To my daughter, Dalia.

IMMUNOCHEMICAL STUDIES ON CALCITONIN

by

SAMIA IBRAHIM GIRGIS
M.B., B.CH., Dip. Med. Sci. Chem. Path.
(University of Assiut, Egypt)

Thesis submitted to the
UNIVERSITY OF LONDON
for the degree of
DOCTOR OF PHILOSOPHY

Endocrine Unit,
Department of Chemical Pathology,
Royal Postgraduate Medical School,
London 1980.

ABSTRACT

The work presented in this thesis falls into three sections.

Section I (Chapter 1) is a literature review of calcitonin from its discovery in 1962 as a hypocalcaemic factor, until the recent evidence showing its presence in the chordate nervous system perhaps suggesting a neurotransmitter function.

Section II (Chapter 2) describes methods used for identification of the human calcitonin molecule at picomolar concentration, by means of several region-specific radioimmunoassays and high performance liquid chromatography.

Section III (Chapters 3, 4 and 5) describes: the characterization of normal human calcitonin in plasma and suggests structural identity with calcitonin-M of medullary thyroid carcinoma (Chapter 3); and the identification of a human calcitonin-like molecule in the nervous system of several chordates which throws some light on the evolution of the calcitonins (Chapter 4). The final chapter (Chapter 5) is a conclusion and general discussion.

ACKNOWLEDGEMENTS

It is a pleasure to record my indebtedness to my supervisor, Professor Iain MacIntyre, for his guidance and encouragement throughout the course of this study.

I am most grateful to Dr. Imogen Evans for reading the manuscript. I should like to thank Drs. Michael Szelke and Carrie Hillyard for their collaboration during the early part of this study. It was a pleasure to collaborate with Mr. Colin McMartin of Ciba-Geigy Horsham Research Centre on the HPLC identification of normal human calcitonin, and with Dr. Quentin Bone of the Marine Biological Association, Plymouth and Drs. Fernando Galan Galan, Ray Rogers and Mr. Tim Arnett on the work described in Chapter 4. The immunization of rabbits with human calcitonin fragments was kindly carried out by Mr. Bijleveld of Pollard Wood Research Station, Little Chalfont.

I wish to thank Dr. W. Rittel of Ciba-Geigy, Basle for the generous supply of synthetic human calcitonin, fragments and analogues.

My thanks are due to the Egyptian Ministry for Higher Education, and the Medical School, University of Tanta for granting me a personal scholarship. I should also like to thank the Medical Research Council for supporting this study.

I am especially grateful to Miss Ann Turner for her hard work in typing this thesis. Finally, I wish to thank my husband for his support.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CALCITONIN : LITERATURE REVIEW

1. Introduction

The studies described in this thesis are mainly concerned with the immunochemical characterization of normal human calcitonin and a comparison of this with calcitonin from medullary thyroid carcinoma. Therefore, the literature has been reviewed with respect to calcitonin in general, with some emphasis on medullary thyroid carcinoma.

2. History

In the late 1950's, it was believed that parathyroid hormone was the only important regulator of plasma calcium (Howard, 1957). McLean and Urist (1961) postulated a feedback mechanism, whereby hypocalcaemia stimulates parathyroid hormone secretion and hypercalcaemia inhibits it by direct action on the gland. However, parathyroid hormone is slow acting, and therefore a control system of this kind would lead to oscillation in the plasma calcium level (Rasmussen, 1961); this is not the case as plasma calcium is constant.

In a study of the direct humoral control of parathyroid function, Copp and Cheney (1962) noticed that perfusion of the isolated thyroparathyroid gland complex of dogs with high calcium blood caused a fall in systemic blood calcium which occurred earlier and was of greater magnitude than that produced by total thyroparathyroidectomy. Hypercalcaemic perfusion of the thyroid gland alone had no effect, so they concluded that the source of this hypocalcaemic factor must be the parathyroid glands. They called the factor "calcitonin".

The existence of "calcitonin" in dogs was confirmed by Kumar, Foster and MacIntyre (1963) but their investigations did not provide any evidence to confirm that the parathyroid glands were the source of the hormone. This is because perfusion of the isolated parathyroids in separation from all thyroid tissues is not technically feasible in the dog.

At about the same time, Hirsch, Gauthier and Munson (1963) observed that, in rats, parathyroidectomy by electrocautery produced a greater fall in calcium than did surgical parathyroidectomy. They concluded that cautery of the adjacent thyroid gland during parathyroidectomy released a calcium lowering factor from the thyroid gland. They also found a hypocalcaemic factor in rat thyroid extracts and they called it thyrocalcitonin to distinguish it from "calcitonin".

The initial controversy regarding the source of this hormone - thyroid gland or parathyroid gland - was resolved in 1964 by Foster and his colleagues who demonstrated its thyroid origin by perfusion experiments on goats whose external parathyroid glands have a separate blood supply making isolated parathyroid gland perfusion feasible. Nevertheless, Copp and Henze (1964) and Care and Keynes (1965) produced further evidence from experiments using sheep still claiming that the parathyroids were the gland of origin of calcitonin.

3. Cell of Origin

Further studies by Foster, MacIntyre and Pearse (1964) on dog thyroid glands perfused with high and low calcium blood,

indicated that the mitochondrion-rich cells are responsible for secretion of calcitonin. These cells correspond to the parafollicular cells of Nonidez (1930) and the parenchymatous cells of Baber (1876). Following the original suggestion by Foster, MacIntyre and Pearse (1964) the term C-cells (C for calcitonin) was introduced by Pearse (1966). Demonstration of the calcitonin content within the C-cells was provided by immunofluorescence studies in dogs and pigs by Bussolati and Pearse (1967); and Bussolati, Carvalheira and Pearse (1968).

Using cytochemical methods, Pearse and Carvalheira (1967) and Carvalheira and Pearse (1968) demonstrated that C-cells in mice and rats arise from the last ultimobranchial pouch and migrate into the thyroid. This confirmed the earlier suggestion of Godwin (1937) who noticed that the ultimobranchial cells and some thyroid cells had similar histological features. In sub-mammalian vertebrates (birds, reptiles, fish) the ultimobranchial pouches exist as separate glands in adult life and do not become embedded in the thyroid as occurs in mammals and because of this it was suggested that these glands were producing calcitonin. This was confirmed by Tauber (1967), Copp, Cockcroft and Kueh (1967) and Moseley et al. (1968) who found a hypocalcaemic factor in extracts of the ultimobranchial glands in fish, chickens, pigeons and amphibians.

