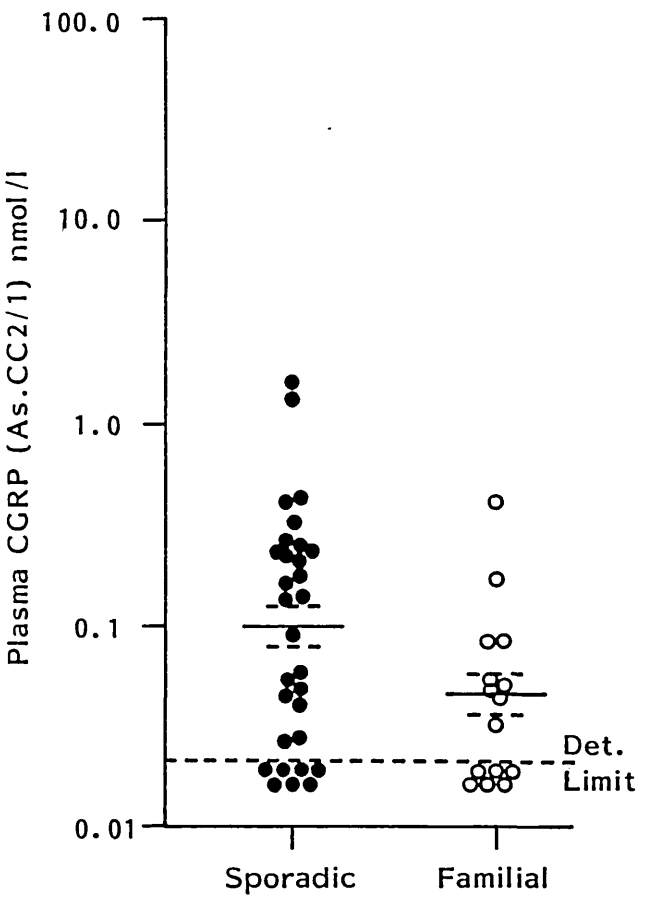

Figure 4.3 Plasma CGRP levels (antiserum CC2/1) in familial and sporadic MTC (mean. SEM)

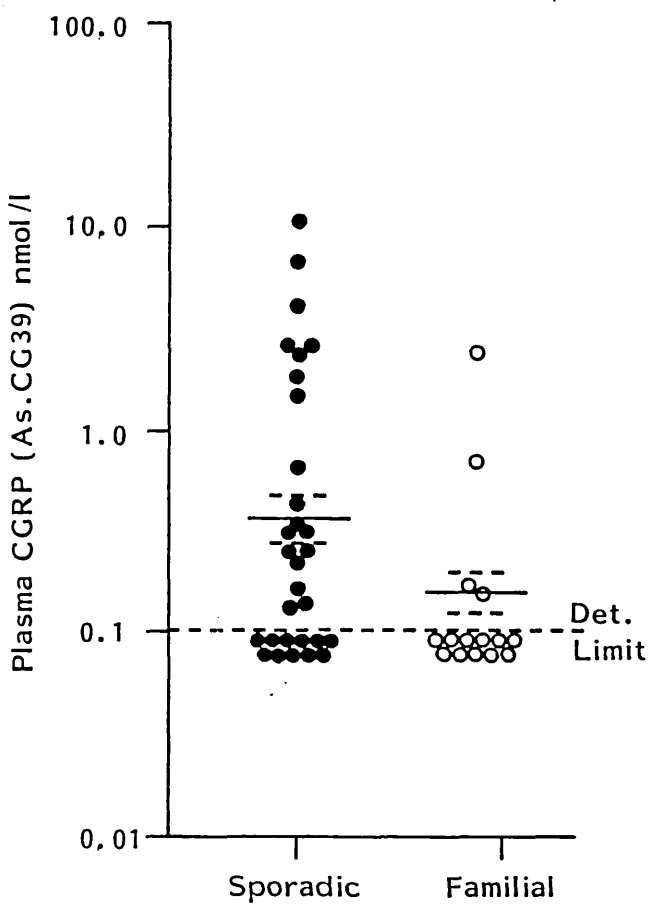

Figure 4.4 Plasma CGRP levels (antiserum CG39) in familial and sporadic MTC (mean. SEM)

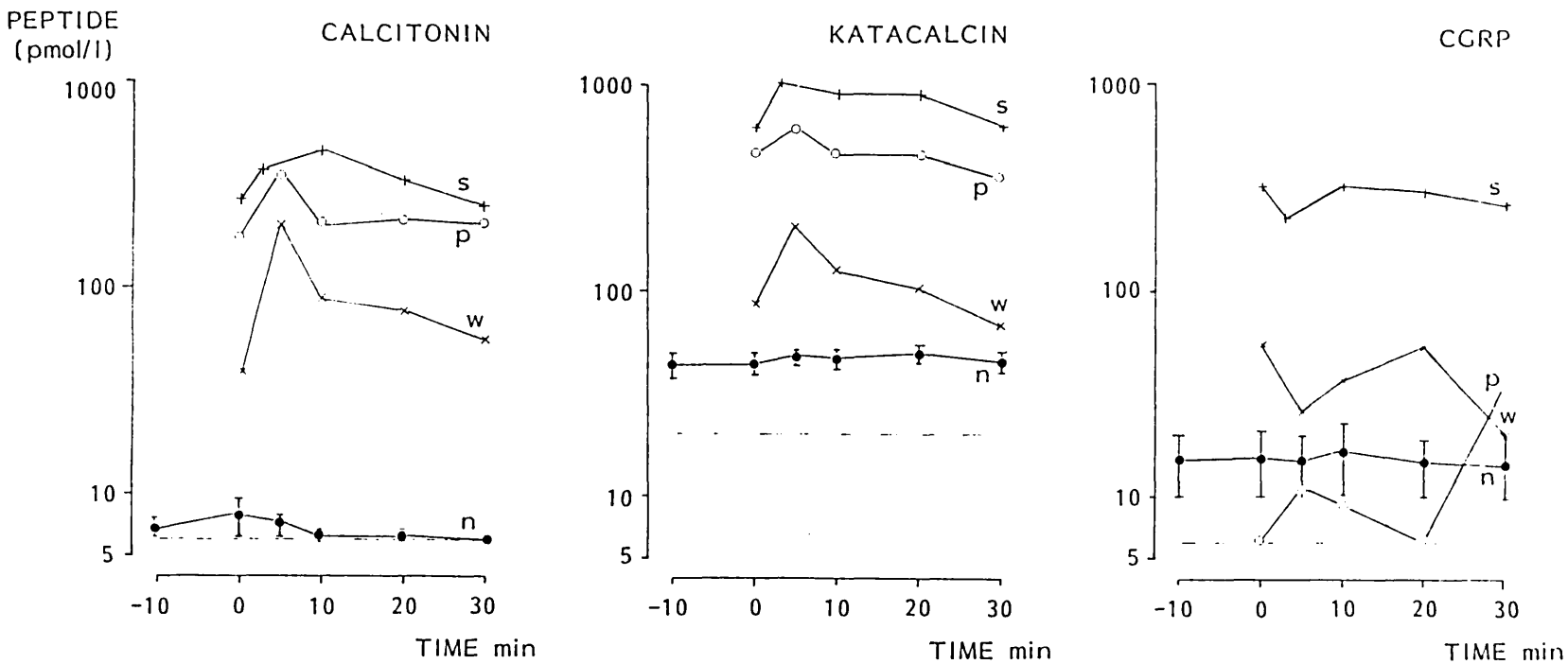

Figure 4.5 Whisky stimulation tests on normal volunteers (n=mean of 5) and MTC patients (S, P and W). (mean. SEM) (Detection limit indicated by dotted line.)

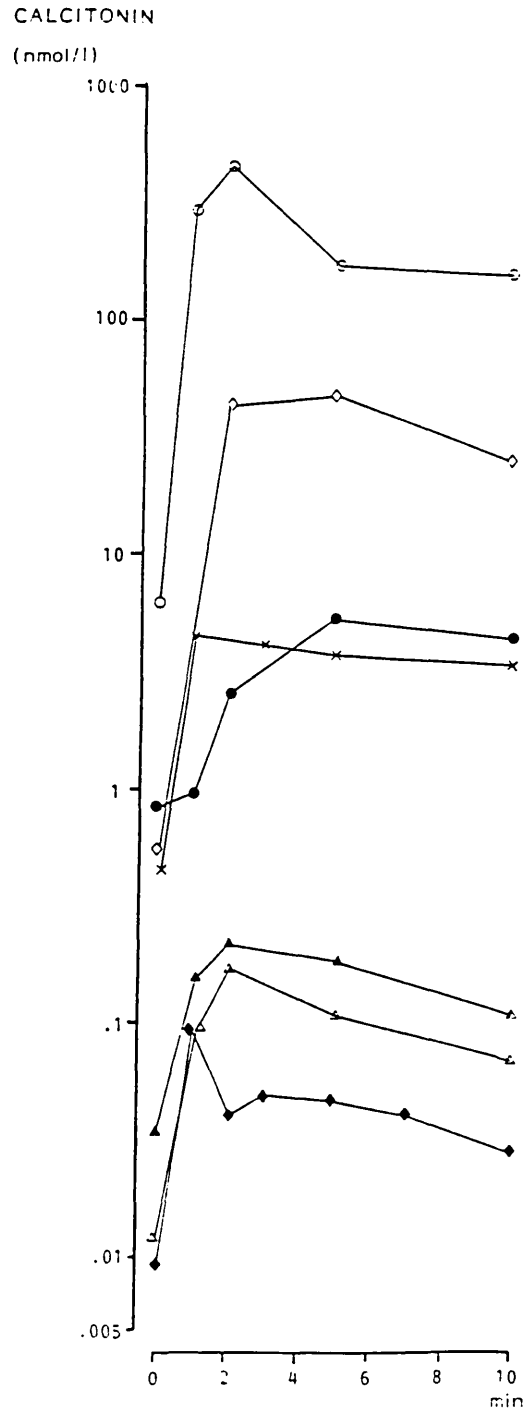

Figure 4.6 Plasma calcitonin levels during pentagastrin and combined pentagastrin/calcium stimulation tests on MTC patients

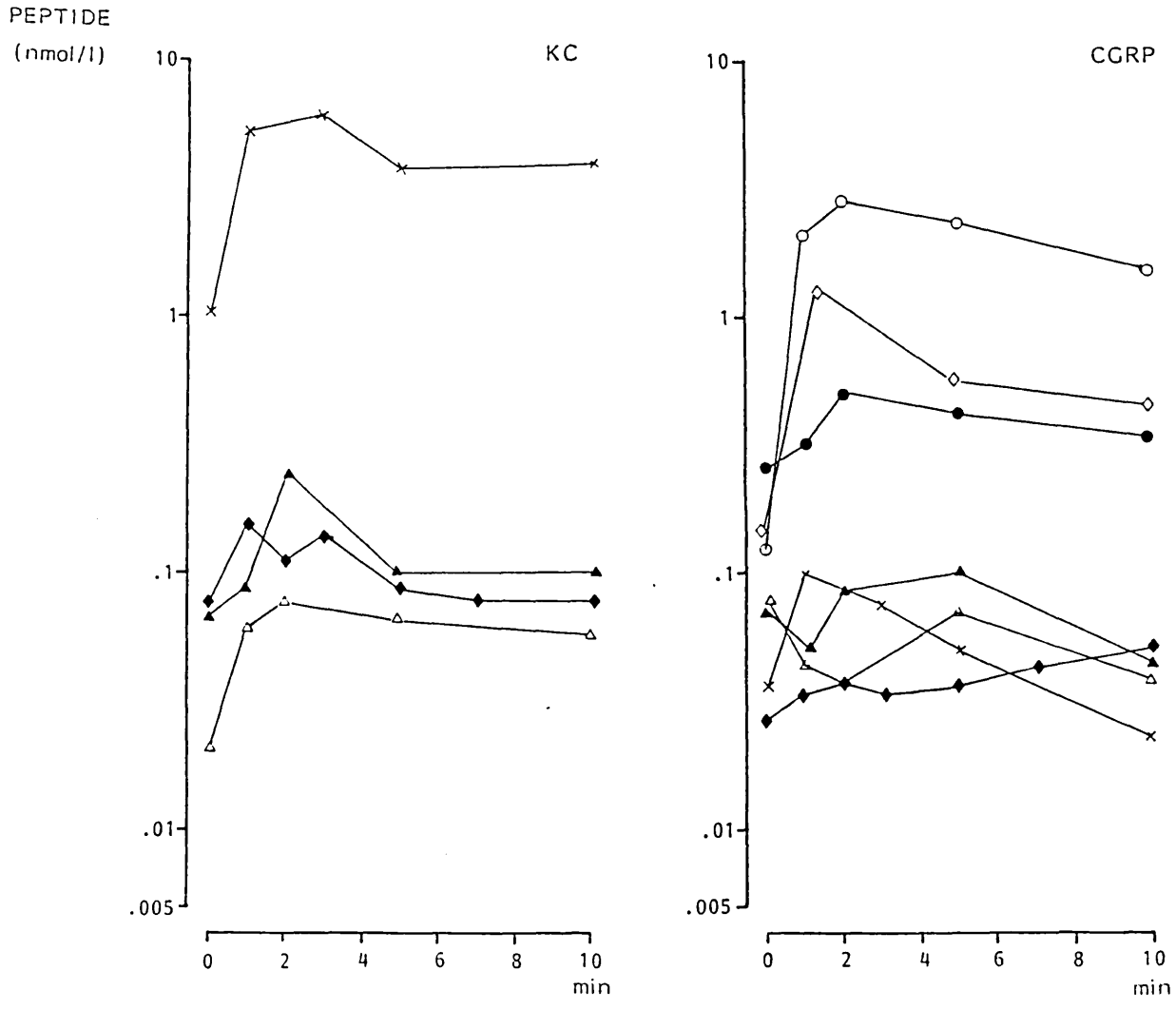

Figure 4.7 Plasma katacalcin and CGRP levels during pentagastrin and combined pentagastrin/calcium stimulation tests on MTC patients

4.3.3 Localisation

The measurements of calcitonin and CGRP on plasma samples taken during selective venous catheterisation on Patient 'H' are shown in Figure 4.8. Calcitonin levels were generally elevated but indicated a possible tumour bearing area of the left thyroid. This finding was corroborated by a thyroid ultrasound which showed an abnormal structure on the left side. CGRP measurements were higher in the vicinity of the left superior thyroid vein and the right adrenal vein, suggesting the presence of CGRP secreting tissue in these areas.

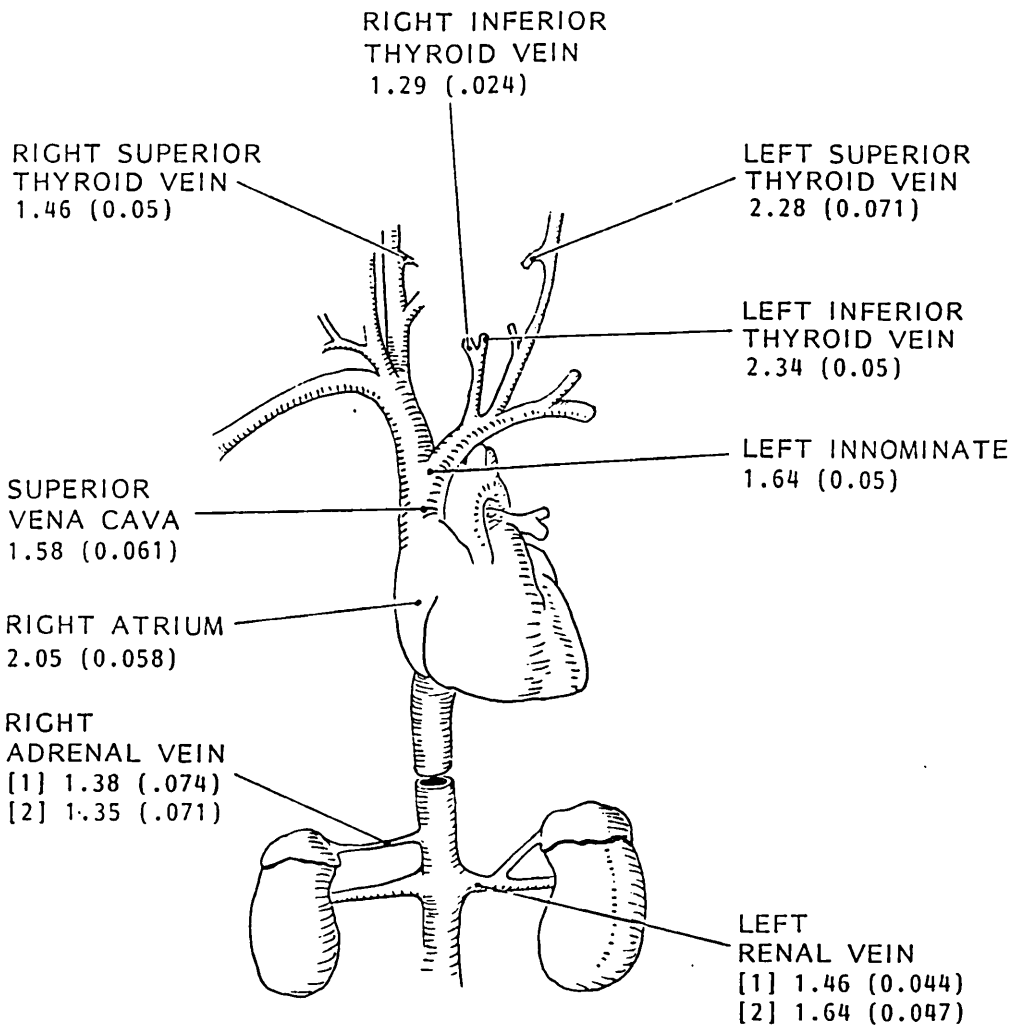

Figure 4.8 Selective venous catheterisation on a patient with MEN II. Calcitonin levels are given at each site with CGRP levels in brackets (nmol/ℓ)

4.4 DISCUSSION

The search for new tumour markers in the diagnosis and follow up of MTC is necessary to enhance the information available from clinical examination and calcitonin measurements. The ideal tumour marker should have the following characteristics:

(i) be related to the viable mass of tumour cells

(ii) be unique to the neoplastic tissue

(iii) be easily measured by specific, precise, inexpensive assays capable of automation.

Additionally the tumour marker may:

(iv) serve as an aid to early cancer detection

(v) locate the neoplasm by demonstrating venous concentration gradients of the given hormone

(vi) detect recurrent tumours.

Calcitonin has long been established as a useful marker for MTC fulfilling many of these criteria.

In the present study of 45 cases of MTC, katacalcin and calcitonin circulated in approximately equimolar amounts (correlation coefficient $r=0.9$) confirming the previous observations of Hillyard et al (1983). As calcitonin and katacalcin are present in the same precursor molecule, preprocalcitonin, then these two peptides may be processed and released into the circulation from the same secretory granules. Furthermore, as several heterologous forms of calcitonin have been reported to be secreted from MTC tumours (Becker et al 1978, Tobler et al 1983), a portion of the immunoreactive calcitonin material in the plasma may be in the form of preprocalcitonin and consequently immunoreactive to antikatacalcin antibodies in an equimolar proportion.

In this study 3 of the patients had undetectable katacalcin levels with detectable calcitonin; however, other workers have observed elevated katacalcin in all their patients (Ittner et al 1985, Hillyard et al 1983). In the provocative tests katacalcin was stimulated with calcitonin. Katacalcin offers an extremely useful alternative to calcitonin as a tumour marker for MTC and the choice of assay for a particular laboratory is probably dependent on the detection limit and quality of the assays available to that laboratory.