Later Pearse and Polak (1971) reported cytochemical evidence for the neural crest origin of mammalian ultimobranchial C-cells.

4. Chemical Structure

4.1 Isolation and sequence analysis

After it became clear that the thyroid was the gland of origin in mammals and similarly the ultimobranchial bodies in submammalian vertebrates, there followed a race to purify the new hormone.

Porcine calcitonin was the first to be isolated (Baghdiantz et al. 1964). Isolation of calcitonin was made possible by the development of a bioassay by Kumar et al. (1965). The assay was based on the fact that when calcitonin is injected into young rats, there is a marked fall in plasma calcium and phosphate. In the bioassay, the hypocalcaemic response seen 30 minutes after intravenous injection of calcitonin is compared with that of M.R.C. porcine standard. The sequence of porcine calcitonin was reported by several groups (Neher et al. 1968a; Bell et al. 1968; and Potts et al. 1968) and synthesis was accomplished in the same year by Rittel et al.

Early attempts, to isolate and sequence calcitonin from normal human thyroid failed, because of the low yield of the hypocalcaemic factor in normal thyroid (Haymovits et al. 1967). However, MacIntyre's group in London, knowing that medullary thyroid carcinoma (MCT) was a C-cell tumour (Williams, 1966), realised that this tumour might contain calcitonin in sufficient quantities to isolate and characterize the tumour hormone. In collaboration with Riniker and colleagues in Basle, they isolated two closely related human calcitonin peptides from one single

mediastinal C-cell tumour (Neher et al. 1968b; and Riniker et al. 1968). The two peptides displayed a monomer-dimer relationship and they were designated calcitonin-M (monomer) and calcitonin-D (dimer). Calcitonin-M (CT-M) was also isolated by the same group, from four other medullary thyroid carcinoma tumours, but calcitonin-D was not present in all cases. In the same year CT-M was synthesized by Sieber and colleagues. However, it was not clear at that time whether CT-M was the same as the normal hormone. It was not until recently with the development of sensitive radioimmunoassays for human calcitonin based on several region-specific antisera that it has been possible to characterize the normal hormone and to obtain evidence which suggests structural identity with CT-M. This will be discussed in Chapter 3.

The isolation and sequence of the various calcitonins is summarized in Table 1.

4.2 Comparative chemistry

The known sequences of the calcitonins are presented in Figure 1 arranged in order of their similarity in structure to CT-M. All calcitonins are composed of a 32 amino acid polypeptide chain with a 1-7 disulphide bridge, and a prolineamide at the C-terminal.

The structure of calcitonin in the various species is quite different. Only 9 residues are preserved in all species, those at positions 1, 3, 4, 5, 6, 7, 9, 28 and 32. Seven of them reside in the N-terminal region of the molecule. However, the

Table 1

Summary of isolation and sequence studies of calcitonins.

Hormone	Source	Isolation	Sequence	Synthesis
Porcine calcitonin (p-CT)	Normal thyroid	Baghdiantz et al. 1964	Neher et al. 1968(a) Bell et al. 1968 Potts et al. 1968	Rittel et al. 1968 Guttmannet al. 1968
Human calcitonin-M (CT-M)	Medullary thyroid carcinoma	Riniker et al. 1968 Neher et al. 1968(b)	Neher et al. 1968(c)	Sieber et al. 1968(b)
Bovine calcitonin (b-CT)	Normal thyroid	Brewer and Ronan, 1969	Brewer and Ronan, 1969	
Ovine calcitonin (o-CT)	Normal thyroid	Potts et al. 1970	Potts et al. 1970	
Salmon calcitonin (s-CT) s-CT I s-CT II, III	Salmon ultimobranchial bodies	O'Dor et al. 1969	Niall et al. 1969 Keutmann et al. 1972	Guttmann et al. 1969
Rat calcitonin (r-CT)	Medullary thyroid carcinoma Normal thyroid	Byfield et al. 1976(a) Raulais et al. 1976	Byfield et al. 1976(b) Raulais et al. 1976	
Eel calcitonin (e-CT)	Eel ultimobranchial bodies	Noda and Narital, 1976	Morikawa et al. 1976	Morikawa et al. 1976

Amino Acid Sequences of the Calcitonins

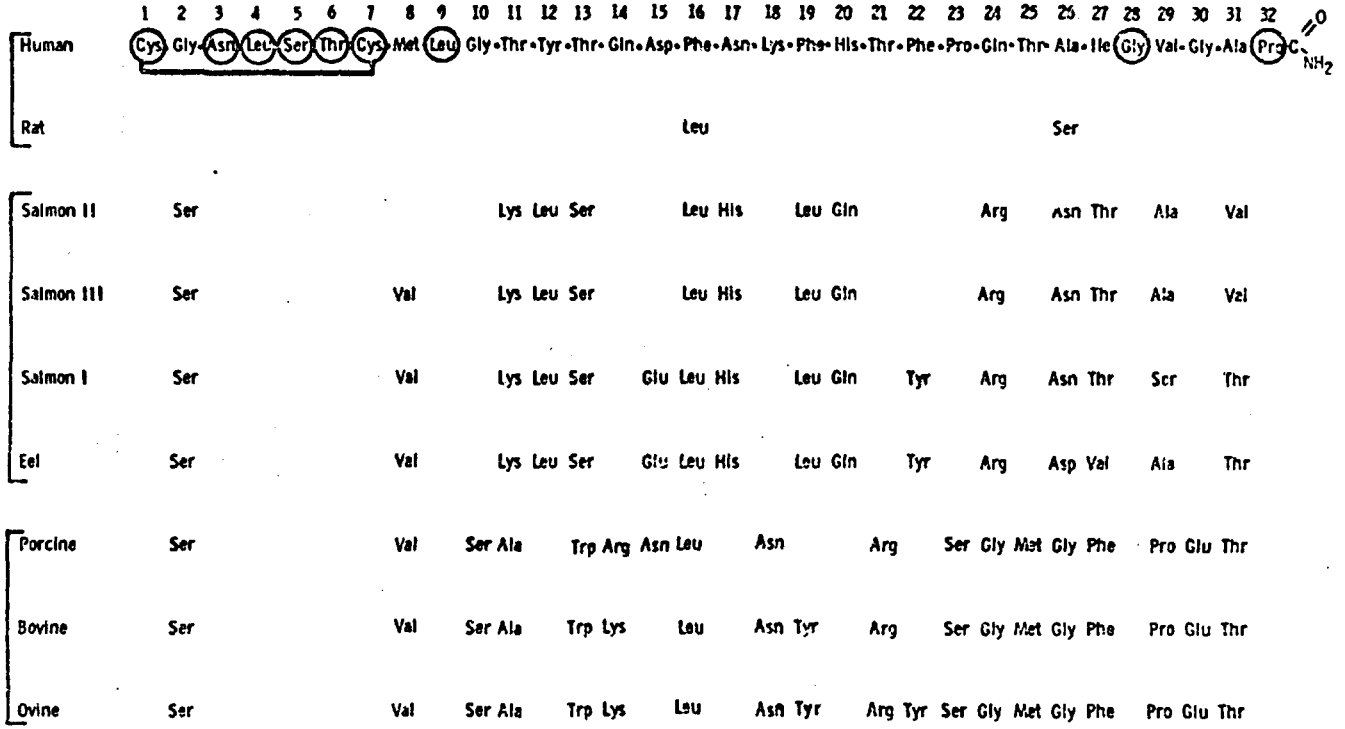


Fig. 1 Amino acid sequences of the calcitonins. The calcitonins from the various species are listed in order of their relatedness to human calcitonin. The amino acids that differ from those of the human hormone are listed; a blank space indicates that the amino acid at that site is the same as that in human calcitonin. The bracket linking the cystein residues at position 1 and 7 indicates the disulphide bridge that is common to all 9 hormones. The data listed in this figure are derived from references shown in Table 1.

sequence variability of the middle region of calcitonin is perhaps more apparent than real. Although no single amino acid in the region of 10-27 inclusive is constant in all the 9 known calcitonins, there is considerable similarity when the comparison is based on the chemical properties of the amino acid side chains. All calcitonins contain at least one acidic amino acid (glutamic acid or aspartic acid at position 15 and in the case of bovine, ovine or porcine another glutamic acid at position 30). Basic residues are confined to a relatively few positions. All calcitonins contain at least two basic residues (arginine, lysine and histidine). Positions 14, 17, 18 and 20 are occupied by either a basic residue or an amide. Hydrophobic residues (leucine, phenyl alanine and tyrosine) are distributed almost regularly along the peptide chain, occupying positions 4, 9, 12, 16, 19 and 27.

The sequences of nine calcitonins so far determined fall into three groups: the primate-rodent group (e.g. man and rat); the teleost group (e.g. salmon and eel); and the artiodactyl group (e.g. ox, sheep and pig). Rat calcitonin differs from human calcitonin-M by only two amino acids at positions 16 and 26, while salmon calcitonin I (the major form of salmon calcitonin) differs from human calcitonin by 16 amino acid substitutions. Porcine and bovine calcitonin differ from CT-M by 18 amino acid substitutions.

4.3 Chemical structure-activity relationships

The complete sequence of 32 amino acids is required for full biological activity of the calcitonins in spite of

differences in their structures. Many amino acid substitutions can be tolerated and some actually increase biological activity (Potts et al. 1971). Seven of the nine conserved residues in all calcitonins reside in the N-terminus suggesting its importance in biological activity. The C-terminal prolineamide is also essential for biological activity as deletion in human calcitonin greatly reduces the hypocalcaemic effect (Rittel et al. 1976) which may suggest that the N- and C-terminals are close to one another in the spatial configuration of the molecule (Byfield et al. 1972). Cleavage of the 1-7 disulphide bond in porcine calcitonin (Sieber et al. 1970) and human calcitonin (Rittel et al. 1976) results in loss of biological activity. Replacement of the disulphide bond (S-S) by an ethylene linkage (C-C) in eel calcitonin, (Asu^{1-7})-eCT, does not significantly affect biological activity (Morikawa et al. 1976).

Oxidation of methionine at position 8 in the human calcitonin molecule destroys biological activity (Sieber et al. 1970) while oxidation of porcine and ovine calcitonin (methionine at position 25) causes no change in biological activity. Furthermore, methionine does not exist at all in the highly active salmon I and III.

The higher biological potency of salmon calcitonin and ultimobranchial calcitonins compared to mammalian calcitonin may be explained primarily by the fact that salmon calcitonin has a greater affinity for receptors in kidney and bone than the porcine calcitonin (Marx, Woodard and Aurbach, 1972), but other factors

include greater resistance to metabolic degradation and consequently longer half-life in the circulation (Habener et al. 1971; and De Luise et al. 1972).

5. Biological Actions

In young mammals, the most obvious effect of calcitonin is hypocalcaemia and hypophosphataemia. The explanation of the hypocalcaemia rather than the hypophosphataemia attracted many investigators. The hypocalcaemic effect might be caused by an action on bone, kidney or gastrointestinal tract. Kidney and gut mechanisms were excluded as possible causes of hypocalcaemia by the demonstration of a calcium-lowering effect in rats after removal of the gastrointestinal tract (Aliopoulios, Savery and Munson, 1965) and also after nephrectomy (Gudmundsson, MacIntyre and Soliman, 1966) and, as calcitonin does not significantly change the calcium content of soft tissues (Kenny and Heiskell, 1965), it seemed logical to conclude that the hypocalcaemic effect is mainly due to the action of calcitonin on bone.

5.1 Action on bone

On the basis of a kinetic study in rats Milhaud, Peilaut and Moukhtar (1965) suggested that calcitonin acted by inhibiting bone resorption. This suggestion was quickly confirmed in bone culture studies when Freidman and Raisz (1965) found that calcitonin inhibited bone resorption directly. This *in vitro* effect was also reported by Aliopoulios, Goldhaber and Munson (1966) and later by Reynolds et al. (1968) who reported that

calcitonin inhibited bone resorption induced not only by parathyroid hormone but also by vitamin A. Perfusion studies using isolated bone, carried out by MacIntyre and Parsons (1966), provided direct evidence that calcitonin increased net retention of calcium in bone. Thus, by studying the effect of calcitonin on isolated rat tibia, they found that calcitonin decreased the calcium content of the effluent blood. In vivo studies of isotope kinetics by Robinson et al. (1967) provided further evidence that calcitonin acted on bone by inhibiting bone resorption. Calcitonin also markedly reduced the urinary excretion of hydroxyproline (Martin, Robinson and MacIntyre, 1966; and Aer, 1968).