Plasma CGRP measurements on the MTC patients in this study showed elevated levels, above the normal range in only about 50% of samples. Whilst there is a weak correlation between circulating CGRP and calcitonin (r=0.55 for CC2/1) this relationship is not equimolar. Elevated CGRP levels were generally observed in those patients with the highest calcitonin levels. Similar observations have been made by Mason et al (1986) and Schifter et al (1986).

The use of provocative tests for calcitonin secretion induced increases in circulating CGRP in advanced cases of MTC. However, those cases with low basal levels of calcitonin did not show a CGRP stimulation with the peak of calcitonin. These findings were consistent with those of Mason et al (1986). CGRP does not therefore represent as good a tumour marker for MTC as either calcitonin or katacalcin, particularly for the early detection of disease. However, CGRP may prove useful in locating the site of suspected thyroid tumours, via selective venous catheterisation, particularly in patients where calcitonin levels are extremely high throughout the circulation.

The presence of CGRP in some patients may be the cause of some of the symptoms of MTC. CGRP has been reported to be a potent vasodilator (Brain et al 1985) and to cause contraction of the guinea-pig ileum (Tippins et al 1984). The cutaneous flushing and diarrhoea associated with MTC may thus be attributable to CGRP, although calcitonin has also been implicated as a major cause of diarrhoea in MTC (Cox et al 1979).

A large proportion of new cases of MTC are diagnosed following histochemical examination of thyroidal material removed during surgery. Investigations are subsequently undertaken to ascertain whether the disease is of a sporadic or familial nature. Whilst a positive family history of MEN lesions can prove familial MTC, this condition cannot be disproved by a negative family history. Extensive screening of the patient's family may be necessary to identify family members at risk of MEN. Such screening is costly, time-consuming and may create needless anxiety in relations of patients with sporadic MTC.

Certain histopathological criteria exist which can give an indication of the nature of the disease (Block et al 1980). Familial MTC is thought to develop from a clone of affected cells (probably via c-cell hyperplasia) and consequently the disease may present as multicentric lesions. Sporadic disease usually presents as unicentric tumours without c-cell hyperplasia. Familial tumours tend to be located in the upper central portion of each lobe whereas sporadic tumours frequently occur in the centre of the thyroid lobe.

The present study on circulating calcitonin gene peptides in MTC has indicated a different pattern of CGRP secretion in patients with sporadic disease when compared to familial disease. These findings may be biased because family screening means that patients with familial MTC are usually diagnosed at an earlier age than sporadic MTC and before clinical signs of the disease appear. Familial MTC patients may therefore have smaller tumours. The ages of the two groups of patients in this investigation were indeed significantly different; however, their calcitonin and katacalcin levels were similar. Since calcitonin levels are thought to correlate well with tumour mass (Wells et al 1978B, Stepanas et al 1979) then the familial and sporadic groups of patients probably had similar tumour loads. The present observation of significantly higher circulating CGRP levels in sporadic MTC when compared with familial MTC may be useful in distinguishing between the two types of disease. A high level of CGRP would suggest the likelihood of sporadic disease particularly in younger patients.

The difference in CGRP levels between those two groups may be related to the different patterns of calcitonin immunostaining in MTC tumours observed by Saad and colleagues (1984B). This group noted that 83% of patients with MEN II had calcitonin-rich tumours, whereas 78% of sporadic tumours were relatively calcitonin-poor, despite the fact that circulating calcitonin levels were not significantly different. The proportion of calcitonin immunostaining in the cells of the tumour may reflect the degree of differentiation of the cells, with MEN II patients having more differentiated calcitonin-rich tumours than sporadic patients.

Sikri et al (1985) have reported immunocytochemical studies on a range of MTC tumours which showed CGRP localisation in secretory granules to be more sporadic than calcitonin localisation. CGRP was present mainly in those cells which were heavily granulated and, although double immunostaining showed CGRP and calcitonin to be localised in the same granules, serial sections indicated that certain cells exhibited more intense CGRP immunoreactivity. Furthermore, the switching of an MTC cell line from high to low calcitonin production led to the discovery of CGRP. Thus the less differentiated cells observed in a large proportion of sporadic tumours could have switched their peptide production to increase the ratio of CGRP to calcitonin or altered their ability to secrete peptides.

Lippman et al (1982), also investigating calcitonin immunostaining in MTC tissue, concluded that cellular heterogeneity was associated with a virulent neoplasm. Trump et al (1979) reported a case of MTC with a low plasma calcitonin in the presence of widespread metastases. Elevated CGRP levels may therefore be related to aggressive metastatic tumours and hence the observed difference in circulating CGRP between sporadic and familial MTC may result from the less aggressive nature of familial MEN II disase rather than its hereditary origin. To differentiate between MEN II and the more aggressive MEN III larger numbers of patients need to be studied.

A long-term, age and sex matched study on sporadic and familial patients is needed to elucidate the role of CGRP in MTC.

Calcitonin and katacalcin remain the principle diagnostic tumour markers for MTC, the choice of peptide being dependent on the available assays. CGRP was inferior to both calcitonin and katacalcin but may prove to be a useful adjunct to calcitonin in selective venous catheterisation. Additionally circulating CGRP may be a prognostic indicator or a means of distinguishing sporadic and familial disease.

CHAPTER 5 CALCITONIN GENE PEPTIDES IN NON-THYROID TUMOURS

5.1 INTRODUCTION

The term Ectopic Hormone was coined with reference to the concept of tumour products which cause remote effects resembling those of known hormones and are inappropriate to the tissue of origin of the tumour.

The thyroidal c-cells are the major site of calcitonin production in man and the eutopic production of calcitonin in tumours of this cell type was described in the previous chapter. The present chapter investigates the possible non-thyroidal (ectopic) production of calcitonin and the related product, CGRP, in cancer. Several tumour types have previously been reported to secrete ectopic calcitonin including midgut carcinoids, phaeochromocytoma, lung and breast cancer (Abe et al 1977, Milhaud et al 1974, Coombes et al 1974).

Carcinoid tumours are endocrine tumours of the gastrointestinal tract which are frequently associated with ectopic hormone secretion (Goedert et al 1980). They are thought to originate in the enterochromaffin cells distributed thoughout the gastrointestianal tract, pancreas, bronchial epithelium and urogenital tract. The hepatomegaly, flushing, diarrhoea, endocardial lesions and broncho-constriction of classical carcinoid syndrome are frequently associated with midgut carcinoids (Kaplan 1978). Some of these symptoms have been attributed to over-production of serotonin (5-hydroxytryptamine, 5HT) by midgut carcinoids.

Phaeochromocytomas usually arise in the adrenal medulla, a tissue of neuroectodermal origin like the thyroidal c-cells. Occasionally phaeochromocytomas also arise in the extra-adrenal paraganglion. Phaeochromocytomas generally occur sporadically; however, bilateral tumours are seen in familial disease which may be present as part of MEN syndrome (see previous chapter) or in association with neuroectodermal dysplasias (Scott 1978). The symptoms of phaeochromocytoma are accredited to the release of excessive amounts of adrenalin and noradrenalin from the tumour. These symptoms include hypertension (persistent or intermittent), headaches, sweating, nervousness and facial pallor.

Ectopic hormone secretion has also been observed in lung cancer (Gropp et al 1980), one of the major causes of cancer-related deaths. There are 4 main histological types of lung cancer (Aisner & Matthews 1985) which have different patterns of disease spread and consequently different treatment programmes and prognoses.

Small cell carcinomas metastasise widely and many patients present with disseminated disease. Whilst small cell carcinoma of lung (SCCL) is frequently inoperable because of its aggressive spread, it usually responds to radiotherapy and chemotherapy.

Non small cell carcinomas (NSCCL) include squamous cell carcinoma, adenocarcinoma and large cell carcinoma which do not metastasise as early as SCCL. Localised tumours offer the opportunity for resection.

Breast cancer is one of the most common malignancies in women. The progression of the disease can be staged according to the size of the tumour and spread of metastases, with stage 1 referring to small tumours less than 2 cm and stage 4 referring to tumours with distant metastases (Keys et al 1983). A tumour marker for the early detection of breast cancer is required to take full advantage of the available treatment at the earliest opportunity.

Several hypotheses have been proposed to explain the phenomenon of ectopic hormone secretion by neoplastic tissue (Rees & Ratcliffe 1974).

The 'sponge' hypothesis proposes that tumours adsorb peptides from the circulation, rather than synthesising new hormone, and that such peptides are released on cell death. Observations on the release of peptides from tumour cells in short and long term culture tend to discredit this theory for certain neoplastic tissues.

The abnormal genome hypothesis requires that extensive base substitution occurs within the nuclear DNA which would conveniently code for peptide sequences with some biological activity. However many ectopically produced hormones have precursor and fragments similar to the hormone produced by the normal endocrine gland.

The endocrine cell hypothesis holds that endocrine tumours arise in cells that previously made a different hormone e.g. APUD cells, and hence are derived from a single cell type. A switch in gene function

within such an endocrine cell would enable it to secrete ectopic hormones. Whilst the extent of diffuse endocrine systems is at present unknown, ectopic hormone production by non-endocrine tumours such as adenocarcinomas and squamous cell tumours does not fit this hypothesis. Furthermore gastrointestinal and pancreatic endocrine cells have not been shown to be of neural crest origin unlike other APUD cells (pituitary, adrenal medulla and thyroid c-cells).

The derepression hypothesis postulates that all somatic cells possess the potential to develop into any cell in the body but that differentiation inactivates or represses certain genes. Thus derepression caused by mutation, rearrangement or deletion of portions of the genome may result in ectopic hormone synthesis. Neoplastic tissue can be classified into 4 main groups with progressively increased derepression:

- Simple hyperfunction; when the hormone release is normally produced by that tissue, e.g. calcitonin in MTC;
- First order regression; where the peptide released is characteristic of another tissue derived from the same embryological cell line, e.g. calcitonin production by phaeochromocytomas;
- Second order regression; where the tumour releases hormone which is normally produced by cells of an unrelated cellular origin, e.g. calcitonin production in breast cancer;
- Primitive expression; where the production of peptides by a tumour which are normally produced in the foetus.

5.2 CARCINOIDS

5.2.1 Patients

Fourteen carcinoid patients were studied (7 male, 7 female; age range 18-73) all of which demonstrated some of the symptoms of carcinoid syndrome. The primary tumour site in 6 of the patients was ileal but was unknown in the remaining patients. Two samples were obtained from patient 5, one of which was taken during a flushing episode, and the other during a phase when no flushing was evident.

Calcitonin gene peptides were measured on the carcinoid patients according to the methods in Chapter 2. Katacalcin was measured only in patients 5-13, insufficient sample was available for the remaining patients.

5.2.1 Results

Table 5.1 lists the patients studied, the location of the primary tumour (where known) and the symptoms of carcinoid syndrome which were reported. The results of the calcitonin and CGRP measurements on the 15 patients are shown in Table 5.1 and Figure 5.1. Three of the patients (Nos 9, 10, 12) had calcitonin levels elevated above normal (20%), one of which (No 12) was grossly increased. Katacalcin results were increased in patients 9, 10 and 12, reflecting calcitonin results. CGRP levels were also elevated in 2 patients: Nos 12 and 14. CGRP and calcitonin levels were indistinguishable in both the flushing and non-flushing samples of patient 5.

No statistically significant relationship was observed between calcitonin and CGRP levels in carcinoid patients following statistical analysis.

Patient information was provided by clinicians via a questionnaire designed by the author.

Table 5.1 Carcinoid patients: site, and peptide levels

PATIENT	PRIMARY TUMOUR	HEPATIC METAS-TASES	DIAR-RHOEA	FLUSH-ING	CARDIAC LESIONS	BROCHO-CONSTRIC-TION	PLASMA CALCITONIN (pmol/l)	PLASMA CGRP (pmol/l)
1	ILEAL	YES	++	++	-	-	<6	42
2	?	YES	++	++	-	-	<6	15
3	ILEAL	YES	++	++	-	+	9	33
4	?	YES	++	+++	++	-	11	26
5	ILEAL	YES	+	+	-	+	10	<10
6	ILEAL	YES	++	++	-	-	22	25
7	?	YES	+++	++	-	-	20	<10
8	?	YES	++	++	+++	-	23	36
9	?	YES	++	+	-	-	67	30
10	?	YES	++	+	-	-	35	33
11	?	YES	++	+	-	-	23	13
12	?	YES	+++	-	++	-	30,430	75
13	ILEAL	YES	++	++	-	-	6	<10
14	ILEAL	?	?	+++	?	?	9	726

Symptoms: + mild
++ moderate
+++ severe
? unknown

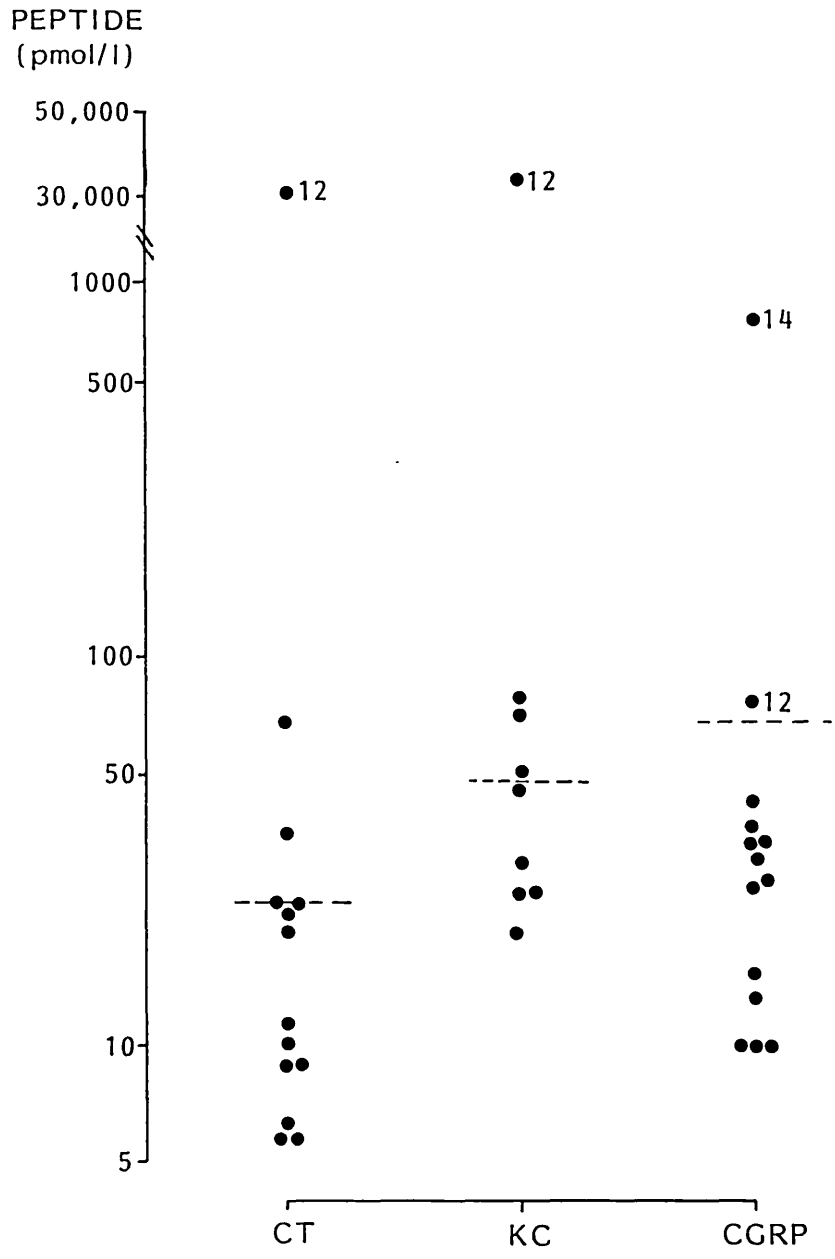

Figure 5.1 Circulating levels of calcitonin gene peptides in carcinoid patients (upper limit of normal shown by dashed line)

5.3 PHAEOCHROMOCYTOMA

5.3.1 Patients

Nineteen patients with phaeochromocytoma and one neuroblastoma were studied (7 male, 13 female; age range 2-71). Tumours from eleven of the patients were located in the adrenal glands, 4 of which were malignant and one bilateral. The remaining tumours were extra-adrenal including a para-vertebral phaeochromocytoma, a chemodectoma and a malignant bladder phaeochromocytoma.

Calcitonin and CGRP were measured in these patients according to the methods in Chapter 2. Katacalcin was not measured because of insufficient sample.

5.3.2 Results

Calcitonin was elevated in 2 patients with phaeochromocytoma. One, with a malignant phaeochromocytoma, had a minor elevation; but the other patient with bilateral adrenal phaeochromocytoma had a calcitonin level markedly greater than the normal limit. CGRP levels were again largely normal with elevated levels present in the neuroblastoma patient and in the patient with a malignant bladder phaeochromocytoma.

The distribution of plasma calcitonin and CGRP levels is shown in Figure 5.2 and Table 5.2. Analysis of the results indicated no correlation for either calcitonin or CGRP with either adrenalin or noradrenalin in these patients. However a correlation between calcitonin and CGRP was just significant ($p=0.05$).

Patient information was obtained from Mr R Causon, Clinical Pharmacology, Hammersmith Hospital.

Table 5.2 Plasma levels of calcitonin and CGRP in patients with phaeochromocytoma

PATIENT	AGE	SEX	TUMOUR	PLASMA CALCITONIN (pmol/l)	PLASMA CGRP (pmol/l)
1	57	f	Extra adrenal phaeochromocytoma	13	20
2	39	m	Chemodectoma	<6	17
3	25	f	Pelvic phaeochromocytoma	<6	24
4	2	m	Neuroblastoma	22	75
5	65	f	Left adrenal phaeochromocytoma	<6	17
6	28	m	Left adrenal phaeochromocytoma	<6	26
7	45	f	Malignant phaeochromocytomas	25	14
8	35	f	Left adrenal phaeochromocytoma	6	10
9	58	f	Malignant phaeochromocytomas	23	26
10	39	m	Malignant phaeochromocytomas	9	<10
11	42	m	Bladder phaeo. + secondaries	21	127
12	27	f	Right adrenal phaeochromocytoma	<6	25
13	67	f	Left adrenal phaeochromocytoma	10	12
14	32	f	Extra adrenal phaeochromocytoma	8	37
15	65	f	Right adrenal phaeochromocytoma	<6	11
16	45	f	Extra adrenal phaeochromocytoma	<6	<10
17	35	m	Bilateral adrenal phaeochromocytomas	320	41
18	71	f	Paravertebral phaeochromocytoma	<6	12
19	58	m	Extra adrenal phaeochromocytoma	<6	17
20	43	f	Malignant phaeochromocytomas	6	32

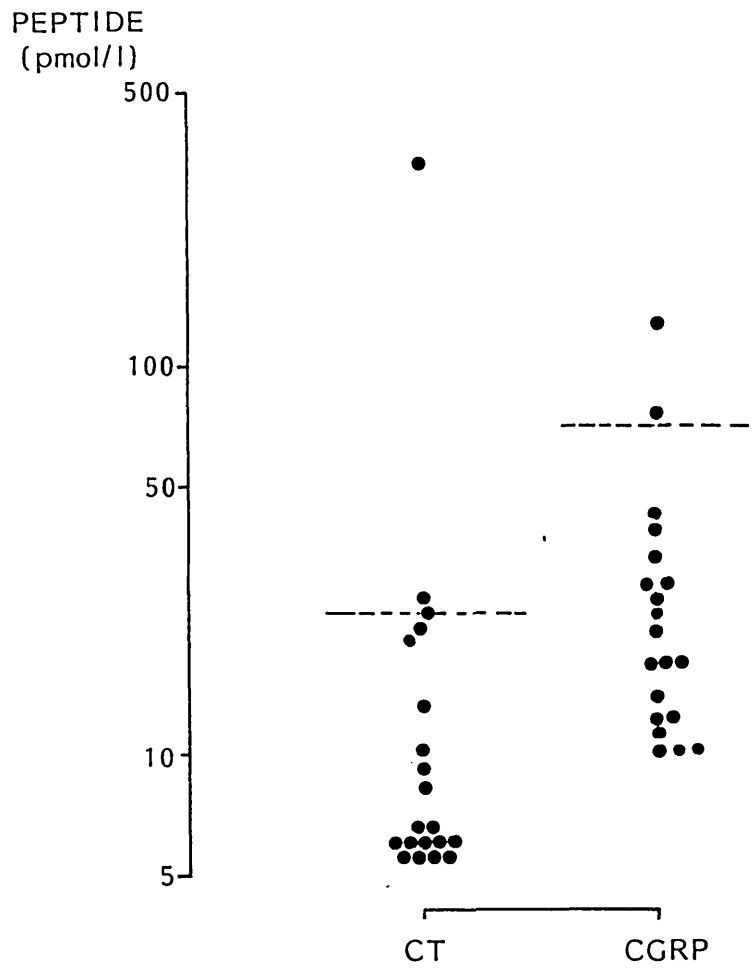

Figure 5.2 Plasma levels of calcitonin and CGRP in patients with phaeochromocytoma (upper limit of normal shown by dashed line)

5.4 LUNG CANCER

5.4.1 Patients

Seventeen patients with lung cancer were studied (11 male, 6 female; age range 44-76) including 5 patients with small cell carcinoma and 12 non-small cell carcinoma.