In addition, Kallio, Garant and Minkin (1972) observed that osteoclasts in cultured mouse calvaria rapidly lost their ruffled borders (the metabolically active area in bone resorption) on the addition of calcitonin to the culture media. Furthermore, Holtrop, Raisz and Simmons (1974) reported that the addition of calcitonin to the culture media for rat fetal long bones (ulna and radius), produced a decrease both in the number of osteoclasts' ruffled borders and in the amount of ^{45}calcium release.

Receptors for calcitonin have been identified in extracts of rat calvarial membranes (Marx, Woodard and Aurbach, 1972). These receptors have higher affinity for salmon calcitonin than for mammalian calcitonins and this may explain in part the greater biological potency of salmon calcitonin.

The biochemical mechanism by which calcitonin inhibits bone resorption is not entirely understood. It is possible that

calcitonin acts by a cyclic AMP mediated process since stimulation of adenylate cyclase activity in bone cell membranes (Heersche, Marcus and Aurbach, 1974) and of cyclic AMP accumulation in isolated bone cells (Wong and Cohn, 1975) have been demonstrated. Although calcitonin and parathyroid hormone produce an additive effect on cyclic AMP (Heersche, Marcus and Aurbach, 1974), they may be acting on separate bone cell populations (Luben, Wong and Cohn, 1976).

5.2 Action on kidney

Robinson, Martin and MacIntyre (1966) showed that infusion of calcitonin into parathyroidectomized rats led to a dose-dependent phosphaturia. However, there is quite a considerable species difference in the renal response to calcitonin. Thus the hormone does not promote phosphate excretion in either the pig (Russel and Fleisch, 1968) or the dog (Clark and Kenny, 1969).

The effect of calcitonin on the kidney may also be mediated by cyclic AMP as calcitonin increases the concentration of cyclic AMP in rat renal cortex in vivo as well as in isolated cortical tubules (Marx, Woodard and Aurbach, 1972); and calcitonin produces an increase in urinary cyclic AMP in thyroparathyroidectomized rats which precedes the increase in urinary phosphate excretion (Kurokawa et al. 1974). In addition Queener, Fleming and Bell (1974) have isolated partially purified adenylate cyclase from porcine renal cortex which specifically binds calcitonin.

5.3 Action on gastrointestinal tract

It has been proposed that a major function of calcitonin is to inhibit postprandial hypercalcaemia (Gray and Munson, 1969). This view was supported by the observation made in pigs, that feeding calcium meals does not change the plasma calcium, but during feeding there is a gradual elevation of calcitonin levels. However, calcium absorption from ligated gut loops is not affected by calcitonin (Robinson, Matthews and MacIntyre, 1969).

The effects of calcitonin on the gastrointestinal tract seem more likely to be pharmacological than physiological. Gray, Beiberdorf and Fordtran (1973) showed that salmon calcitonin (1 MRC unit/Kg/hr) given intravenously (i.v.) to normal subjects markedly increased intestinal secretion of sodium, potassium, chloride and water but did not influence calcium absorption. They suggested that this mechanism may be at least partly responsible for the diarrhoea occurring in patients with medullary thyroid carcinoma. Calcitonin has also been shown to decrease gastric acid secretion (Becker et al. 1973) and gastrin secretion in patients with peptic ulcers or Zollinger-Ellison syndrome and in normal subjects after meals (Becker et al. 1974).

5.4 Action on nervous system

Only in recent years has interest been focussed on the effect of calcitonin on the nervous system and there is some evidence to suggest that calcitonin may be a neurotransmitter.

This will be discussed in Chapter 5, but some of the relevant work will be reviewed here. During the assessment of the pharmacological and toxological characteristics of salmon calcitonin Pecile et al. (1975) noticed that the hormone in a 2 µg dose elicited a clear cut analgesic effect when injected intracerebroventricularly (i.c.v.) in conscious rabbits. The effect was equivalent to that produced by 40 µg of morphine, and although the mechanism of this central analgesia was unknown it was later found to be independent of opiate receptors (Braga et al. 1978).

At about the same time Nakhla and Nandi Majundu (1978) noticed that i.v. infusion of calcitonin in rats, in addition to lowering plasma calcium and free tryptophan, produced a concomitant increase in brain 5-hydroxytryptamine and cerebral acetyl cholinesterase activity. It is unclear from this experiment whether the effect was due to the lowering of plasma calcium or a direct effect of calcitonin. Further studies by Iwasaki et al. (1979) showed that i.c.v. injection of calcitonin into anaesthetised male rats resulted in a significant dose-related increase in plasma prolactin. This effect was not observed following intravenous injection of calcitonin. However, calcitonin mildly stimulated prolactin release from anterior pituitary cells in culture, and again, in this experiment, the effect of calcitonin may be a direct one on the pituitary cells or may be mediated through a neurotransmitter action of calcitonin. In addition, Freed, Perlow and Wyatt (1979) have observed that subcutaneous and intracerebral injections of calcitonin inhibit feeding in rats.

6. Biosynthesis

Roos, Okano and Deftos (1974), studied the biosynthesis of calcitonin in trout ultimobranchial cells *in vitro*. They incorporated ^{14}C-leucine into the cell proteins and, by chromatographic study of the cell extract, showed two immunoassayable and immunoprecipitable calcitonin peaks, of 3,000 and 7,000 M.W. Furthermore, the relative incorporation of ^{14}C-leucine into the higher and lower molecular weight peaks during pulse-chase experiments was consistent with a precursor-product relationship between them. Similarly Moya, Nieto and Candela (1975) provided evidence for a calcitonin precursor of 13,000 M.W. by studying calcitonin biosynthesis *in vitro* in chicken ultimobranchial glands. However, Cutler, Habener and Potts (1978) could not obtain evidence of a biosynthetic precursor of calcitonin from chicken ultimobranchial glands, either *in vitro* or *in vivo*.

In mammals, studies on calcitonin biosynthesis were precluded, partly due to lack of suitable tissue in which to perform these studies, as the C cells in the thyroid are widely dispersed and represent only a small fraction of the more numerous follicular cells. To overcome this problem, recent investigations into calcitonin synthesis have made use of tissue obtained from patients with medullary thyroid carcinoma. Using this approach, Van der Dork et al. (1978) have reported the existence of a large precursor of calcitonin of apparent molecular weight 65,000 by translation in the frog oocyte, of messenger RNA prepared from human and rat medullary thyroid carcinoma as well as

from a large variety of other tumours such as insulinoma, parathyroid carcinoma, gastrinoma and renal carcinoma (Lips et al. 1978). More recently, Goodman, Jacobs and Habener (1979) used wheatgerm and reticulocyte lysate cell-free systems for the translation of polyadenylated RNA from human medullary thyroid carcinoma. They found that the major product of translation was a protein of 15,000 M.W. which was specifically immunoprecipitable with an antiserum to synthetic human calcitonin and they concluded that this 15,000 M.W. product is likely to be the biosynthetic precursor of calcitonin. This view is strengthened by the studies of Jacobs et al. (1979) in which similar molecular weight products were specifically immunoprecipitated with calcitonin antisera as a major product of the in vitro translation of polyadenylated RNA isolated from rat medullary thyroid carcinoma and cod fish ultimobranchial glands.

7. Calcitonin and the Pituitary Gland

Recently, Deftos and colleagues (1978a,b) reported the localization of immunoreactive calcitonin in all cells of the intermediate lobe, and throughout the anterior lobe of the pituitary gland. They postulated that calcitonin may be part of the 31,000 molecular weight precursor molecule of adrenocorticotrophin and β-lipotrophin (31K) and they suggested a possible physiological role of calcitonin in the pituitary. Despite the intense immunofluorescent staining of the intermediate lobe, Deftos and co-workers (1978b) could not measure or detect immunoreactive calcitonin in rat pituitary extracts, a finding

which alone would cast some doubts on the nature of the immunofluorescent staining. Furthermore, MacIntyre's group in collaboration with Professor Lelio Orci, and using several well characterized specific calcitonin antisera, could not confirm the localization of calcitonin in the pituitary gland (MacIntyre et al. 1979). Similarly, Weber et al. (1979) could not localize calcitonin in the rat pituitary and they could not immunoprecipitate any calcitonin-like molecule from the 31K precursor molecule or its fragments. Moreover, analysis of the tryptic digest of the 31K precursor indicated that calcitonin is not contained within the common precursor 31K molecule. Thus the staining reported by Deftos and colleagues (1978a,b) probably represents some artefact. Despite this, the presence of calcitonin-like immunoreactivity in the rat pituitary was reported by Margulies et al. (1979) who found levels in the pituitary of about 0.1% that of the thyroid, and elevation of immunoreactive calcitonin content in the thyroid and pituitary of genetically obese rats. Furthermore, MacIntyre's group found the immunoreactive calcitonin content of rat pituitary to be less than 0.2% of that in the thyroid (MacIntyre et al. 1979). However, the exact nature of this material remains to be clarified.

8. Calcitonin in Man

8.1 Normal

The physiological role of calcitonin in man is far from clear. Since it acts by inhibiting bone resorption it would be expected that its major effect on plasma calcium would be at

those times at which bone resorption is contributing significantly to the maintenance of plasma calcium i.e. when rapid growth is taking place. Thus, in normal adult subjects injection of calcitonin has no effect on plasma calcium (Foster et al. 1966; and Martin and Melick, 1969), whereas it invariably lowers plasma calcium in children (McCredie et al. 1971), and in adults with increased bone resorption from thyrotoxicosis or Paget's disease of bone (Bijvoet, Van der Sluys Veer and Jansen, 1968).

Calcitonin levels are elevated during pregnancy and lactation (Stevenson et al. 1979; and Samaan et al. 1973) and it is suggested that a physiological role of calcitonin may be the protection of the maternal skeleton.

More recently, Talmage and co-workers (1980), have proposed an important physiological role for calcitonin which is summarized as follows: calcitonin, secreted in response to food intake, stimulates rapid storage of a portion of the absorbed calcium in bone fluid. This calcium is stored in a labile form in combination with phosphate in the bone fluid. On cessation of calcium absorption from the gut, with the accompanying decrease in circulating levels, the labile form of stored calcium is gradually released into the extracellular fluid. This process decreases postprandial calcium loss in the urine, and supplies calcium to maintain the plasma concentration during fasting periods. The net result is conservation of calcium postprandially and a decrease in parathyroid hormone-induced bone destruction during subsequent periods of fasting. Supporting evidence for

this proposal was presented based on experiments in rats (Talmage et al. 1980). However, in man the evidence for this proposal is not sufficient, i.e. a postprandial increase of calcitonin secretion has not been established (Deftos, 1978).

8.2 Disorders of calcitonin secretion

8.2.1 Medullary thyroid carcinoma

A) History

Medullary thyroid carcinoma is a tumour of the calcitonin-producing C cells of the thyroid gland. Hazard, Hawk and Crile (1959) recognised it as a distinct pathological entity that could be distinguished from other thyroid tumours by its histological features which include uniformity of cell type, a moderate number of mitoses as compared to the undifferentiated or anaplastic carcinoma, a uniform arrangement of cell sheets separated by stroma, and, especially, the presence of amyloid in the tumour. The clinical evidence of a relatively long patient survival, despite the undifferentiated appearance of the neoplasm, established it as a clinicopathological entity. In 1966, Williams suggested that the C cells might be the cells of origin of medullary thyroid carcinoma and his hypothesis was proved to be correct in 1968 when several investigators demonstrated by bioassay the presence of abnormal calcitonin concentrations in the plasma and tissues of patients with MCT (Cunliffe et al. 1968; Meyer and Abel-Bari, 1968; Melvin and Tashjian, 1968; and Milhaud

et al. 1968). In fact, the high concentrations of this hormone in medullary thyroid carcinoma permitted the isolation, purification and sequencing of human calcitonin (Neher et al. 1968b,c). These findings were confirmed by specific radioimmunoassay of human calcitonin in tumour and blood by Clark et al. (1969) and by histochemical and immunohistochemical studies (Kalina et al. 1970).

B) Incidence

Medullary thyroid carcinoma is not a common thyroid tumour, comprising about 7 - 10% of thyroid carcinomas; a range of 3.5 to 11.9% was recorded in the extensive review by Ljungberg (1972). The occurrence of medullary carcinoma as the most frequent type with a familial association was established by Schimke and Hartmann (1965). It is inherited as an autosomal dominant, and Williams (1965) reported the association of medullary thyroid carcinoma with phaeochromocytomas. Prior to this Sipple (1961) had described the association of phaeochromocytomas with thyroid tumours. The association of medullary thyroid carcinoma, parathyroid adenopathy and phaeochromocytoma and the familial relationship was reported by Steiner, Goodman and Powers (1968) who designated it as multiple endocrine neoplasia type II, in contrast to the type with anterior pituitary, parathyroid and pancreatic islet cell adenopathy which had been recognised by Wermer in 1963 and Ballard, Frame and Hartsock in 1964 and is now identified as type I. And another type of familial multiple endocrine adenopathy associated with medullary thyroid carcinoma was also recognised at that time by

Schimke et al (1968) and Gorling et al (1968) in which there were phaeochromocytomas and multiple mucosal neuromas, at times associated with a marfanoid habitus.