Calcitonin and CGRP measurements were performed according to the method in Chapter 2. Katacalcin was not measured because of insufficient sample.

5.4.2 Results

The results of calcitonin and CGRP measurements on lung cancer patients are shown in Figure 5.3 and Table 5.3. Of the 17 patients studied, 41% had an elevated calcitonin level and 41% showed CGRP levels elevated above normal. Analysis of the results failed to show a significant correlation between calcitonin and CGRP levels, whilst no significant difference was observed between peptide levels in SCCL and NSCCL.

Patient information was obtained from medical records.

Table 5.3 Plasma levels of calcitonin and CGRP in patients with lung cancer

PATIENT	AGE	SEX	TUMOUR	PLASMA CALCITONIN (pmol/ℓ)	PLASMA CGRP (pmol/ℓ)
1	57	m	SSC	143	119
2	57	m	"	26	53
3	63	m	"	20	36
4	53	m	"	9	66
5	58	m	"	<6	52
6	72	m	NSSC	23	79
7	76	m	"	<6	63
8	50	m	"	147	42
9	59	f	"	26	45
10	50	m	"	50	74
11	60	f	"	28	108
12	58	m	"	20	13
13	65	f	"	<6	65
14	55	f	"	<6	114
15	66	f	"	<6	55
16	58	m	"	<6	72
17	44	f	"	82	93

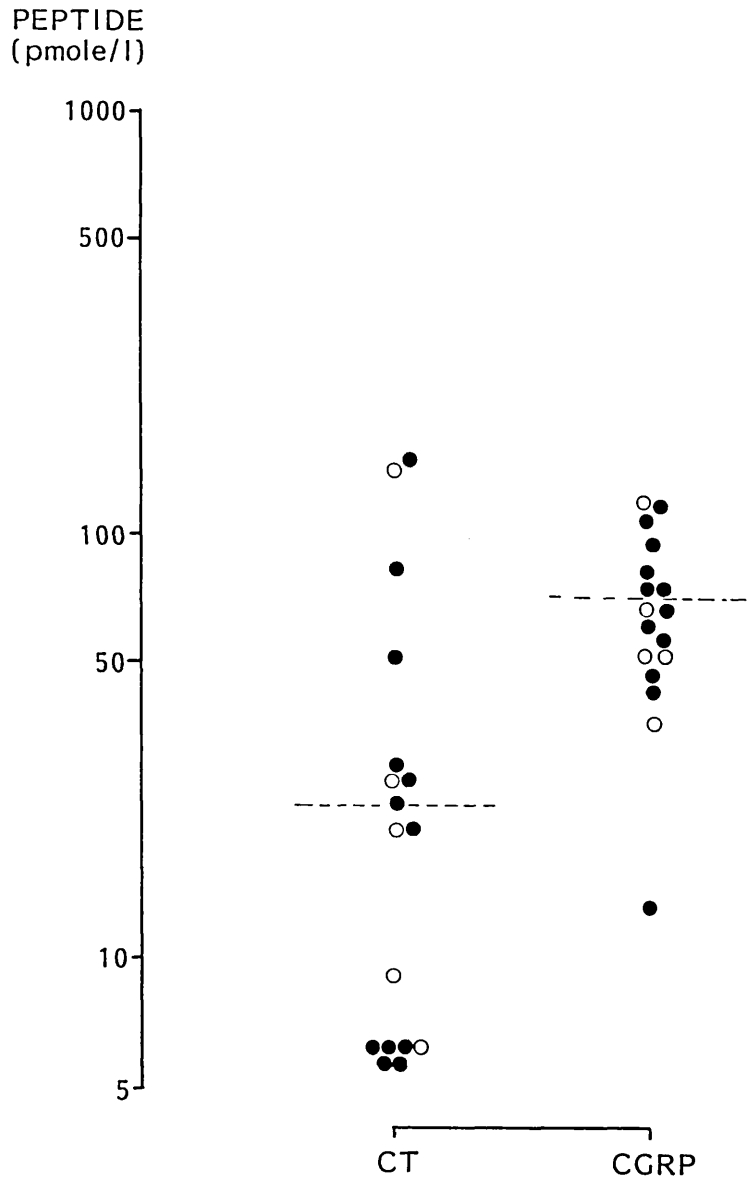

Figure 5.3 Plasma levels of calcitonin and CGRP in patients with lung cancer (SSC (○), NSSC (●)). Upper limit of normal shown by dashed line.

5.5 BREAST CANCER

5.5.1 Patients

Samples from patients with breast cancer were obtained and classified according to the extent of disease. The patients included 20 at Stage I (age range 36-77), 15 at Stage II (age range 47-75), 9 at Stage III (age range 42-78) and 17 at Stage IV (age range 39-72).

Calcitonin, katacalcin and CGRP measurements were performed on plasma samples obtained from these patients as described in Chapter 2.

5.5.2 Results

The calcitonin, katacalcin and CGRP levels in the plasma of patients with breast cancer (four stages) are illustrated in Figures 5.4, 5.5 and 5.6 respectively, as well as Table 5.4. Calcitonin was elevated in 22% of the patients, katacalcin in 63% and CGRP in 37%. Statistical analysis of the results using one-way analysis of variance indicated a significant difference in calcitonin results between the four groups ($p<0.05$) largely due to differences between Stage I and IV. No significant differences were observed in katacalcin or CGRP results. A significant correlation was observed between calcitonin and CGRP levels in breast cancer ($p<0.01$) although no relationship existed between katacalcin and calcitonin or between CGRP and katacalcin.

Patient information was provided by clinicians via a questionnaire.

Table 5.4 Plasma levels of calcitonin and CGRP in patients with breast cancer

PATIENT	AGE	DISEASE STAGE	PLASMA CALCITONIN (pmol/ℓ)	PLASMA CGRP (pmol/ℓ)	PLASMA KATACALCIN (pmol/ℓ)
1	36	1	<6	<10	44
2	46	1	15	195	69
3	56	1	15	<10	25
4	51	1	23	26	30
5	77	1	23	<10	65
6	68	1	17	115	105
7	42	1	<6	63	61
8	64	1	<6	10	64
9	47	1	8	59	119
10	66	1	12	34	112
11	46	1	22	54	94
12	40	1	10	<10	50
13	64	1	10	100	50
14	50	1	<6	19	64
15	60	1	<6	31	40
16	71	1	<6	50	51
17	70	1	<6	20	43
18	66	1	<6	37	61
19	52	1	<6	29	66
20	52	1	60	82	71
21	49	2	21	43	85
22	57	2	12	<10	30
23	64	2	9	87	25
24	74	2	9	53	90
25	61	2	32	87	25
26	57	2	59	65	130
27	47	2	14	<10	56
28	63	2	<6	27	61
29	75	2	<6	22	77
30	65	2	<6	<10	36
31	63	2	189	147	30
32	63	2	10	69	30
33	74	2	18	219	30
34	62	2	15	118	84
35	62	2	15	<10	30
36	70	3	25	55	53
37	62	3	10	92	139
38	45	3	9	48	61
39	78	3	<6	<10	46
40	78	3	10	119	90

continued ...

Table 5.4 (continued)

PATIENT	AGE	DISEASE STAGE	PLASMA CALCITONIN (pmol/ℓ)	PLASMA CGRP (pmol/ℓ)	PLASMA KATACALCIN (pmol/ℓ)
41	75	3	8	111	30
42	42	3	<6	<10	46
43	72	3	10	<10	40
44	61	3	10	190	49
45	68	4	307	82	263
46	54	4	12	75	44
47	68	4	18	26	80
48	41	4	29	113	38
49	48	4	<6	75	43
50	40	4	<6	<10	67
51	62	4	12	170	41
52	57	4	21	52	126
53	72	4	21	67	130
54	69	4	15	24	69
55	63	4	<6	<10	52
56	71	4	60	120	30
57	46	4	78	42	25
58	64	4	51	69	30
59	70	4	40	<10	30
60	65	4	22	53	77
61	39	4	13	<10	43

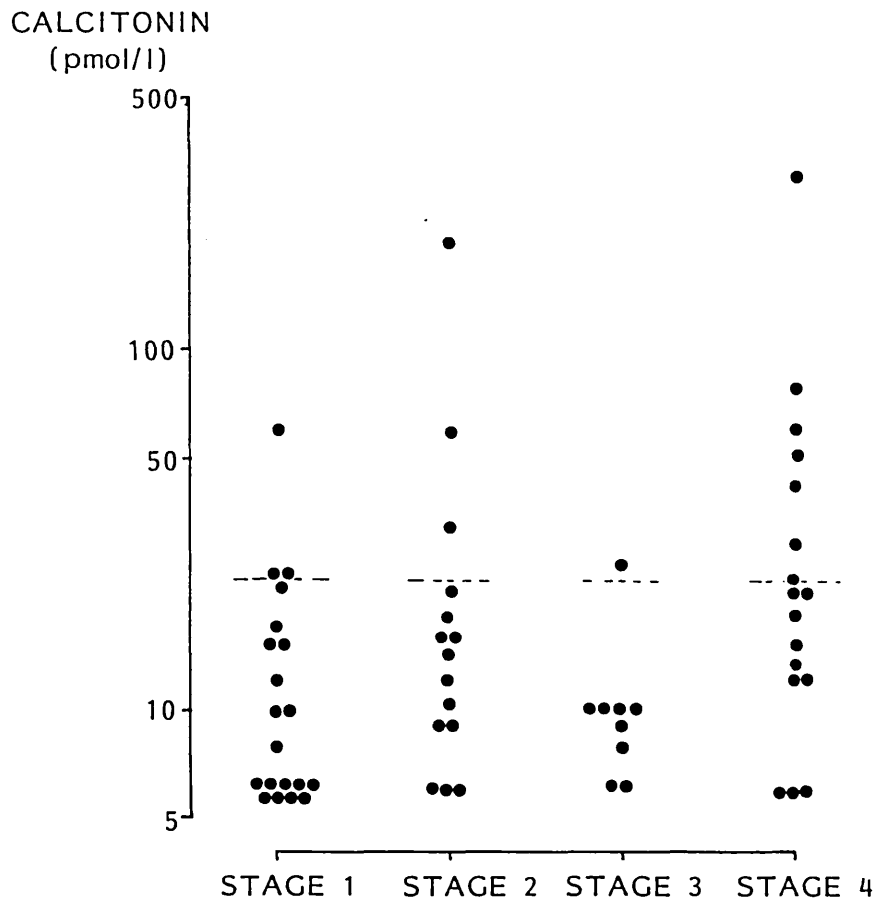

Figure 5.4 Plasma levels of calcitonin in patients with breast cancer (different stages). Upper limit of normal shown by dashed line.

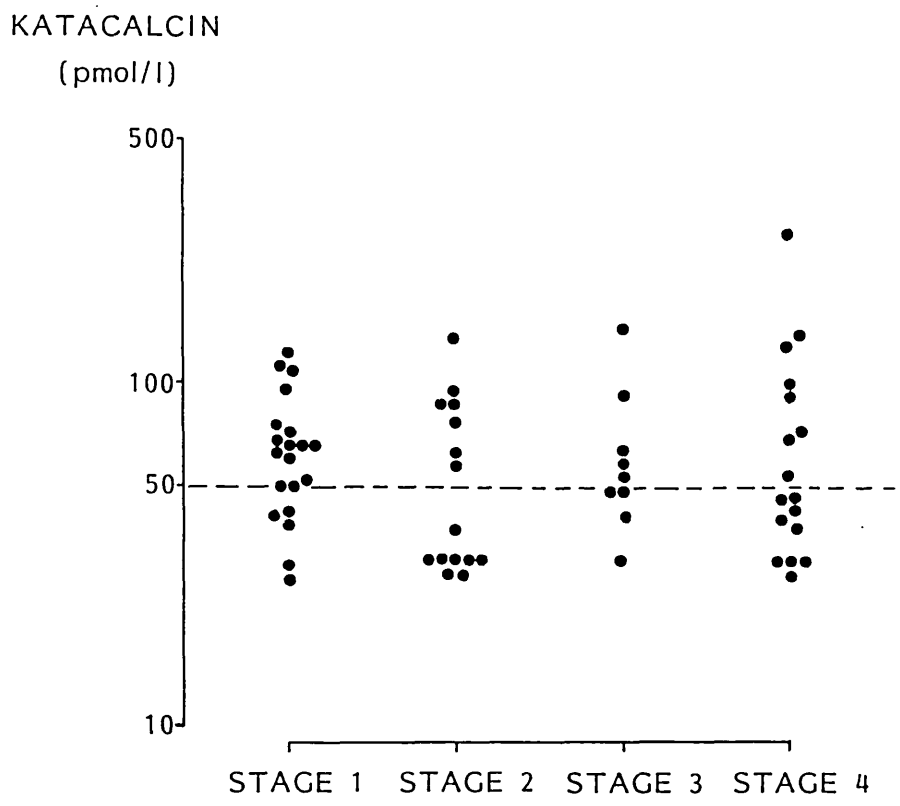

Figure 5.5 Plasma levels of katacalcin in patients with breast cancer (different stages). Upper limit of normal shown by dashed line.

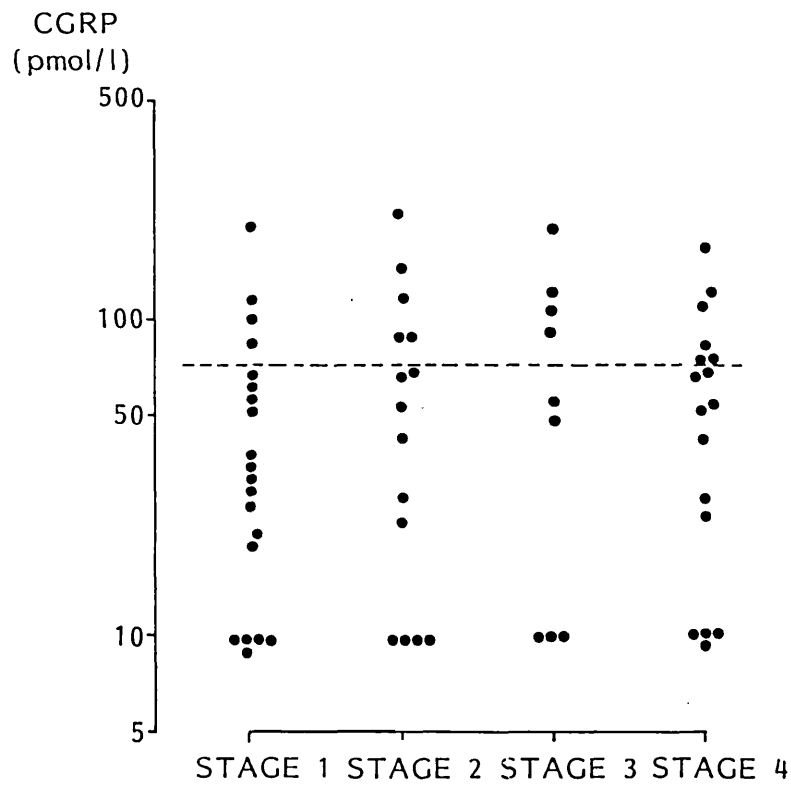

Figure 5.6 Plasma levels of CGRP in patients with breast cancer (different stages). Upper limit of normal shown by dashed line.

5.6 SQUAMOUS BRONCHIAL CARCINOMA CELL LINE ('BEN')

5.6.1 Method

Cells of the squamous bronchial carcinoma cell line, 'BEN', were grown in monolayer culture. The culture medium used was composed of 50% Medium 199 and 50% Dulbecco's Modification of Eagle's Medium, supplemented with 2% Foetal Calf Serum (FCS), Glutamine, Fungizone and 75 units/ml Penicillin/Streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 and air.

The cells were subcultured (by trypsinisation) 3 days prior to the experiment and placed in 8 small flasks with a 25 cm^2 surface area. On the day of investigation the culture medium was replaced with 5 ml of fresh medium. Four flasks were incubated for 6 hours and the remaining four for 24 hours. At the end of the incubation period the medium was aspirated and frozen for assay. The cells were washed with Dulbecco's Phosphate Buffered Saline (PBS) containing 0.2% ethylenediaminetetracetic acid (EDTA). A trypsinisation solution of 0.0125% trypsin in PBS/EDTA was then added to the cells for about 2 minutes until the cells lifted from the surface. The cells were centrifuged immediately and placed in medium containing 5% FCS. The number of cells from each flask was then determined.

Calcitonin and CGRP measurements were then performed on the aspirated medium using blank culture medium in the standard curve.

5.6.2 Results

The measurements of calcitonin and CGRP in culture medium exposed to 'BEN' cells for 6 and 24 hours are shown in Figure 5.7. These results indicate that this squamous lung cancer cell line secretes both calcitonin and CGRP.