C) Diagnosis

The value of the calcitonin radioimmunoassay in the diagnosis of medullary thyroid carcinoma is well established (Clark et al. 1969; Tashjian et al. 1970; and Deftos, 1971). Most patients with this tumour have diagnostically elevated levels of calcitonin in basal plasma samples (Melvin et al. 1972; Samaan et al. 1973). However, during the early stages of tumour growth the basal levels of calcitonin may be indistinguishable from normal levels and provocative tests of calcitonin secretion are necessary to establish the diagnosis, especially among relatives of an index case when the familial form is suspected.

D) Provocative tests of calcitonin secretion

i) Calcium

The most widely used secretagogue for calcitonin in medullary thyroid carcinoma, has been calcium given by intravenous infusion. In early studies calcium infusion at the rate of 3-5 mg/Kg body weight/h for 2 - 4 hours consistently produced elevation of calcitonin levels during or at the end of the infusion period (Deftos, 1974; and Tashjian et al. 1970). The disadvantage of this method is that the large amount of calcium infused often raises the

blood calcium by several milligrams within a short period of time and the calcium remains elevated for several hours; this can produce nausea and vomiting. Because of the length of the infusion this may necessitate admission to hospital for the procedure, so it is not convenient or feasible to screen large numbers of patients. For this reason Parthemore et al. (1974) utilized a shorter calcium infusion in which 150 mg calcium was infused over 5-10 minutes and a significant elevation of calcitonin was observed. However, the degree of stimulation was not as marked as with the standard calcium infusions.

ii) Pentagastrin

Another widely used provocative agent for calcitonin secretion in patients with medullary thyroid carcinoma is pentagastrin (Hennessy et al. 1973; 1974). When administered i.v. at a dose of 0.5 µg/Kg pentagastrin produces a rapid, dramatic increase in plasma calcitonin some 2 minutes after injection. Because the test has the attraction of being quick and easy to perform, and more importantly provoked the secretion of calcitonin in 3 patients in whom calcium stimulation was not effective, pentagastrin stimulation has proved to be a more reliable test than calcium infusion. However, the majority of patients noticed epigastric distress and malaise 30 seconds after the injection which usually persisted for 1-2 minutes.

iii) Glucagon, Gastrin

Although glucagon can stimulate calcitonin secretion (Deftos et al. 1971), it is not a reliable stimulus (Deftos, 1974) because its effect can be variable. Glucagon can also stimulate catecholamine release, which could be potentially dangerous in patients with medullary thyroid carcinoma, because of the high incidence of associated phaeochromocytomas. As in the case of glucagon, Deftos et al. (1973) noted that gastrin is not a reliable secretagogue for calcitonin in medullary thyroid carcinoma patients.

iv) "Alcohol"

Whisky was used as a provocative test for calcitonin stimulation following the observation of Cohen et al. (1973) that the social ingestion of alcohol consistently precipitated flushing and diarrhoea in a patient with medullary thyroid carcinoma. Dymling et al. (1975) found that oral administration of 50 ml of whisky was effective in stimulating calcitonin release when screening families of patients with medullary thyroid carcinoma. The rise in calcitonin usually occurs within 15 minutes and the test has the advantage that whisky is given orally.

E) Immunochemical heterogeneity

Plasma calcitonin exists in multiple immunochemical forms (Singer and Habener, 1974; Sizemore et al. 1975; and Deftos et al. 1975). When the plasma of a patient with MCT is immunoassayed after gel filtration chromatography, multiple peaks of immunoreactive calcitonin can be seen. Although the precise nature of these peaks is not fully investigated, some may reflect the biosynthesis, secretion and metabolism of calcitonin.

8.2.2 Ectopic secretion of calcitonin

There are several documented cases of ectopic calcitonin production. In one normocalcaemic patient with an oat cell lung carcinoma (Silva et al. 1973), serum calcitonin levels fell following chemotherapy and an arterio-venous calcitonin gradient was shown across the tumour bed. In a second patient, a high calcitonin concentration was detected in the tumour tissue (200 ng/g wet weight) as compared with a level of 1 ng/g in the thyroid gland (Silva et al. 1974a). Moreover, an oat cell bronchogenic carcinoma, taken from a patient with hypercalcaemia and maintained in proliferative cell culture, has been shown to secrete immunoreactive calcitonin indistinguishable from CT-M (Coombes et al. 1976). Similarly, one monolayer culture of breast carcinoma cells released immunoreactive calcitonin for 10 weeks. The production of calcitonin by oat cell bronchogenic carcinomas may be explained by the APUD theory (Pearse, 1968) as these cells are derived from neural crest origin. However, the

most likely explanation for the production of calcitonin by breast carcinoma tissue, which is not embryologically derived from neural crest tissue, is the gene derepression theory (Gelhorn, 1963). Furthermore, Coombes et al. (1976) have reported that a poorly differentiated epidermoid bronchial carcinoma, in culture, produced calcitonin which was different from CT-M both immunologically and chromatographically.

Elevated plasma calcitonin levels in patients with non-thyroid tumours were reported by several groups. Coombes et al. (1974) reported a series of 46 patients with non-thyroid carcinomas. They found elevated levels in 21, of whom 8 had oat cell lung carcinomas and 8 breast carcinomas. The plasma calcitonin in these patients was immunologically indistinguishable from CT-M. Milhaud et al. (1974) reported raised levels of plasma calcitonin in 100% of patients with oat cell carcinomas and in 50% with carcinoid tumours.

Hypercalcitoninaemia has been reported in patients with hypercalcaemia (Tashjian et al. 1970), renal failure (Silva et al. 1974b), acute pancreatitis (Canale and Donabedian, 1975) and Zollinger-Ellison syndrome (Sizemore et al. 1973).