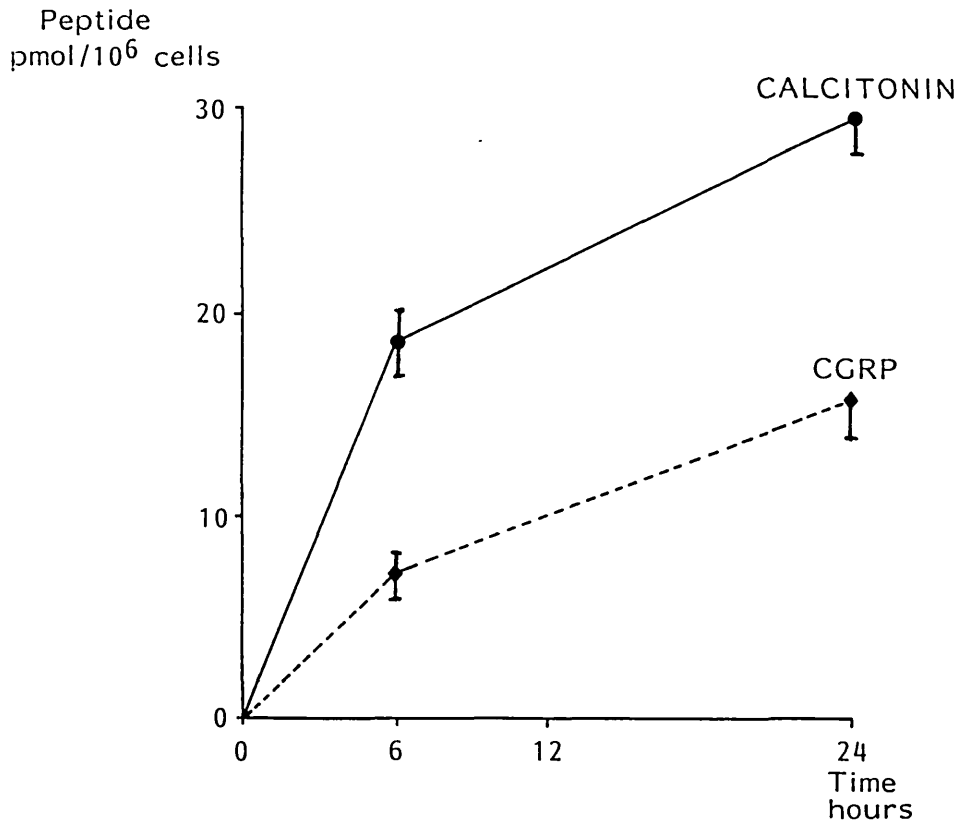

Figure 5.7 Secretion of calcitonin and CGRP by 'BEN' cells (mean, SEM)

5.7 DISCUSSION

The work described in this chapter has shown circulating levels of calcitonin and CGRP to be generally within normal reference values in patients with non-thyroid carcinomas. However in a number of these patients minor elevations in these peptides were observed.

The finding of elevated calcitonin in at least 3 of the carcinoid patients studied is consistent with the observations of other workers who have demonstrated ectopic calcitonin secretion associated with carcinoid tumours (Deftos et al 1976, Kaplan et al 1973). Kaplan et al also drew a comparison between carcinoid and MTC tumours because of the humoral similarities between the two types of tumour. A calcitonin level of the magnitude of that found in patient 12 is usually diagnostic of MTC. Although a thyroid scan on this patient appeared normal this does not exclude the possibility of microscopic foci of neoplastic c-cells. Unfortunately a post-mortem was not carried out on this patient and the site of calcitonin secretion remains unknown.

Carcinoid tumours have been reported in association with MEN II (O'Dorisio and Vinik 1985) and the true incidence of this combination may be obscured because both MTC and primary carcinoids are frequently symptomless. Primary gastrointestinal carcinoids are drained by the portal venous sytem to the liver where most of the 5HT secreted by the tumour is metabolised (Kaplan 1978). Following the occurrence of extensive liver metastases (hepatomegaly) the secretory products of both the primary tumour and metastases can reach the systemic circulation giving rise to some of the classical symptoms of carcinoid syndrome.

The symptoms of flushing and diarrhoea observed in carcinoid syndrome have previously been ascribed to kallikrein and 5HT respectively (Goedert et al 1980). However Lucas and Feldman (1986) found no significant difference in kallikrein levels between carcinoids and controls, while Norheim et al (1986) have observed increased tackykinins during carcinoid flushes. The gastric and vasodilator activity of CGRP suggested the possible involvement of this peptide in carcinoid syndrome.

Of the 14 patients studied, Number 12 with suspected MTC had a minor elevation in circulating CGRP and patient 14 had considerably elevated levels. The remaining patients all showed normal circulating levels of CGRP; CGRP is therefore not a useful marker for carcinoid syndrome. However the flushing attacks and diarrhoea are frequently episodic rather than persistent (Kaplan 1978), often being stimulated by alcohol or food intake. Furthermore CGRP is thought to exert its vasodilator actions via an endothelial factor (Brain et al 1985). Thus CGRP, released in response to a food stimulus, may precede the onset of flushing or diarrhoea and consequently sampling between attacks would mask the involvement of CGRP.

Phaeochromocytomas have previously been reported to secrete calcitonin (Abe et al 1977) and CGRP has been immunohistochemically localised to cells of both familial and sporadic phaeochromocytomas (Johanssen et al 1986). The results of calcitonin and CGRP measurements on the series of patients in this study suggest that ectopic secretion of these peptides is relatively rare in phaeochromocytoma. In the patient with elevated calcitonin MTC was excluded but renal disease was present. Elevated plasma calcitonin levels have been reported in patients with renal failure (Ardaillou et al 1975) and therefore the phaeochromocytoma may not be the source of calcitonin in this patient. The ability of certain phaeochromocytomas to produce calcitonin and CGRP may reflect the close embryological relationship between c-cells and the adrenal medulla (both arising from neural crest tissue).

The ectopic production of calcitonin in lung cancer is now well established (Silva et al 1974, Coombes et al 1974) and various cell cultures of lung tumour tissue have been shown to secrete calcitonin (Ellison et al 1976). A proportion of patients and cultured tumour tissue from all the four main histological types of lung cancer have been observed to secrete calcitonin although SCCL usually shows the largest percentage of calcitonin secretors (McKenzie et al 1977). More recently imunoreactive CGRP has also been demonstrated in extracts of primary lung cancer (Yamaguchi et al 1985) and in the media of SCCL cell lines (Edbrooke et al 1985).

The present study confirms previous findings of ectopic calcitonin secretion in lung cancer and demonstrates elevated circulating levels of CGRP. No relationship has been observed between calcitonin levels and disease stages (Gropp et al 1980) but calcitonin measurements may be useful for monitoring therapy in patients with lung cancer (Silva et al 1979).

The production of calcitonin by lung tumours was previously considered to be ectopic; however, the lung has now been suggested to be an endocrine organ (Ben-Harari and Youdim 1983) and diffuse Kulchitsky or endocrine cells are known to be present in foetal and adult lung (Gail and Lenfant 1983). Immunochemical studies have indicated calcitonin immunostaining in these endocrine cells (Becker et al 1980) whilst CGRP has also been localised to such cells (Uddman et al 1985). The function of calcitonin, CGRP and other peptides in the lung is unknown but the lung is ideally equipped and positioned to modify the hormonal composition of the systemic blood supply.

Bronchial carcinoids and SCCL are both thought to originate from lung endocrine cells (Gail and Lenfant 1983) which may explain the occurrence of elevated calcitonin levels in patients with both these diseases. However this does not account for the elevated calcitonin levels observed in other histological types of lung cancer. Many lung cancer patients are heavy smokers possibly with chronic obstructive pulmonary disease and/or smoking related bronchitis which may be associated with increased blood levels of calcitonin (Becker 1985). CGRP and calcitonin secretion need not originate from the tumour; however, studies in this chapter on the squamous bronchial carcinoma cell line have indicated that lung tumour tissue may freqently be the site of peptide production. An alternative perspective on this problem was taken by Aisner and Matthews (1985) when reviewing lung tumours of combined histological type and cultured tumours which transform from one cell type to another. Their studies lead to the suggestion that various lung cells are interrelated and that certain lung neoplasms are derived from progenitor cells capable of differentiating along differing pathways. Such observations also led Baylin (1985) to propose theoretical pathways for lung cell differentiation, involving totipotential cells capable of developing

into various cell types in the bronchial epithelium by transition through an endocrine-like cell. Cell renewal following injury could then result in an expanded population of transitional endocrine cells susceptible to neoplastic transformation and able to differentiate to various cell types.

In the group of patients with breast cancer several patients had elevated levels of one or more of the calcitonin gene peptides. No obvious relationship existed between the circulating levels of these peptides and the stage of disease although calcitonin levels were high in Stage IV breast cancer. Nielsen and Gadeberg (1980) proposed that physiological calcitonin secretion from the thyroid may be increased in widespread breast cancer to prevent osteolysis by bone metastases. Whilst Coombes et al (1975) also observed greater calcitonin levels in patients with metastatic disease, the secretion of calcitonin by 8 of 15 breast tumours maintained in monolayer culture has demonstrated breast cancer cells to be secreting this peptide.

The production of calcitonin gene peptides by breast cancer tissue is an example of peptide production by cells which are not usually recognised to have endocrine function. However Wilander et al (1984) observed endocrine-like cells in certain breast tumours but additionally noted a sparse distribution of such cells in normal mammary tissue. Bucht et al (1986) have demonstrated that the calcitonin present in human milk is not of thyroid origin and may therefore be secreted by breast endocrine cells. Whether those cells are a further extension of a diffuse endocrine system remains to be seen, as does the question of whether peptide secreting breast tumours arise from such cells or rather from non-endocrine mammary tissue.

From the observations made in this chapter abnormal plasma levels of calcitonin gene peptides appear to be an occasional feature of solid tumours from both endocrine and non-endocrine cell types.

A hypothesis has now been proposed (LeRoith and Roth 1985) that synthesis of hormonal peptides is not unique to glandular tissue but that many types of cell are capable of making these peptides (in small amounts). The distinction between classical endocrine tissue and non-endocrine tissue may then be quantitative rather than qualitative, such that normal non-endocrine, endocrine and malignant cells show a range of abilities to synthesise, store and secrete peptide hormones. The definitions of ectopic and eutopic, endocrine and non-endocrine may therefore be somewhat misleading.

CHAPTER 6 HAEMATOLOGICAL DISORDERS AND THE CALCITONIN GENE PEPTIDES

6.1 INTRODUCTION

Circulating ectopic hormones have been reported in a variety of tumours, particularly those of endocrine origin (Coombes et al 1974, Abe et al 1977). However reports of ectopic hormone production in haematological malignancies and disorders have been infrequent. Hillyard et al (1979) described calcitonin measurements in patients with various leukaemias and myelofibrotic disorders. This work was later confirmed (Pfluger et al 1982, Foa et al 1982) and immunoreactive calcitonin was demonstrated in media from patient leukaemic cells maintained in short term culture. Immunoreactive calcitonin has also been measured in the medium of the established promyelocytic leukaemia cell line, HL60 (Oscier et al 1983).

In addition to ectopic calcitonin production in leukaemia, cases of inappropriate parathyroid hormone have been reported in acute lymphoblastic leukaemia (ALL), (Ramsay et al 1979) and chronic lymphocytic leukaemia (CLL), (Laugen et al 1979). The secretion of ectopic adrenocorticotrophic hormone (ACTH) has also been described in a patient with acute myeloblastic leukaemia (AML), (Pfluger et al 1981).

The studies described in this chapter involve investigations on the haematological diseases: leukaemia, lymphoma and myeloproliferative disorders.

Leukaemia is characterised by an accumulation of abnormal white cells in the bone marrow with the possible consequences of bone marrow failure, an increase in circulating white cell numbers and infiltration of other organs. ALL probably arises from cells of the lymphoid development series whilst AML is thought to originate from cells of the myeloid development line. Chronic myeloid leukaemia (CML), including chronic granulocytic leukaemia (CGL) is probably derived from an early stem cell and may transform to ALL or AML. CLL, however, originates from peripheral lymphocytes.

Lymphomas, usually classified as Hodgkin's disease and non-Hodgkin's lymphoma (NHL), involve the replacement of normal lymphoid structure by collection of abnormal cells. The abnormal cells in Hodgkin's disease are Reed-Sternberg (monocyte type) cells whereas abnormal lymphocytes are involved in NHL.

Myeloproliferative disorders are a group of interrelated diseases in which there is proliferation of haemopoietic cells in bone marrow and possibly spleen and liver. These disorders occur in various myeloid cell types and include myelofibrosis, polycythaemia vera and essential thrombocythaemia.

6.2 PATIENTS AND METHODS

Plasma samples were obtained from various patients with haematological disorders. The samples were stored at -20°C and assayed for calcitonin, katacalcin and CGRP as described in Chapter 2.

6.2.1 Leukaemia and Myeloproliferative Disorders

Forty-two patients with myeloid leukaemia were studied, 29 suffering from CGL (18 male, 11 female; age range 18-73) and 13 patients with AML (7 male, 6 female; age range 21-63). A further 13 patients with lymphoid leukaemia were also studied (7 male, 6 female; age range 9-65) including patients with both chronic and acute lymphoid leukaemia. Eleven patients with myeloproliferative disorders were investigated (6 male, 5 female; age range 35-85).

6.2.2 Lymphoma

Twenty-one patients with lymphoma were studied, of which 8 had Hodgkin's disease (6 male, 2 female; age range 22-79). The remaining 13 suffered from NHL (7 male, 6 female; age range 29-93). Katacalcin was not measured for these patients because of insufficient sample.

Information on patients with both leukaemia and lymphoma was obtained from medical records.

6.3 RESULTS

6.3.1 Leukaemia and Myeloproliferative Disorders

The results of calcitonin measurements on the leukaemic patients showed elevated levels in 38% of CGL patients and 69% of AML patients. Only one patient with lymphocytic leukaemia had a calcitonin level above normal whilst 3 patients with MPD had abnormal levels (Figure 6.1). Plasma CGRP levels were elevated in 31% of CGL and 23% of AML patients. All patients with lymphoid leukaemia had normal CGRP levels and only one patient with MPD had an elevated level (Figure 6.2). Katacalcin was elevated in 59% of the samples measured from patients with myeloid leukaemia and MPD.

Haematological information on patient haemoglobin, white blood cell count and platelets was obtained where possible and used for statistical analysis with the peptide results. Analysis of the results revealed a negative correlation between haemoglobin levels and calcitonin in myeloid leukaemia ($p<0.05$) and between haemoglobin and white blood cell count ($p<0.01$). A positive correlation existed between calcitonin and katacalcin levels in the myeloid leukaemia and MPD patients (Figure 6.3). One way analysis of variance on the calcitonin results from the 4 groups of patients (CGL, AML, MPD and lymphoid leukaemia) revealed a significant difference between the groups, largely due to high calcitonin levels in AML ($p<0.01$). Analysis of variance on the CGRP results also indicated a significant difference between the groups ($p<0.001$) with CGRP levels being greatest in CGL patients, decreasing through AML, MPD to the lowest levels in lymphoid leukaemia.

6.3.2 Lymphoma

CGRP was not elevated above normal in either Hodgkin's disease or NHL but minor increases in calcitonin levels were observed in 33% of all the lymphoma patients (Figure 6.4).

Table 6.1 Plasma levels of calcitonin and CGRP in patients with leukaemia and myeloproliferative disease

PATIENT	DISEASE	PLASMA CALCITONIN pmol/ℓ	PLASMA CGRP pmol/ℓ	PATIENT	DISEASE	PLASMA CALCITONIN pmol/ℓ	PLASMA CGRP pmol/ℓ
1	CGL	<6	<10	34	AML	94	87
2	CGL	27	<10	35	AML	18	23
3	CGL	8	<10	36	AML	73	179
4	CGL	31	<10	37	AML	60	<10
5	CGL	12	36	38	AML	120	37
6	CGL	<6	47	39	AML	168	53
7	CGL	<6	224	40	AML	77	8
8	CGL	<6	<10	41	AML	67	16
9	CGL	<6	45	42	AML	13	100
10	CGL	<6	106	43	MPD	133	19
11	CGL	9	58	44	MPD	186	50
12	CGL	9	53	45	MPD	35	29
13	CGL	38	26	46	MPD	<6	40
14	CGL	56	89	47	MPD	9	<10
15	CGL	108	792	48	MPD	<6	<10
16	CGL	61	121	49	MPD	11	<10
17	CGL	20	132	50	MPD	19	<10
18	CGL	76	106	51	MPD	<6	22
19	CGL	70	343	52	MPD	<6	46
20	CGL	28	98	53	MPD	12	54
21	CGL	59	317	54	CCL	7	10
22	CGL	35	13	55	CCL	6	10
23	CGL	20	84	56	CCL	6	10
24	CGL	<6	60	57	CCL	<6	10
25	CGL	<6	34	58	CCL	8	10
26	CGL	<6	23	59	CCL	<6	10
27	CGL	8	29	60	CCL	<6	10
28	CGL	7	23	61	ALL	20	11
29	CGL	22	68	62	ALL	12	10
30	AML	12	12	63	ALL	59	21
31	AML	47	22	64	ALL	8	10
32	AML	19	33	65	ALL	<6	<10
33	AML	246	44	66	ALL	129	17

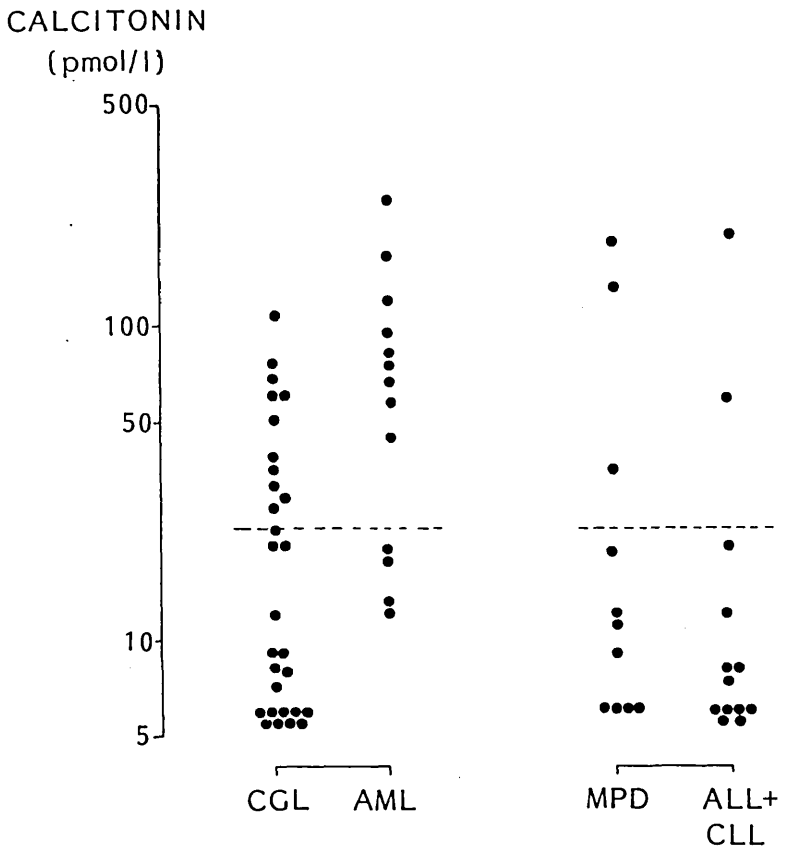

Figure 6.1 Plasma calcitonin levels in patients with haematological disorders (upper limit of normal indicated by dashed line)

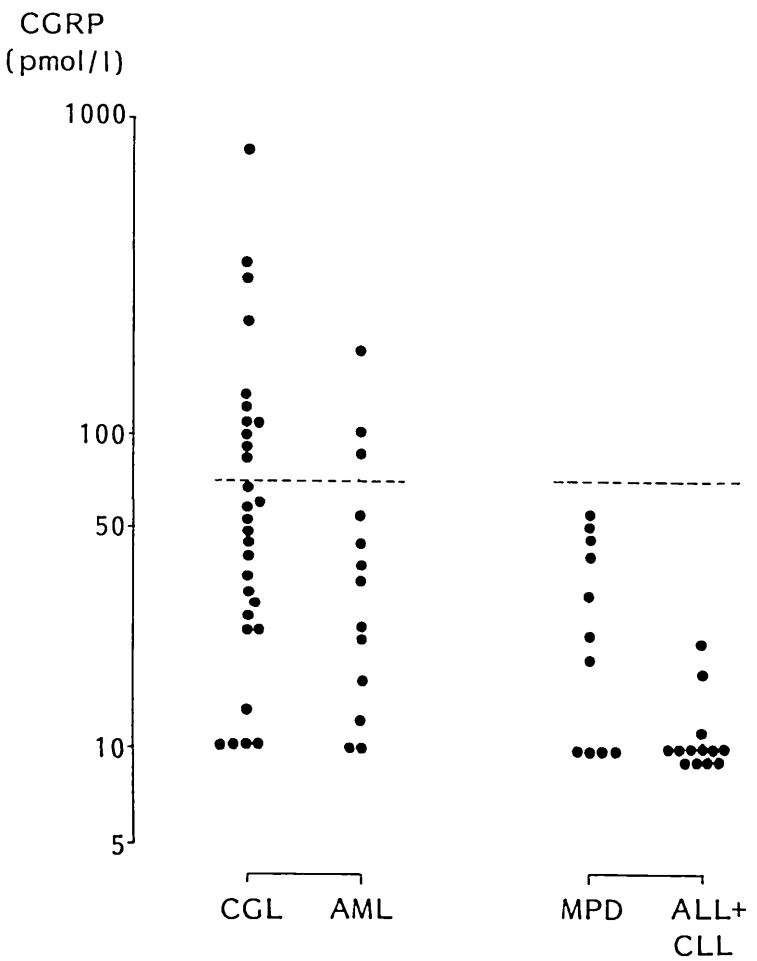

Figure 6.2 Plasma CGRP levels in patients with haematological disorders (upper limit of normal indicated by dashed line)

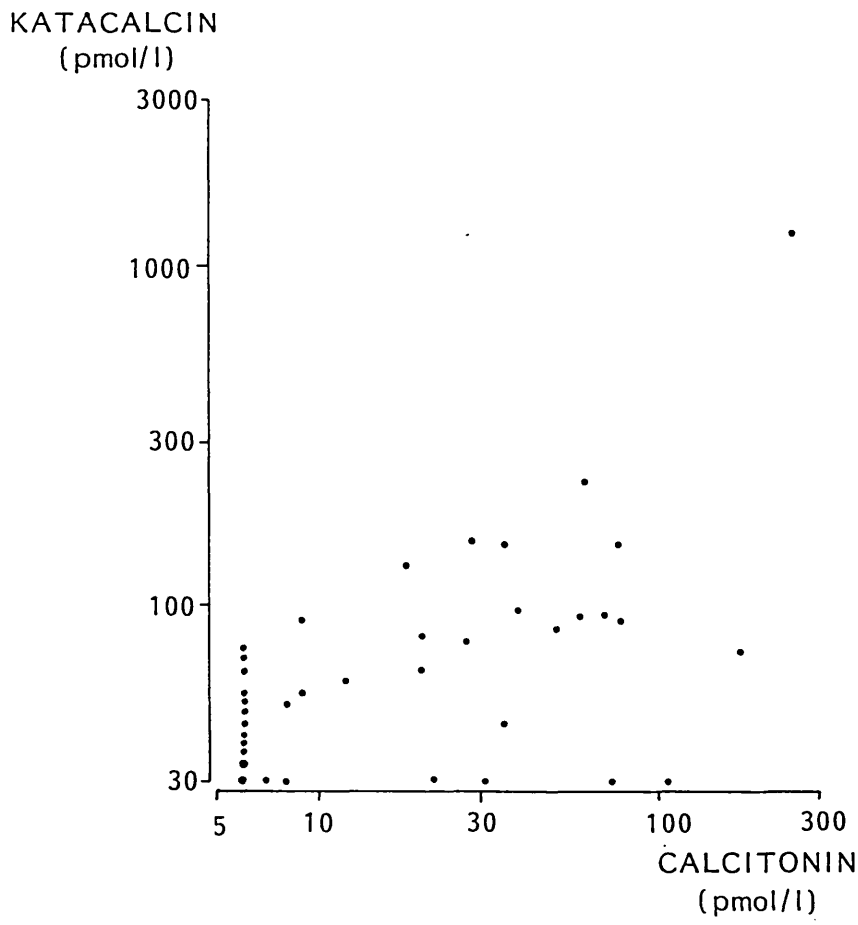

Figure 6.3 Correlation between plasma calcitonin and katacalcin in patients with myeloid leukaemia and MPD

Table 6.2 Plasma levels of calcitonin and CGRP in patients with lymphoma

PATIENT	AGE	SEX	DISEASE	PLASMA CALCITONIN (pmol/ℓ)	PLASMA CGRP (pmol/ℓ)
1	22	m	HD	50	40
2	49	f	HD	14	40
3	41	m	HD	10	42
4	32	f	HD	10	16
5	79	m	HD	20	15
6	40	m	HD	8	30
7	53	m	HD	55	26
8	50	m	HD	29	16
9	70	f	NHL	11	17
10	56	f	NHL	9	29
11	64	f	"	21	62
12	29	m	"	35	61
13	75	m	"	29	32
14	64	m	"	94	42
15	93	f	"	8	<10
16	60	m	"	8	<10
17	61	m	"	23	<10
18	75	m	"	10	58
19	77	f	"	23	13
20	74	f	"	10	42
21	66	m	"	27	<10

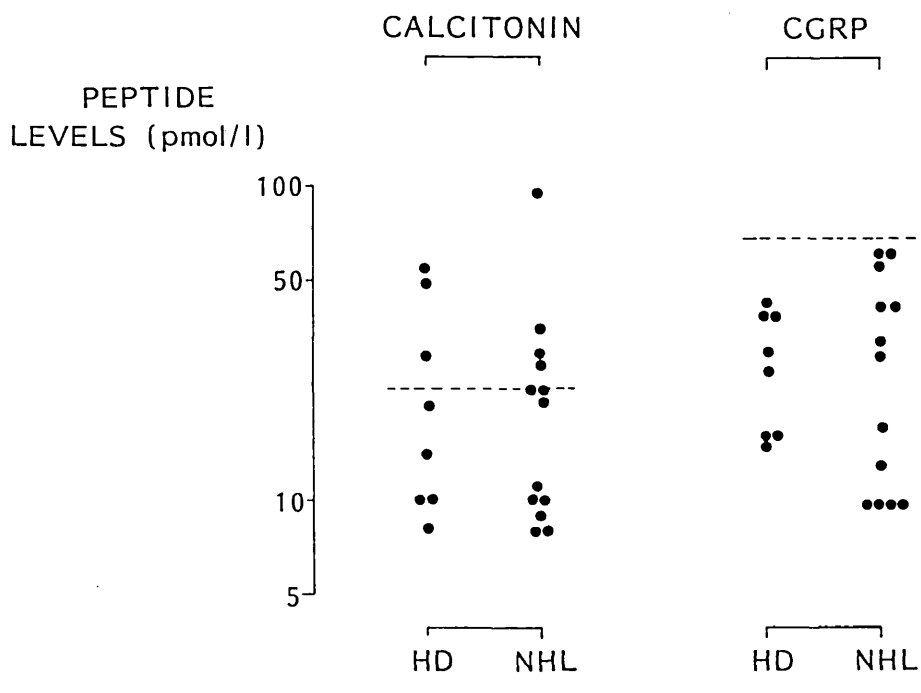

Figure 6.4 Plasma calcitonin and CGRP levels in patients with lymphoma (Hodgkin's disease and non-Hodgkin's lymphoma, NHL)

6.4 DISCUSSION

The observations described in this chapter of elevated calcitonin gene peptides in myeloid leukaemia are compatible with the previous findings of Hillyard et al (1979) and Pfluger et al (1982).

The origin of elevated calcitonin gene peptides in leukaemia is unclear. Martin et al (1981) have proposed that calcitonin released from certain malignant cells may have been bound to calcitonin receptors and then slowly released into the circulation. The reports of calcitonin receptors on cultured human lymphocytes (Marx et al 1974) and of a cAMP response to calcitonin in lymphocytes may support this theory. However observations by Oscier et al (1983) have demonstrated calcitonin secretion by the promyelocytic leukaemia cell line, HL60, in long term culture. Calcitonin may alternatively be involved in inter-cell communication and a grossly expanded population of white cells would then increase the total circulating white cells and hence their secretions. Theoretically, over-production of a peptide by a malignant tissue should not be described as ectopic until all sources of that peptide are known.

Leukaemia is a clonal disease and expansion may occur in a clone of cells which have a differentiation arrest e.g. acute leukaemia. A theory has been proposed suggesting that stem cells, present in normal tissue but which are not fully differentiated, may be the target of carcinogenic agents (Ruddon, 1982). The pluripotent stem cell, from which all haematological cell types derive, (or a committed precursor) may thus be susceptible to carcinogenic insult, being prevented from further differentiation whilst multiplying uncontrollably. The consequence of carcinogenic damage could lead to disturbances in oncogene activity, examples of which have been reported in connection with leukaemic cells. The c-myc oncogene has been found to be amplified in both the promyelocytic cell line HL60 and in the primary leukaemic cells of the same patient (Dalla Favera et al 1982). Frequent chromosomal rearrangements and translocations have been observed in malignant haematological disorders (Dewald et al 1985). Such translocations can alter the expression of oncogenes e.g. the Philadelphia Chromosome associated with CGL (t(9;22)(q34;q11)).

The c-abl oncogene is translocated from the long arm of chromosome 9 to the long arm of chromosome 22 (Deklien et al 1982) where an alteration in the c-abl protein results in abnormal tyrosine kinase activity (Konopka et al 1984). Changes in oncogene products may then give rise to derepression of genes which normally suppress calcitonin gene peptide production.

The secretion of calcitonin gene peptides in myeloid leukaemia and MPD may thus be a reflection of that in normal mature leukocytes or immature precursors. Alternatively derepression of peptide genes may have resulted from the oncogenic disturbances associated with leukaemia.

CHAPTER 7 STUDIES ON THE EXPRESSION OF CALCITONIN AND CGRP mRNA IN MEDULLARY THYROID CARCINOMA

7.1 INTRODUCTION

The observation that MTC occurred in a familial pattern as well as a sporadic pattern has sparked considerable interest in the genetics of this disease. Both MEN II and III are inherited in an autosomal dominant manner but the age of MEN II expression is variable whilst MEN III may present earlier in life and has a more aggressive course (Emmertsen 1985).

No recognisable defects have yet been identified for MEN II and III although there have been several studies to discover such a defect or a closely linked genetic marker. Van Dyke et al (1984) reported a deletion of Chromosome 20 (probably sub-band 20p12.2) in 9 out of 10 families with MEN II and in 3 out of 5 MEN III families. These findings were in contrast to those of other groups (Hsu et al 1981, Goodfellow et al 1985, Emmertsen et al 1983B). Several other chromosomal markers have been investigated but no linkage could be demonstrated with MEN II (Emmertsen et al 1983B, Ferrel et al 1985). However, a linkage has recently been reported between the retinol binding protein gene and the MEN II locus (Mathew et al 1987, Simpson et al 1987). There have also been reports of a preponderance of chromosomal instability in cultured lymphocytes from patients with MEN II and MTC (Hsu et al 1981, Samaan et al 1984).

Both the α and β calcitonin/CGRP genes have been mapped to the short arm of chromosome 11, the α calcitonin gene to the region 11p13-15 (Przepiorka et al 1984) and the β gene to 11p12-14.2 (Alevizaki et al 1986). Several other genes have also been localised to the short arm of chromosome 11 including catalase, parathyroid hormone, the β-globin gene cluster, the oncogene c-Ha-ras, insulin-like growth factor II and insulin (Kittur et al 1985). Linkage experiments by Kidd and colleagues (1984) have, however, excluded the distal region of the

short arm of chromosome 11 as the site of the MEN II abnormality in the family they studied. Chromosome 10 is now believed to be the locus for MEN II in certain families (Mathew et al 1987, Simpson et al 1987) because of the linkage of MEN II with retinol binding protein gene.

As described in Chapter 1, the α-calcitonin gene encodes an N-terminal peptide, calcitonin, katacalcin and CGRP. Figure 7.1 illustrates the arrangement of exons within the α-calcitonin/CGRP gene and the processing of the primary transcript. The exons within the β-CGRP gene are referred to by the number of the equivalent exon in the α-gene. There is approximately 67% homology in the nucleotide sequence for the calcitonin (exon 4 region) of both genes, with 92% homology between the two exon 5 regions (CGRP coding) and 65% between the α and β exon 6 region which is CGRP specific but non-coding (Figure 7.2).

Following the observation that there may be a difference in the circulating CGRP levels between familial and sporadic cases of MTC (Chapter 4) investigations were undertaken into the expression of calcitonin and CGRP mRNA in MTC tissue.

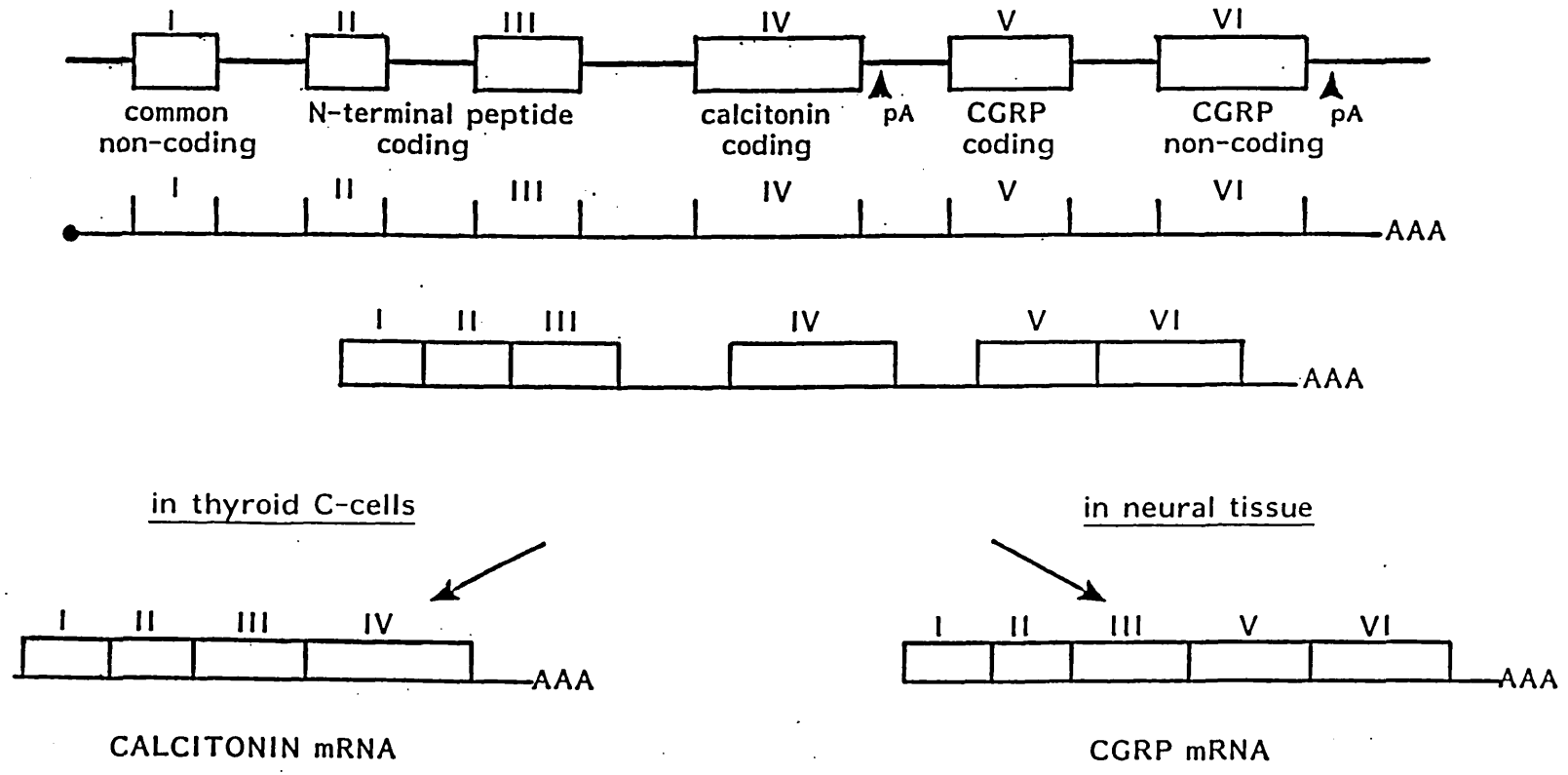

Figure 7.1 The α calcitonin/CGRP gene

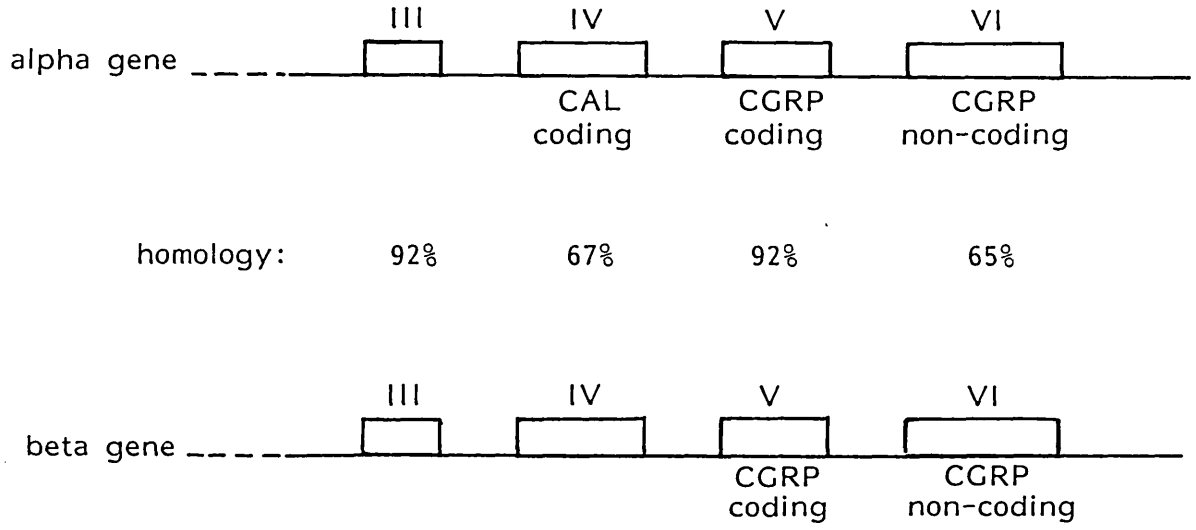

Figure 7.2 Homology between the calcitonin/CGRP genes

7.2 METHODS

7.2.1 Patient Material

Medullary thyroid carcinoma tissue, taken during surgery, was frozen and stored in liquid nitrogen. Material from seven primary tumours was investigated including tumour from 1 MEN III patient, 3 MEN II patients and 3 sporadic MTC patients (F1, F2, F3, F4, S1, S2 and S3 respectively). Additional control material was also used including 'normal' thyroid tissue from a patient with multinodular goitre (N) and cells from the squamous bronchial carcinoma cell line, 'BEN'.

7.2.2 Analysis of mRNA

7.2.2.1 Isolation of Poly A mRNA

- Extraction of mRNA

Approximately 1-2 g of each tumour was pulverised under liquid nitrogen and the resulting powder homogenised in 20 ml of guanidinium mixture (see Appendix).