8.2.3 Therapeutic uses

Calcitonin has proved to be very effective in the treatment of Paget's disease of bone (Bijvoet, Van der Sluys Veer and Jansen, 1968; Haddad, Birge and Avioli, 1970; Bell, Avery and Johnston, 1970; Woodhouse et al. 1971; Singer et al. 1972; Rojanasathit, Rosenberg and Haddad, 1974; Nagant de Deuxchaisnes et al. 1977; Evans et al, 1977) and some hypercalcaemic states. The therapeutic applications of calcitonin utilize its major biological actions: to inhibit bone resorption

and to lower blood calcium. Four forms of calcitonin have been used: extracted porcine, synthetic salmon, synthetic human and synthetic eel.

Paget's disease of bone is a non-metabolic bone disease, whose aetiology remains unknown, although the possibility of a viral aetiology was raised (Rebel et al. 1976; Singer, Melvin and Mills, 1976). It is characterized by excessive rates of bone resorption and formation and an increase in the number of osteoclasts and osteoblasts with eventual formation of structurally weakened bone tissue. The disease is often familial and usually asymptomatic but severe bone pain may be the presenting feature. Biochemically, serum alkaline phosphatase levels are elevated and urinary hydroxyproline is increased.

Calcitonin relieves bone pain and reduces skin temperature over affected bones. The elevated levels of serum alkaline phosphatase and urinary hydroxyproline fall. Radiological and histological regression has been documented (Haddad et al. 1970; Woodhouse et al. 1971; Evans et al. 1977).

Antibodies to porcine and salmon calcitonins are encountered in over 50% of patients (Dubé et al. 1973; Haddad and Caldwell, 1972; and Singer et al. 1972). In about 10% of cases the antibodies are of sufficiently high titre to induce clinical and biochemical relapse. However, even long term treatment with synthetic human calcitonin does not promote antibody formation (Evans et al. 1977; Greenberg et al. 1974; Burckhardt et al. 1977).

An advantage of calcitonin over mithramycin and the diphosphonate (EHDP) for the treatment of Paget's disease is the relative freedom from side effects.

CHAPTER 2

IMMUNOCHEMICAL METHODS AND THE DEVELOPMENT OF REGION-SPECIFIC RADIOIMMUNOASSAYS FOR HUMAN CALCITONIN

1. Immunological Methods

1.1 Radioimmunoassay technique

1.1.1 Introduction

The development of radioimmunoassay (RIA) by Berson and Yalow during the late 1950's represents a major advance. Over the past two decades this technique has made an important impact on endocrinology and other areas of medical investigation. The method offers a technique for assaying materials otherwise unmeasurable or detectable only with difficulty.

1.1.2 Basic principles

The method is based upon the competition between unlabelled antigen and labelled antigen for a limited number of specific binding sites, thereby diminishing the binding of the labelled antigen (Berson and Yalow, 1957; and Yalow and Berson, 1959, 1960). For the determination of the concentration of antigen in unknown samples, the degree of competitive inhibition observed in the unknown is compared with that obtained in a known standard solution.

1.1.3 Requirement for RIA

The development of a radioimmunoassay for any antigen depends on the availability of the appropriate components of the radioimmunoassay system. These are:

A) Specific antisera to the antigen.

B) Pure radiolabelled antigen.

C) Standard preparation of antigen.

D) A method for the separation of bound and free antigen.

A) Antisera

To be useful in an assay system, antisera must contain antibodies of appropriate specificity, with an affinity constant high enough to be sensitive in the range of interest and with a titre sufficient for a reasonable number of assays (Odell et al. 1971). Titre may be defined as the optimal concentration of the antibody in the assay tube, or more commonly, the final dilution used in the assay system. The titre is determined by incubating serial dilutions of the antiserum with a fixed amount of labelled antigen. Usually the dilution chosen for radioimmunoassay is that which binds 40-50% of the labelled antigen in the absence of unlabelled antigen.

Sensitivity: Having selected the proper dilution of an antiserum, the ability to detect small changes in the substrate concentration can be evaluated by incubating fixed amounts of antiserum (at appropriate dilutions) with fixed amounts of labelled antigen and different known concentrations of a cross-reacting substrate (the antigen or an immunologically similar compound). The slope of this dose response (standard) curve reflects the sensitivity.

Specificity: Specificity depends on the inherent ability of the antiserum to detect subtle differences in molecular structure, e.g. the alteration of a single amino acid sequence in porcine and human insulin (Berson and Yalow, 1959). Substances showing identical dose response curves are immunologically similar, but this does not necessarily indicate that they are identical.

B) Radiolabelled antigen

The most widely used radionuclides for labelling proteins and peptides to high specific activity are ^{125}I and ^{131}I. They are commercially available as sodium iodide. ^{125}I is usually preferred because of its longer half life (60 days compared with 8 days for ^{131}I), greater isotopic abundance, and higher counting efficiency in a well-type crystal.

i) Iodination methods

The method of Hunter and Greenwood (1962) is commonly used. It is based on the use of chloramine T as an oxidizing agent, oxidizing the sodium iodide ($Na^{125}I$), with the subsequent incorporation of ^{125}I into the aromatic ring within the tyrosyl moiety. Sodium metabisulphite is used to reduce excess chloramine T and limit chemical change. The ratio of iodide to peptide is dependent on the desired specific activity of the product and the number of the tyrosyl groups in the molecule being labelled.

The enzymatic iodination using peroxidase (Marchalonies, 1969; Morrison, Bayse and Webster, 1971) has the advantage of gentle treatment of the antigen as shown by retention of biological activity with little chemical "damage".

Other methods include the use of chlorine or sodium hypochlorite as an oxidant (Redshaw and Lynch, 1974) and more recently the use of iodogen (1, 3, 4, 6 tetrachloro-3α, 6α phenylglycoluril) has been described by Salminki et al. (1979) as a simple alternative to other methods.

For peptides and proteins lacking a phenolic group (e.g. secretin) (Mutt et al. 1970) and those highly susceptible to damage during chloramine T oxidation (e.g. parathyroid hormone), Bolton and Hunter (1973) have described a method of labelling by conjugation of the peptide to a ^{125}I-labelled acylating agent.

ii) Purification of labelled antigen

Following most of the iodination methods, purification of the radiolabelled antigen is necessary. The methods of purification commonly used are basically the same as those for separation of bound and free tracer which are described below in the separation methods.