BEN cells, grown in monolayer culture on 576 cm^2 plates as described in Chapter 5, were washed with saline (150 mmol/ℓ). Guanidinium mixture was then poured on to the cells which were scraped from the culture plates and homogenised.

- Centrifugation

Caesium chloride was added to the guanidinium mixture (4 g for every 10 ml) which was then layered over an 8 ml cushion of caesium chloride (9.6 g in 10 ml of 100 mmol/ℓ ethylenediaminetetraacetic acid, EDTA). The RNA extract was then centrifuged overnight at 24,000 rpm (20°C).

The following morning the supernatant was discarded and the RNA pellet resuspended in TRIS/EDTA (10 mmol/ℓ TRIS, 0.1 mmol/ℓ EDTA, TE) with 0.1% sodium dodecylsulphate (SDS) containing Proteinase K.

Following a 30 minute incubation at 37°C the RNA was shaken with an equal volume of phenolchloroform (see Appendix) and centrifuged. The aqueous phase was then shaken with an equal volume of ether and the RNA remaining in the aqueous phase precipitated at -20°C by adding 1/7th volume of sodium acetate (2 mol/ℓ) and 2.5 volumes of ethanol.

- Purification of Poly A mRNA

The total RNA was purified on a sepharose poly-u column, with a bed volume of 3 ml, to isolate poly A mRNA. Three buffers were prepared (load, wash and elute) containing increasing amounts of sodium chloride (see Appendix).

The precipitate of total RNA was dissolved in load buffer (without sodium chloride), heated to 60°C, cooled and then sodium chloride added. The column was equilibrated with 5 ml of load buffer, then the total RNA passed through, followed by a further 5 ml of load buffer. Wash and elute buffer were then run through the column sequentially and 1 ml aliquots of the eluate collected. A spot (2 μl) from each fraction was placed on an ethidium bromide gel (20 g/ℓ) and monitored under an ultraviolet light to identify the RNA fractions.

Ammonium acetate was added to the pooled poly A RNA fractions to give a concentration of 1 mol/ℓ and the RNA precipitated with 2.5 volumes of ethanol. The poly A RNA was resuspended in 1 ml of water and the absorbance determined at 260 nm. The RNA was then re-precipitated, resuspended at 1 mg/ml and stored at -20°C.

7.2.2.2 Northern Blotting

- Electrophoresis

Agarose (1.4 g) was boiled in 87.5 ml of water and then 5 ml of 20xMOPS buffer (see Appendix) and 7.5 ml of 40% formaldehyde added (gel buffer). Sample buffer was added to the RNA to give a final concentration of 50% formamide, 6% formaldehyde and 1xMOPS buffer. After heating the RNA to 60°C for 2 minutes an orange dye marker was added. The samples were then loaded into wells in the gel, submerged in gel buffer and electrophoresed at about 80 mA.

- Transfer to Nylon Filter

The portion of gel behind the origin was discarded prior to blotting the RNA on to a nylon filter (Amersham 'HYBOND'). Blotting was performed using a solution of 20x SSC (175.3 g/ℓ sodium chloride, 88.2 g/ℓ trisodium citrate dihydrate pH 7.0) which was placed in a blotting tank with a double wick arranged over the lid. The blotting apparatus was then set up as illustrated in Figure 7.3 with the gel inverted and surrounded at the edges with polythene wrap to prevent the system drying out. The system was left to blot overnight before dismantling the following day and baking the filter for 2 hours at 80°C.

7.2.2.3 Hybridisation of Probes to Filters

- Probes (Dr Maria Alevezaki)

In all cases inserts from double stranded M13 clones were excised and used for labelling.

CALCITONIN: A 500 base pair BglII-BglII genomic fragment containing most of exon 4 from the α calcitonin/CGRP gene.

CGRP (α): A 700 base pair RSA-RSA genomic fragment containing the whole of exon 6 from the α calcitonin/CGRP gene and approximately 50 base pairs of flanking intron sequences on each side.

CGRP (β): A 750 base pair HaeIII-HaeIII genomic fragment containing the whole of exon 6 from the β CGRP gene and approximately 100 base pairs of intron sequence from the 5' end.

- Random Primer Labelling

The probe, 10 ng in a 15 μl volume, was boiled for 10 minutes to denature the DNA and then transferred to a 37°C water bath for a further 10 minutes. Bovine serum albumin (10 μg in BRL buffer), nucleotide buffer (see Appendix), 10 μCi of dCTP and 1 unit of Klenow enzyme were then added and the reaction incubated for 90 minutes at 37°C. A small Sephadex G50 column was prepared and equilibrated with TE/0.1% SDS. The labelled probe was then passed down the column to remove unincorporated nucleotides, and eluted in 200 μl fractions.

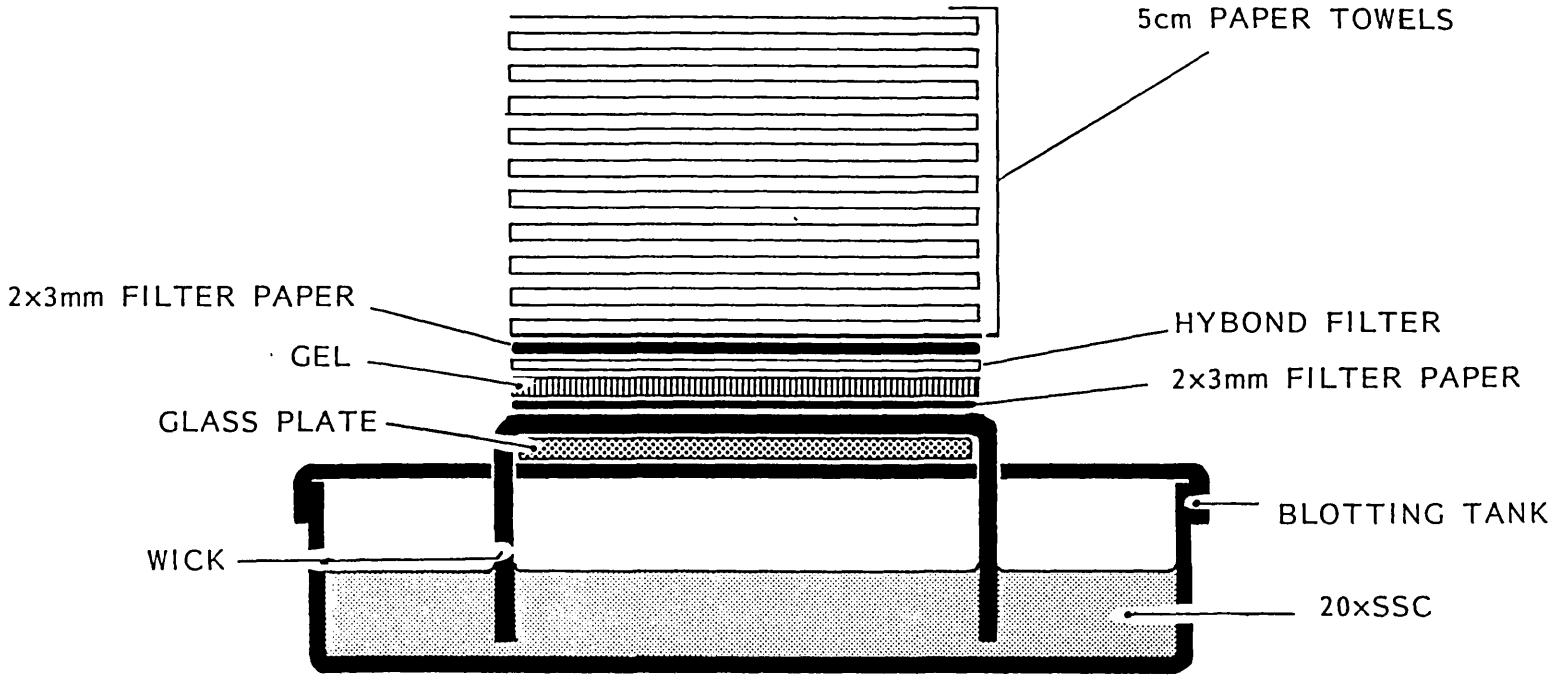

Figure 7.3 RNA blotting apparatus

- Hybridisation

The baked filter was wetted in distilled water and prehybridised at 42°C for several hours in 50% formamide, 5x SSPE (see Appendix), 0.1% dried milk, 0.2% SDS. The filter was then incubated overnight at 42°C in hybridisation buffer containing the boiled probe. The following morning the filter was rinsed in 0.2x SSPE with 0.1% SDS and then washed twice at 42°C for 30 minutes and once at 60°C for 30 minutes.

After washing the filter was sealed in a plastic bag before exposure to a pre-flashed X-ray film (Kodak XAR5) for varying periods of time.

- Re-Use of Filters

Prior to re-use of filters the probe was removed by washing for 2 hours at 65°C in 5 mmol/ℓ TRIS/HCl (pH 8.0) with 2 mmol/ℓ EDTA and 0.1x Denhart's solution (see Appendix). Prehybridisation and hybridisation were then carried out as described previously.

- Oligo dT Labelling

For oligo dT labelling a reaction mixture of 0.4 μg of oligo dt in BRL buffer was prepared with 1 μmol/ℓ of mixed deoxynucleotides, 10 μCi of ^{32}pdCTP and 10 units of terminal transferase. Following a 2 hour incubation at 37°C, the labelled oligo dT was purified on a Sephadex G50 column equilibrated with TE/0.1% SDS.

- Oligo dT Hybridisation

The filter was prehybridised at room temperature for several hours in 5x SSPE with 0.2% dried milk and 0.2% Nonidet P40 (NP40). An overnight hybridisation was then carried out at room temperature in hybridisation solution containing the labelled oligo dT. The following morning the filter was washed at room temperature twice in 5x SSPE (0.1% NP40) for 2 minutes and twice in 2x SSPE (0.1% NP40) for 20 minutes. After washing the filter was exposed to preflashed X-ray films for varying periods of time.

7.2.3 Experiment 1

Approximately equal amounts of poly A mRNA from each tissue together with 0.1, 0.02 and 0.01 $\mu g/\mu l$ of BEN cell mRNA (B1, B2 and B3 respectively) were treated as described above and then placed in the wells of 3 replicate gels. After blotting, probes for α calcitonin, α CGRP and β CGRP were hybridised to the filters. Following exposure to X-ray films the β CGRP was stripped and reprobed with oligo dT to confirm the amount of RNA used in each track.

7.2.4 Experiment 2

Poly A mRNA from selected samples was used in the second experiment and diluted to give calcitonin signals of approximately equal density on the X-ray films. This was to allow a comparison of the relative expression of exon 4α, exon 6α and exon 6β between different tumours. Again 3 gels were prepared, one for α calcitonin, α CGRP and β CGRP. On this occasion BEN cell mRNA was used at concentrations of 0.5, 0.2 and 0.1 $\mu g/\mu l$ (B1, B2 and B3 respectively).

7.3 RESULTS

7.3.1 Experiment 1

The results of Northern blotting of mRNA from various MTC tumours using exon 4α, exon 6α and exon 6β probes are shown in Figures 7.4, 7.5 and 7.6 respectively. Oligo dT probing of one of the filters revealed that the mRNA from 2 of the tumours (F1 and S1) was partially degraded.

The majority of the exon 4 probe hybridised to the mature calcitonin mRNA of 1.0 kilobases (kb). Careful examination of the X-ray films revealed the presence of large mRNA species which are probably precursors of mature calcitonin mRNA. At least 2 large mRNA bands were visible in the tracks of F3 and S2 with one in the track of F2. After correcting for mRNA concentration, using oligo dT, the amount of calcitonin mRNA present in the 1.0 kb band of each tissue was expressed as a ratio of that present in the 'normal' tissue (Table 7.1). This table indicates that calcitonin mRNA in these MTC tumours is between 5 and 90 times that present in the normal thyroid tissue.

The exon 6α probe hybridised principally with the 1.1 kb mature CGRP mRNA species in 4 of the MTC tumours. Two larger exon 6α reactive species were present in samples F3 and F2. The exon 6β probe was clearly hybridised to a mature mRNA in the MTC sample with the highest calcitonin mRNA content (F2) whilst lesser amounts were present in tracks F3, F4, S2 and S3.

7.3.2 Experiment 2

The second experiment was set up to gain a clearer indication of the relative ratios of the 3 mature mRNA species within each tissue. The amount of mRNA applied to the gel was designed to result in calcitonin signals of approximately equal density on the X-ray films, where possible, and enabled comparison between tumours. Figure 7.7, 7.8 and 7.9 show the results of these Northern blots using probes for exon 4α, exon 6α and exon 6β respectively.

On this occasion large exon 4 reactive mRNA species were not clearly visible in the tumours studied. However, one or more exon 6α reactive species were apparent in 4 out of 5 of the MTC tissues as well as BEN cell extracts. Up to 4 such large mRNA species were present in the track of sample S2. In accordance with the complete absence of exon 6α reactive mRNA in S3 of the first experiment, only a very small proportion of mature CGRP mRNA was present in the second experiment. CGRP (β) mRNA was expressed in very minor amounts; however, a large mRNA species was present in both 'normal' and S2 samples. This band was present at an equal or greater density than the mature β-CGRP mRNA. The expression of the 3 mature mRNA species, relative to one another, is shown in Table 7.2. These approximate ratios were calculated with reference to BEN cell mRNA, assuming the probes to have equal specific activity and show equal hybridisation.

Key to Northern Blots in Figures 7.4, 7.5, 7.6

	TISSUE	mRNA (mg/ml)
B1	BEN cells	0.1
B2	BEN cells	0.02
B3	BEN cells	0.01
N	'Normal' thyroid	1.0
F1	Familial MTC (MEN III)	1.0
F2	Familial MTC (MEN II)	1.0
F3	Familial MTC (MEN II)	1.0
F4	Familial MTC (MEN II)	1.0
S1	Sporadic MTC	1.0
S2	Sporadic MTC	1.0
S3	Sporadic MTC	1.0

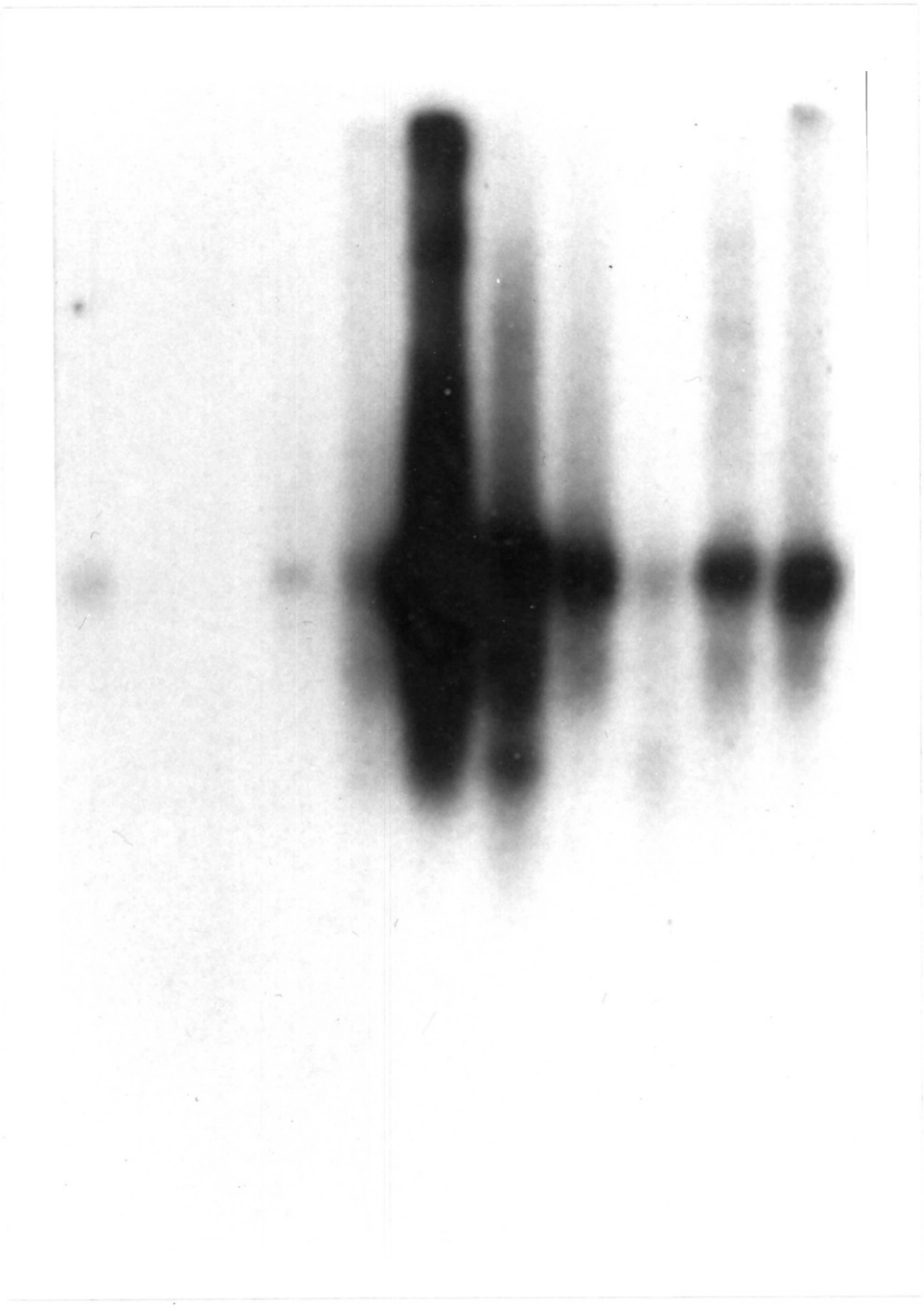

Figure 7.4 Northern blot of MTC RNA, Experiment 1, probed with exon 4α (calcitonin). Left to right: B1, B2, B3, N, F1, F2, F3, F4, S1, S3, S3.

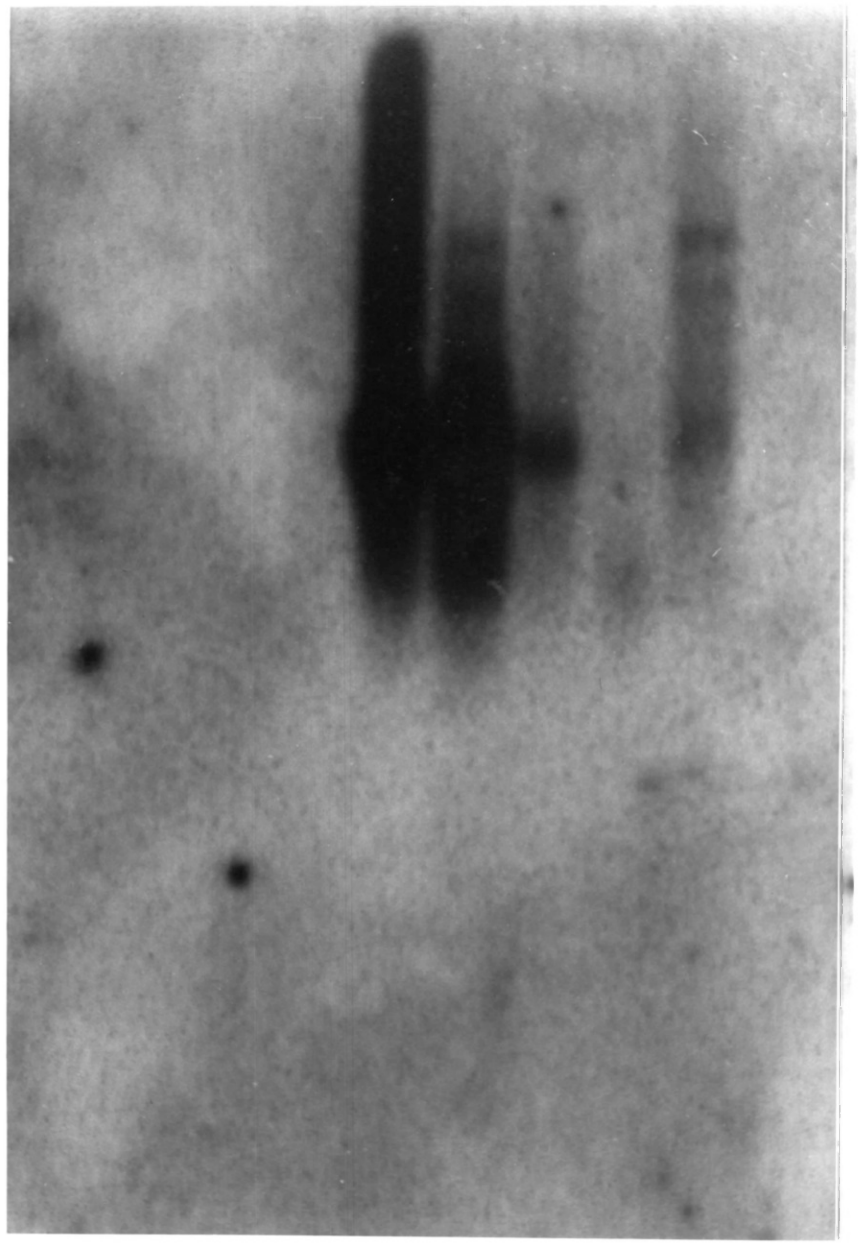

Figure 7.5 Northern blot of MTC RNA, Experiment 1, probed with exon 6α (CGRP specific). Left to right: B1, B2, B3, N, F1, F2, F3, F4, S1, S2, S3.

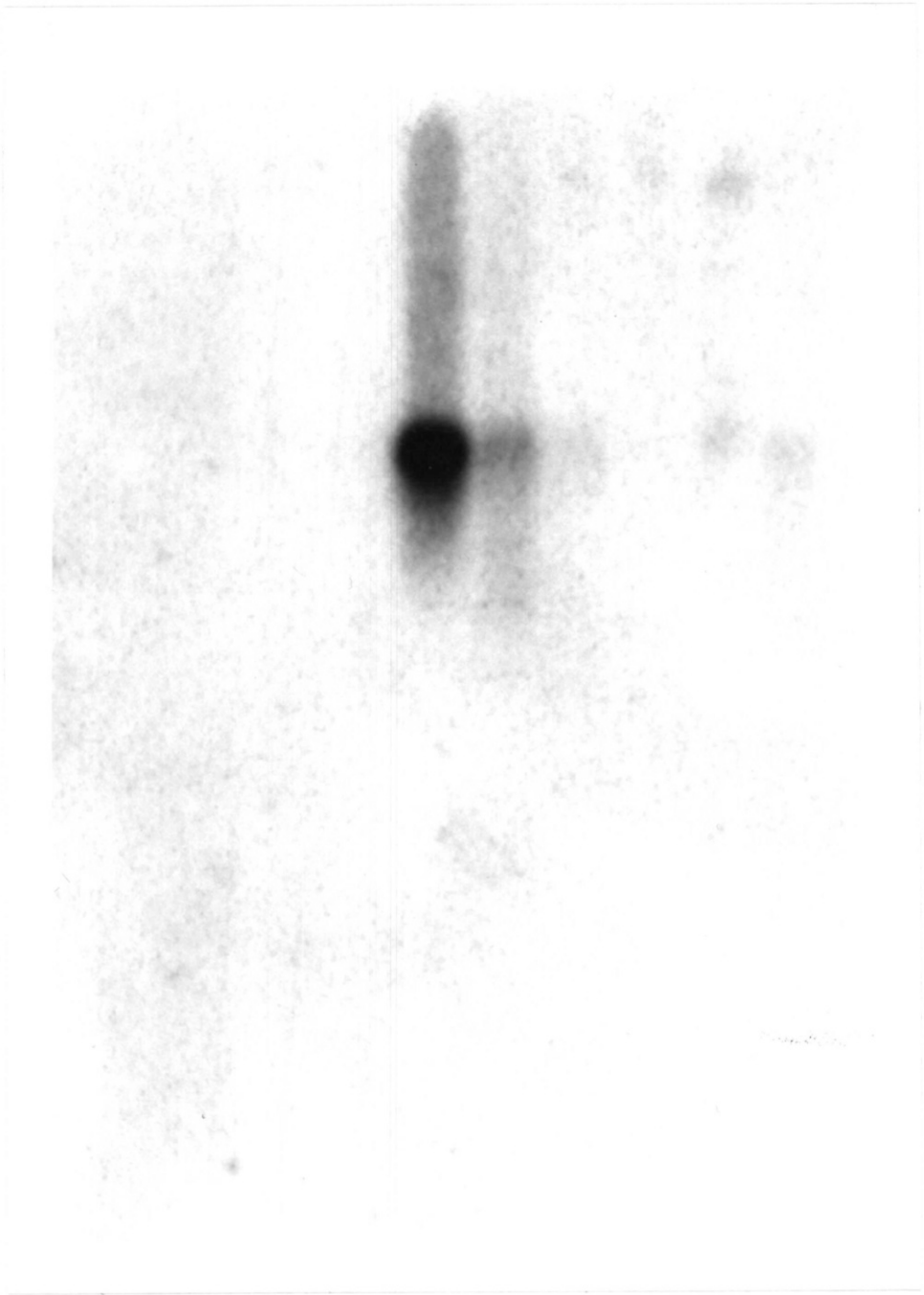

Figure 7.6 Northern blot of MTC RNA, Experiment 1, probed with exon 6β (CGRP specific). Left to right: B1, B2, B3, N, F1, F2, F3, F4, S1, S2, S3.

Table 7.1 Relative expression of exon 4α in each tumour (constant amount of mRNA)

Tissue	Ratio of exon 4 expression in each tissue to normal expression
BEN	5
	..
NORMAL	1
	..
FAMILIAL 1	50
	..
FAMILIAL 2	90
	..
FAMILIAL 3	60
	..
FAMILIAL 4	10
	..
SPORADIC 1	5
	..
SPORADIC 2	50
	..
SPORADIC 3	50

Key to Northern Blots in Figures 7.7, 7.8, 7.9

	TISSUE	mRNA (mg/ml)
B1	BEN cells	0.5
B2	BEN cells	0.2
B3	BEN cells	0.1
N	'Normal' thyroid	1.0
F2	Familial MTC (MEN II)	1.0
F3	Familial MTC (MEN II)	1.0
F4	Familial MTC (MEN II)	1.0
S2	Sporadic MTC	1.0
S3	Sporadic MTC	1.0

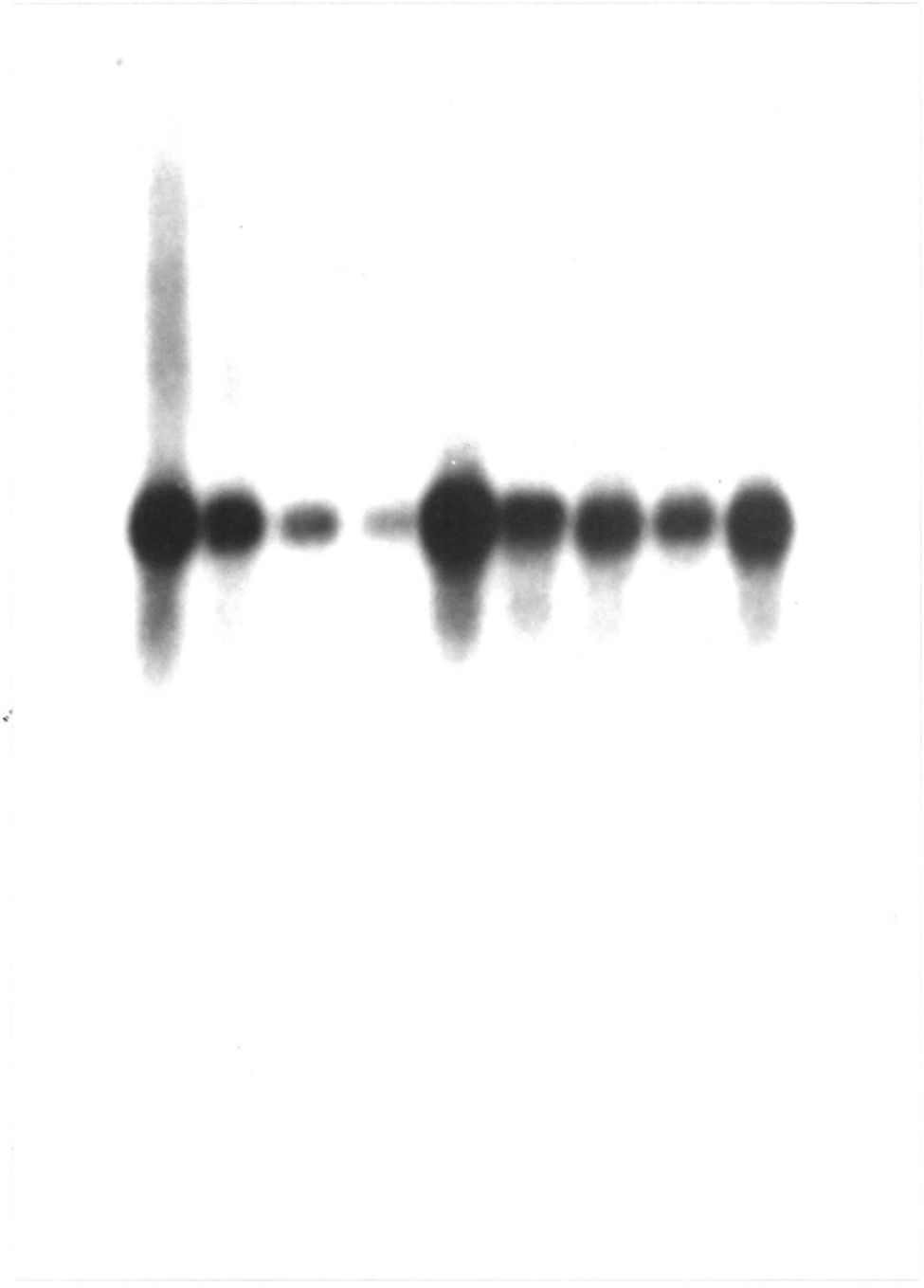

Figure 7.7 Northern blot, Experiment 2, probed with exon 4α.
Left to right: B1, B2, B3, N, F2, F3, F4, S2, S3.

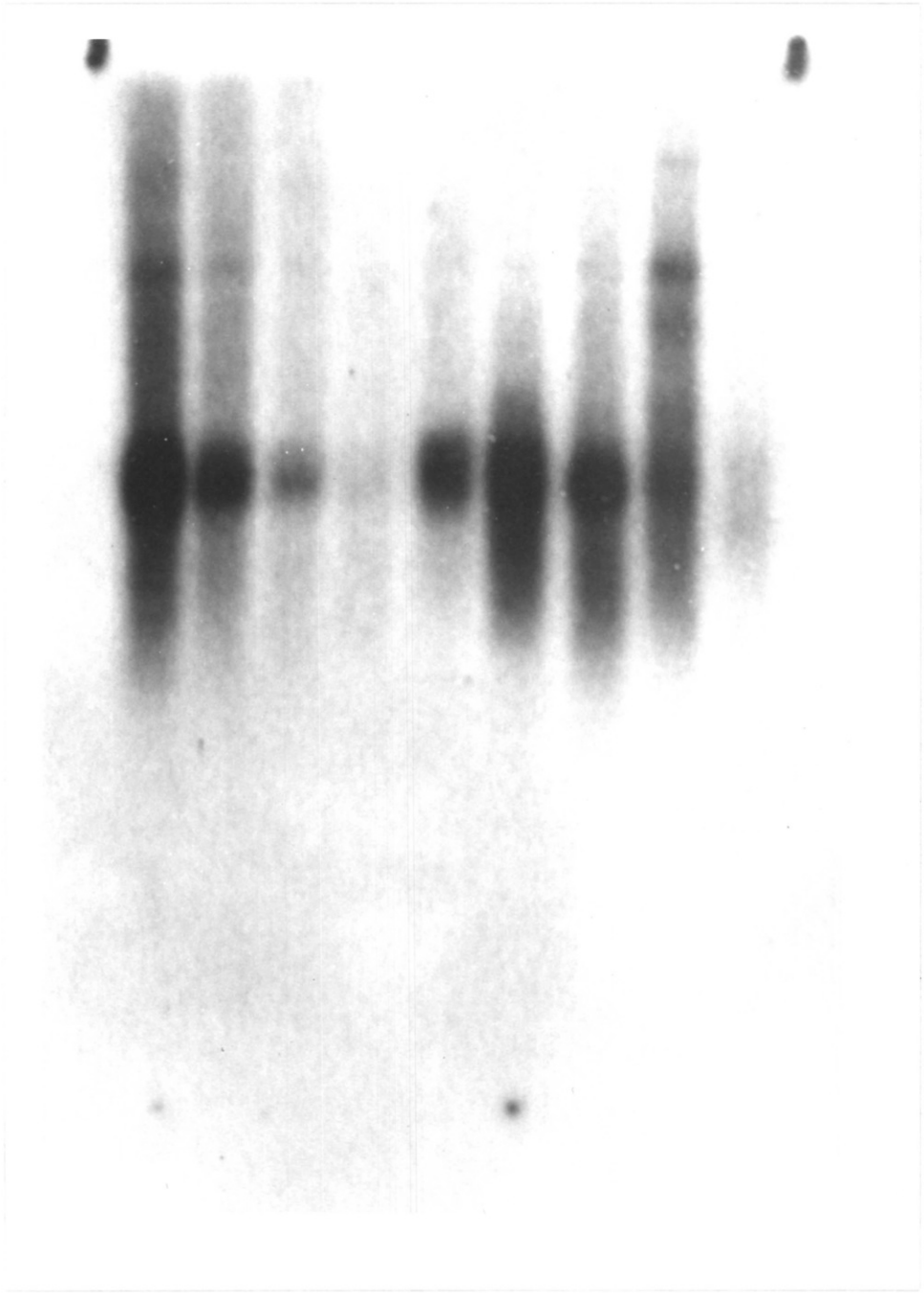

Figure 7.8 Northern blot, Experiment 2, probed with exon 6α.
Left to right: B1, B2, B3, N, F2, F3, F4, S2, S3.

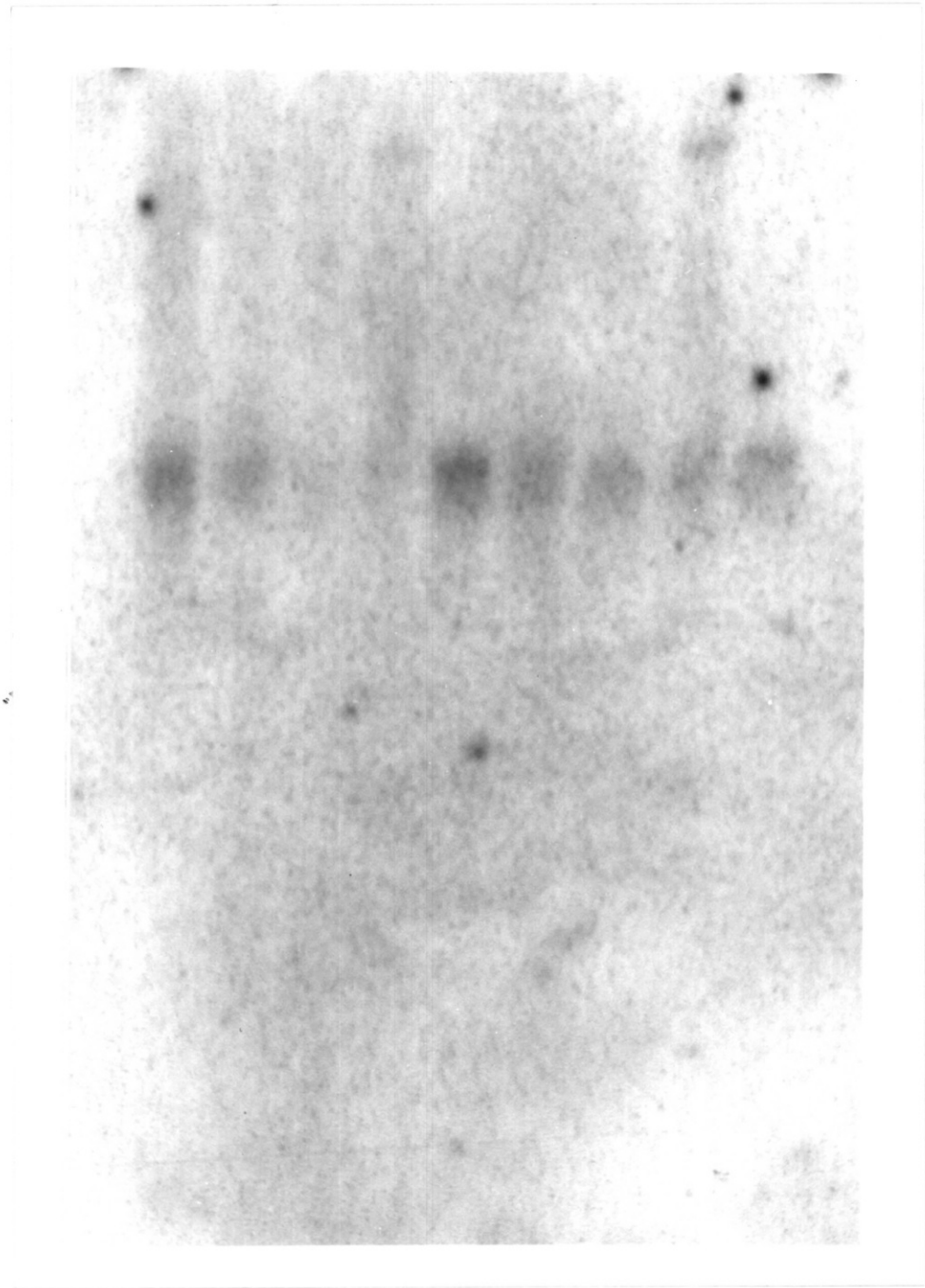

Figure 7.9 Northern blot, Experiment 2, probed with exon 6β.
Left to right: B1, B2, B3, N, F2, F3, F4, S2, S3.

Table 7.2 Relative expression of exon 4α, exon 6α and exon 6β in MTC tumours

Tissue	RATIO exon 4α:6α	RATIO exon 4α:6β	RATIO exon 6α:6β
BEN	7:1	180:1	26:1
NORMAL	35:1	90:1	2.6:1
FAMILIAL 2	35:1	360:1	10.4:1
FAMILIAL 3	4.1:1	180:1	39:1
FAMILIAL 4	6.1:1	315:1	52:1
SPORADIC 2	7:1	180:1	26:1
SPORADIC 3	70:1	450:1	6.5:1

7.4 DISCUSSION

Following the realisation that MTC was a tumour of the calcitonin secreting, thyroidal c-cells (Cunliffe et al 1968) considerable interest has centred on MTC tissue to investigate expression of the calcitonin gene. This work led to the identification of an alternative splice product from the calcitonin gene, encoding the predicted peptide CGRP (Amara et al 1982), and subsequently to the discovery of a second CGRP gene. The studies described in this chapter have confirmed the expression of both α and β CGRP mRNA in human MTC tissue and the squamous carcinoma cell line, 'BEN'. Various other groups have also corroborated these findings (Steenbergh et al 1985, Edbrooke et al 1985) and β CGRP mRNA has recently been observed in Ewing sarcoma cell lines (Hoppener et al 1987).

Large mRNA species, reactive for α exon 4 or exon 6, were observed on the X-ray films of several of the tumours as well as BEN cells. Bovenberg et al (1986), studying mRNA obtained from human MTC tissue and a cell line, have proposed a model for the processing of the calcitonin gene including several intermediate RNA species. Their studies revealed the presence of high molecular weight poly A RNAs of various sizes greater than mature calcitonin and CGRP mRNAs. The model deduced from their findings suggests that introns 1, 2 and 5 (not involved in alternative processing) are spliced from the 5.7 kb (kilo base) RNA to give a 3.3kb RNA . Subsequent processing of the 3.3 kb RNA will either be by removal of exon 3 with polyadenylation of exon 4 to give calcitonin mRNA or by splicing all the intron 3-exon 3-intron 4 region to give CGRP mRNA. The high molecular weight RNA bands observed in the MTC tumours studied in this chapter are presumably similar intermediates in calcitonin/CGRP mRNA processing. The mechanisms of β-CGRP gene processing require further elucidation.

The results from investigations in this chapter were not able, however, to demonstrate any difference in expression of the α-calcitonin gene between familial and sporadic tumours. Studies on plasma and tumours from a large group of patients will be required to establish whether any difference exists between familial and sporadic

MTC in either mRNA procesing or peptide secretion. Furthermore there were differences within the two groups of tumours in the proportion of calcitonin to CGRP mRNA and in the presence of large mRNA species. This finding indicates considerable variation in the expression of the calcitonin gene in MTC tissue.

Work by Baylin et al (1978) on MTC patients who were heterozygous for glucose-6-phosphate dehydrogenase (G6PD) isoenzymes A and B, produced some interesting findings. Such individuals had a mosaic pattern of G6PD types A and B in their normal tissues whilst separate tumour nodules within the thyroid of one patient with familial MTC were found to contain different G6PD isoenzymes. Baylin then postulated that the inherited mutation of MEN II produced multiple clones of defective cells.

Knudson (1971) had earlier proposed a 'two hit' theory for the development of malignant tumours which suggests that two mutational events are necessary for the initiation of neoplasia. In sporadic neoplasia both these mutations would occur in the somatic cells leading generally to solitary tumours. In hereditary neoplasms the first event is a genetic mutation which would render many cells susceptible to a second somatic mutation with the possible consequence that more than one clone of cells would be expanded leading to bilateral or multiple tumours. C-cell and medullary hyperplasia in familial MTC and phaeochromocytoma have been suggested as the primary mutational event in these diseases.

Genetic heterogeneity within the population may mean that for both familial and sporadic MTC mutational events occur at different loci. The process of neoplastic change will then be through a variety of pathways for different individuals or families, and even within individuals with c-cell hyperplasia. The expanding tumours would then select for different changes in calcitonin gene processing and hence produce the variety of calcitonin gene expression observed in the patients of this study.

MTC tumours apparently differ considerably from each other in expression of the calcitonin gene and caution should be used when interpreting investigations which have been performed on MTC tissue or

cell lines. The extrapolation of findings in MTC to the normal expression of the calcitonin gene may not be valid and consideration of processing in more than one tumour may be necessary before definite conclusions are drawn concerning expression of the α-calcitonin/CGRP gene.

Whilst the processing of the calcitonin gene in MTC has sparked considerable interest, the suggestion by Kidd et al (1984) that the MEN II locus can be excluded from the distal region of chromosome 11, means it is unlikely that the calcitonin locus is involved in the occurrence of MEN II in the kindred studied. The idea that regulatory factors may be involved in the control of peptide hormone gene expression has been suggested for some time (Rees, 1976). The gene producing the regulatory factor may be adjacent to or distant from the site of the hormonal gene but mutations affecting the regulatory gene would lead to alterations in expression of the peptide hormone gene. A regulator for the control of calcitonin gene expression at the mRNA level has also been proposed (Leff et al 1987) and investigations into the nature of such factors may prove fruitful in the continuing study of MTC tumour genesis.

CHAPTER 8 OVERALL DISCUSSION AND CONCLUSIONS

CHAPTER 8 OVERALL DISCUSSION AND CONCLUSIONS

8.1 THE MEASUREMENT OF CIRCULATING CALCITONIN GENE PEPTIDES

In order to investigate the production of calcitonin gene peptides in health and malignant disease, it was necessary to develop sensitive and specific immunoassays. These assays were required to distinguish between the normal circulating levels of calcitonin gene peptides, the minor increases seen in ectopic hormone production and the high levels associated with MTC.

The development of a radioimmunoassay for CGRP using an antiserum raised against human CGRP proved to be considerably more sensitive than the assay using an antiserum raised against rat CGRP. The main alteration made to the existing calcitonin and katacalcin RIAs was to purify the radioiodinated peptide on HPLC. HPLC purification enabled the separation of monoiodinated peptide from diiodinated peptide, possible peptide fragments and unlabelled peptide. The resulting label greatly improved the sensitivity of the calcitonin RIA allowing a useful comparison with the newly developed AEIA. The principle advantages of the AEIA were the non-isotopic label and the ease of reading results. Unfortunately the cost of amplifier reagents and the possibility of edge effects in plate coating meant that the calcitonin RIA was used in clinical studies. However, non-isotopic assays will increasingly be used in the future and the careful selection of monoclonal antibodies should produce extremely specific immunometric assays.

Using these RIAs a normal range for plasma CGRP was established whilst the calcitonin normal range was found to be lower than previously thought. Future work on the normal physiology of calcitonin gene peptides should be facilitated by these sensitive immunoassays.

The RIAs also enabled the measurement of circulating calcitonin gene peptides in MTC and non-thyroid malignancies. Studies on plasma from MTC patients have confirmed both calcitonin and katacalcin to be useful tumour markers for this disease; however, CGRP was elevated

above normal in only about 50% of patients. Calcitonin gene peptides were abnormal in a selection of the patients with non-thyroid tumours, particularly myeloid leukaemia, lung and breast cancer. However, calcitonin and CGRP were infrequently elevated in carcinoid and phaeochromocytoma patients, whilst in lymphoma and lymphoid leukaemia calcitonin was only occasionally elevated and CGRP always found to be within the normal range.

8.2 THE EXTENT OF PEPTIDE PRODUCTION IN MALIGNANT DISEASE

Hormone production by malignant tumours is generally described as being either appropriate to the tissue in which the tumour arises (eutopic) or inappropriate to the tissue of origin of the tumour (ectopic).

The real extent of abnormal peptide production in malignant disease can be obscured in a number of ways. Patients with clinical endocrinopathies allow the identification of tumours secreting biologically active pepties; however, biologically inactive precursors or hormone fragments may also be secreted as well as certain peptides which do not produce obvious clinical syndromes e.g. calcitonin. Furthermore, the secretion of biologically active peptides e.g. those produced in classical carcinoid syndrome, may detract from an investigation of inactive peptides.

The true incidence of aberrant hormone secretion can be disguised by cyclic or episodic release of the hormone from the tumour. Certain physiological stimuli can induce peptide secretion and cause the clinical symptoms of endocrinopathies. Examples of such responses are the flushing attacks observed in carcinoids following ingestion of food and paroxysmal attacks in phaeochromocytoma patients following exertion or emotional stress (Chapter 5). Additionally, most hormones are not unique to neoplastic tissue and therefore peptides of neoplastic origin wil be released against a background of normal circulating levels. Physiological feedback mechanisms could also reduce normal secretion of a hormone such that the malignant secretion falls within the normal range.

Peptide production by neoplastic tissue may thus be more widespread than is currently perceived. The mis-timing of sample collection and choice of the wrong spectrum of assays for screening the sample may result in an incomplete picture of peptide secretion in a given patient. The secretion of as yet unidentified peptides from malignant tissue will also obscure the true extent of inappropriate hormone production in cancer.

8.3 CALCITONIN/CGRP GENE EXPRESSION IN TUMOURS

Investigations into the expression of the calcitonin/CGRP genes in MTC have indicated considerable variability between tumours at the mRNA levels. These differences were in both the amount of mature calcitonin mRNA in each tumour and in the ratios of mature calcitonin mRNA to mature α-CGRP mRNA and β-CGRP mRNA. Additionally there were differences in the presence or absence of high molecular weight mRNAs.

From these results it is clear that the end result of neoplastic transformation (in this case a tumour in the thyroidal c-cells) does not always produce the same biochemical alterations. The tumours may not, therefore, all arise by the same mechanism of malignant change. Certain types of cancer are now thought to occur as the result of mutation, rearrangement, translocation or other derangement of mitogenes (the normal counterparts of oncogenes). Mitogenes were found to be substances involved in cell division e.g. growth factors, receptors, intermediates in the transduction of signals within the cell and DNA binding proteins (Gordon 1985). Alterations of such mitogenes to oncogenes would result in a loss of control of mitosis with consequent expansion of a clone of cells. An increase in calcitonin gene peptide production and secretion by tumour cells of either thyroid or non-thyroidal origin may occur as a by-product enhanced by activation of oncogenes. Alternatively the movement of cells into a state of mitosis, through the switching of oncogenes, may represent the switching of cells to an earlier stage of development. A loss of DNA suppression, or more likely increased mRNA processing, would result in production of peptides normally associated with tissues of the same cell series e.g. APUD cells, or those usually observed in foetal development.

8.4 IS THE PRODUCTION OF ABNORMAL AND EXCESSIVE AMOUNTS OF PEPTIDE HORMONE OF BENEFIT TO TUMOUR CELLS?

The expansion of a clone of cells (or several clones) probably occurs via a series of steps so that a tumour arises from those cells with a competitive advantage (Shields 1978). The production of increased amounts of peptide hormones by the tumour could provide a selective advantage if the peptide were acting as a growth factor via an autocrine mechanism. Alternatively the peptide could cause the synthesis of growth factors elsewhere or increase the number of receptors on the cell surface to take maximum advantage of the available growth factors.

The secretion of biologically active peptides by a tumour may also result in changes at the normal target organ of that peptide which are advantageous to the tumour. For example, the secretion of CGRP by a tumour may produce paracrine effects on the local vasculature with consequent vasodilation and increased blood supply to the tumour. The rate of tumour growth might otherwise be restricted by a shortage of blood factors or by accumulated metabolities. Calcitonin has been reported to influence transcellular movement of calcium (Yamaguchi et al 1975). The production of calcitonin by malignant cells may thus induce changes in cell calcium and mediate beneficial alternations in cell function at either autocrine or endocrine levels. Becker (1985) has also suggested that calcitonin may influence the growth of certain tissues.

The production of abnormal and excessive amounts of peptide hormones by tumours may thus reduce serum dependence, increase the availability of growth factors, or cause advantageous changes to the environment of the tumour.

8.5 THE FUTURE ROLE OF CALCITONIN GENE PRODUCTS AS TUMOUR MARKERS

Peptide hormones which are overproduced by malignant tissue are frequently useful as biochemical tumour markers. Ideally a tumour marker should occur with sufficient frequency to make measurements worthwhile, but the practical value of a marker will depend on the availability of effective treatment for the cancer.

Measurements of circulating calcitonin and katacalcin in MTC are widely used in the diagnosis and follow-up of this disease, allowing surgery to be undertaken before any other clinical signs are apparent. As MTC can occur in familial forms as well as sporadic tumours, sensitive calcitonin and katacalcin immunoasays are useful for screening family members at risk of MTC, using stimulation tests to identify those patients with latent c-cell hyperplasia.

Elevated circulating CGRP levels have not proved to be diagnostic of MTC. However, the results in Chapter 4 have indicated significantly different CGRP levels in patients with sporadic and familial MTC. CGRP may therefore be useful in distinguishing between these 2 groups of patients.

The precise rôle of CGRP in differentiating between familial and sporadic MTC requires further study. Clarification of this rôle would probably require a long-term prospective study of circulating CGRP levels in all new patients before removal of the tumour and diagnosis of tumour type.

Both calcitonin and CGRP were found to be elevated in a number of the non-thyroid malignancies studied, particularly myeloid leukaemia, lung and breast cancer. The frequent success of 'lumpectomy' as a treatment for breast cancer means that an early tumour marker for this disease would be valuable. Unfortunately neither calcitonin nor CGRP were consistently elevated for a particular tumour type and their use as tumour markers may be restricted to patient follow-up and monitoring of treatment. The finding of elevated calcitonin levels in non-thyroid malignancies indicates that care should be taken when interpreting calcitonin measurements on suspected MTC patients, as the oversecretion of this peptide is not restricted to MTC alone.

The abnormal calcitonin levels measured in the patients with non-thyroid malignancies may not have been a consequence of ectopic hormone secretion by the tumour. Calcitonin may have been secreted as a physiological response to prevent osteolysis by bone metastases (Neilsen and Gadeberg 1980) or as a response to hyper-calcaemia caused by the secretion of factors such as PTH or "PTH-like" material by the tumour (LeRoith and Roth 1985). However, the decision point for abnormal calcitonin has been set at 23 pmol/ℓ for this calcitonin assay which is more than twice the observed upper normal limit (10 pmol/ℓ, Chapter 3). Although physiological increases in calcitonin secretion in response to these conditions remain a possibility, they are unlikely to exceed 23 pmol/ℓ.

Future studies on the secretion of calcitonin gene peptides in non-thyroid malignancies could be extended to include other endocrine and non-endocrine tumours. An investigation into the prodution of calcitonin gene peptides by neurological tumours would be of particular interest, since immunoreactive calcitonin has been described in the neural tissue of lower chordates whilst CGRP immunoreactivity has been localised to mammalian nerves. The molecular biology techniques of RNA extraction and Northern blotting could also be applied to studying non-thyroid tumours, confirming these tumours as the source of calcitonin gene peptides. Additional information on the relative expression of calcitonin and CGRP mRNA in non-thyroid tumours may also be gained. Furthermore these techniques are able to identify the production of both α and β CGRP mRNA by tumours whist the RIAs cannot distinguish between α and β CGRP immunoreactivity.

If the profile of peptides secreted by a tumour can be shown to associate with a specific mechanism of malignant transformation this may allow new approaches to the classification of neoplasms. A classification of this nature, based on their pathogenesis rather than morphology, could possibly provide an alternative rationale for therapy. Whilst surgery will probably continue as the first line of defence for localised tumours, biochemical treatment, aimed at blocking receptor function and reversing other aspects of oncogene activity, may be added to chemotherapy and radiotherapy to restrict residual malignant tissue. Monitoring the products of neoplastic change e.g. calcitonin gene peptides in certain tumour types, would indicate the success of this treatment.

APPENDIX

IMMUNOASSAYS

TRIS Buffered Saline (Amplified Immunoassay)

50 mmol/ℓ TRIS
150 mmol/ℓ sodium chloride
0.02% Tween 20
Trasylol

Phosphate Buffer (Radioimmunoassay)

4 volumes 50 mmol/ℓ disodium hydrogen orthophosphate
1 volume 50 mmol/ℓ hydrogen disodium orthophosphate, pH 7.4
0.01% EDTA
0.02% sodium azide
20,000 U/ℓ Trasylol

Phosphate Buffer 0.25 mol/ℓ (^{125}I-Labelling)

4 volumes 0.25 mol/ℓ disodium hydrogen orthophosphate
1 volume 0.25 mol/ℓ hydrogen disodium orthophosphate, pH 7.4

MOLECULAR BIOLOGY

Guadinium Mixture

4 mol/ℓ guadinium isothiocyanate
50 mmol/ℓ TRIS HCl (pH 7.6)
10 mmol/ℓ EDTA
144 mmol/ℓ β-mercaptoethanol
2.1% Sarkosyl (sodium lauryl sarkosinate)

Poly-U Sepharose Column

	Load	Wash	Elute
Formamide	25%	50%	97.5%
Pipes	10 mmol/ℓ	10 mmol/ℓ	10 mmol/ℓ
EDTA	1 mmol/ℓ	1 mmol/ℓ	1 mmol/ℓ
SDS	0.1%	0.1%	0.1%
NaCl	700 mmol/ℓ	500 mmol/ℓ	0

MOPS Buffer (20x)

1 mol/ℓ sodium chloride
0.1 mol/ℓ sodium acetate
20 mmol/ℓ EDTA
0.1% formaldehyde, pH 7.0

Nucleotide Buffer (final reaction concentration)

5 mmol/ℓ magnesium chloride
50 mmol/ℓ TRIS HCl, pH 8.0
10 mmol/ℓ β-mercaptoethanol
100 μmol/ℓ of each deoxynucleotide (dATP, dTTP, dGTP)
1 mmol/ℓ HEPES, pH 6.6
650 μg/ml random oligonucleotides

S5PE (20x)

3.6 mol/ℓ NaCl
0.2 mol/ℓ sodium phosphate buffer
20 mmol/ℓ EDTA

BRL Buffer (5x)

1 mol/ℓ potassium cacodylate
125 mmol/ℓ TRIS HCl (pH 6.9)
10 mmol/ℓ dithiothreitol
2.5 mmol/ℓ cobalt chloride

Denhart's Solution

0.02% FICOLL
0.02% polyvinyl pyrolidone
0.02% BSA

Phenol/Chloroform

50% liquefied phenol buffered with TE pH 8.0 and
0.1% 8-hydroxyquinalene
50% chloroform:isoamyl alcohol, 24:1

MATERIALS - RIA AND AEIA

Peptides

Calcitonin - a gift from Dr W Rittel, CIBA-GEIGY, Basle, Switzerland.

Katacalcin - PDN-21 - Peninsula Laboratories, Lot No 004690

CGRPα - SANDOZ Lot No 14498

CGRPβ - Peninsula Laboratories, Lot No 008352.

Chemicals

Trasylol (aprotinin protease inhibitor) - Bayer, Berkshire

^{125}I - Amersham International, Buckinghamshire

Phosphate buffer and iodination reagents - BDH, Poole

Activated charcoal - Sigma, Hertfordshire

TRIS buffer reagents - Sigma, Hertfordshire

Sodium bicarbonate - BDH, Poole

Lactose - BDH, Poole

Tween 20 - Sigma, Hertfordshire

Substrate and Amplifier Buffers for AEIA - IQ(Bio), Cambridge

Other Proteins

Sheep anti-rabbit γ-globulin antiserum, cellulose bound - IRE, Belgium

Human plasma protein fraction BP - Blood Products Laboratory, Hertfordshire

HSA Fraction V) - Sigma, Hertfordshire

BSA Fraction V)

Other Equipment

Microtitre plates - Nunc, Denmark

Titertek plate sealers - Flow Laboratories

Titertek Multiskan MCC - Flow Laboratories

1261 MultiGamma Counter - LKB Wallac, Wallac Oy, Turku, Finland

MATERIALS - MOLECULAR BIOLOGY

Guanidium isothiocyanate (FLUKA) - Fluorochem Ltd, Derbyshire

Caesium chloride - Rose Chemicals, London

Sodium dodecylsulphate - Cambridge Biotechnology Laboratories, London

Formaldehyde - Koch-Light Ltd, Suffolk

Hybond Filters - Amersham International, Buckinghamshire

^{32}p dCTP - Amersham

Kodak XAR5 Film - Kodak, Hemel Hempstead

MATERIALS - TISSUE CULTURE

Gibco, Uxbridge, Middlesex

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