C) Standard preparation of antigen

For proper validation of radioimmunoassay procedures, the hormone in the standard must be immunologically identical to the hormone in the unknown sample (Berson and Yalow, 1966). It is not necessary that the standard and unknown be chemically identical or that they have identical biological potency. When standard reference preparations are not available, crude glandular extracts or plasma containing a high concentration of the hormone, permit evaluation of the relative concentration of hormone in unknown samples.

D) Separation method of bound from free

Methods for separating bound and free tracer are also used for purification of radiolabelled antigen. These are:

i) Differential migration of bound and free antigen.

ii) Precipitation methods.

iii) Adsorption methods.

iv) Solid phase antibody systems.

i) Differential migration

a) Chromatoelectrophoresis

This method was first used by Yalow and Berson (1960) for the first reported RIA. It depends on

selecting suitable paper (such as Whatman 3 MM) that adsorbs undamaged free hormone at its site of application, while the damaged hormone and free iodide move from the origin under the influence of an electric current and buffer flow caused by evaporation. However, the method is expensive, time consuming and cannot be automated. Thus it is now rarely employed for separation except as a reference technique and in the assessment of labelled antigen.

b) Gel filtration

Because antigen-antibody complexes (Ag^{*} Ab) are much larger than free antigen, it is possible to separate them adequately by the use of gel filtration on cross-linked dextrans. Genuth, Frohman and Lebovitz (1965) used micro columns of sephadex G-75 for separation of free and bound insulin in their radioimmunoassay, but this method is time consuming and has a low sample capacity. However, gel filtration is the most widely used method for the purification of iodinated antigens after radiolabelling.

ii) Precipitation methods

a) Non specific precipitation of the antigen-antibody complex

A number of workers have proposed radioimmunoassays based on the non specific precipitation

of Ag^* Ab under conditions which leave Ag^* in solution. These include ammonium sulphate (Goodfriend,1968), sodium sulphate (Grodsky and Forsham, 1960), ethanol (Heding, 1966) and polyethylene glycol (Desbuquois and Aurbach, 1971).

b) Immunoprecipitation of the antigen-antibody complex

The method is based on the precipitation of the soluble antigen-antibody complexes formed in the first antibody reaction by the addition of a second antibody directed against the first antibody to yield insoluble immune complexes.

The method was first applied to RIA by Utiger, Parker and Daughaday (1962) for GH and by Morgan and Lazarow (1963) and Hales and Randle (1963) for insulin. This method is currently one of the most widely used separation techniques.

iii) Adsorption methods

Adsorption methods, usually applied to the free fraction, are widely employed in RIA because of their simplicity, speed, economy and large sample capacity. It is essential that the conditions for use of these methods are optimized experimentally for each antigen. These conditions include: relative surface area of adsorbant,

nature and concentration of competing proteins, temperature, ionic strength and pH.

a) Charcoal

Herbert et al. (1965) introduced charcoal for RIA of insulin and recommended coating the charcoal with dextran, of an appropriate molecular size, to provide pores for penetration of free peptide and to exclude larger bound complexes. However, in the presence of an adequate concentration of serum (10-30%) dextran is unnecessary (Ekins, 1969; Palmieri, Yalow and Berson, 1971; Binoux and Odell, 1973).

b) Silicate

Peptide hormones adsorb readily to glass surfaces and silicates can be used to bind free hormone. Talc and Quso G-32 have been used particularly in the RIA of ACTH, PTH, insulin and calcitonin (Rosselin et al. 1966; Palmieri, Yalow and Berson, 1971).

iv) Solid phase antibodies

Immunoadsorbents (i.e. antigens or binding reagents coupled to an insoluble polymer) have been used for phase separation in two forms: one, the antibodies may be adsorbed to polystyrene or polypropylene discs or

tubes (Catt and Tregear, 1967); or two, the antibodies may be coupled to a cyanogen bromide activated dextran or cellulose particles (Wide and Porath, 1966; Axen, Porath and Ernback, 1967; Wide, 1969).

1.2 Radioimmunoassay for human calcitonin

1.2.1 Method of RIA

A) Source of peptide

Synthetic CT-M (monomer), synthesized by Ciba-Geigy, Basle, Switzerland, is used for labelling with radioiodine. As standard in the radioimmunoassay, MRC human synthetic calcitonin, tumour sequence (ref. 70/50) (National Institute for Biological Standards and Control, London) is used.*

B) Antiserum

Antibodies to human calcitonin have been prepared in rabbits, guinea pigs, goats and mice. Both material extracted from medullary thyroid carcinoma tissue (Clark et al. 1969; Tashjian et al. 1970; Deftos, 1971) and the synthetic CT-M (Dietrich and Rittel, 1970; Deftos, 1971; Dietrich, 1975; Hillyard, 1979) have been used as immunogen.

The antiserum used in this study for monitoring the separation of peptides by chromatographic methods was antiserum 827/4 and this was used in combination with other antisera

---

* The full addresses of all manufacturers of chemicals and equipment are listed in Appendix 1.

described later in this chapter for the immunological studies. This antiserum (827/4) was produced in a rabbit immunized by subcutaneous injection of synthetic human calcitonin by Drs. Court and Hurn (Wellcome Reagents). The antiserum was obtained six months after the initial immunization and was selected from five other antisera because of its higher titre and avidity (Figs. 2 and 3).

C) Preparation and purification of labelled calcitonin

Synthetic human CT is radioiodinated by minor modifications of the chloramine T method of Hunter and Greenwood (1962). One millicurie of ^{125}I (Radiochemical Centre, Amersham) is used to iodinate 2 µg of CT-M in 0.5 M phosphate buffer, pH 7.4 (25 µl). 40 µg chloramine T in 0.05 M phosphate buffer pH 7.4, (25 µl) is added. The mixture is agitated for 10-15 seconds, and 80 µg of sodium metabisulphite ($Na_2 S_2 O_5$) in 25 µl of 0.05 M phosphate buffer, pH 7.4, is added and mixed. The reactants are then run through an Amberlite CG400 anion-exchange column (25 x 1 cm), in 0.05 M acetate buffer pH 4.8. Eight fractions are collected, fractions 1 and 8 containing 2 ml and fractions 2-7, 1 ml. Each fraction is checked by counting 50 µl and the fraction with the highest counts (usually fraction 4) is diluted with 0.05 M phosphate buffer pH 7.4 (containing 1% calcitonin-free human plasma) to obtain 6,000-10,000 counts per minute (c.p.m.)/50 µl and tested with excess antibody as well as with an overnight standard curve.

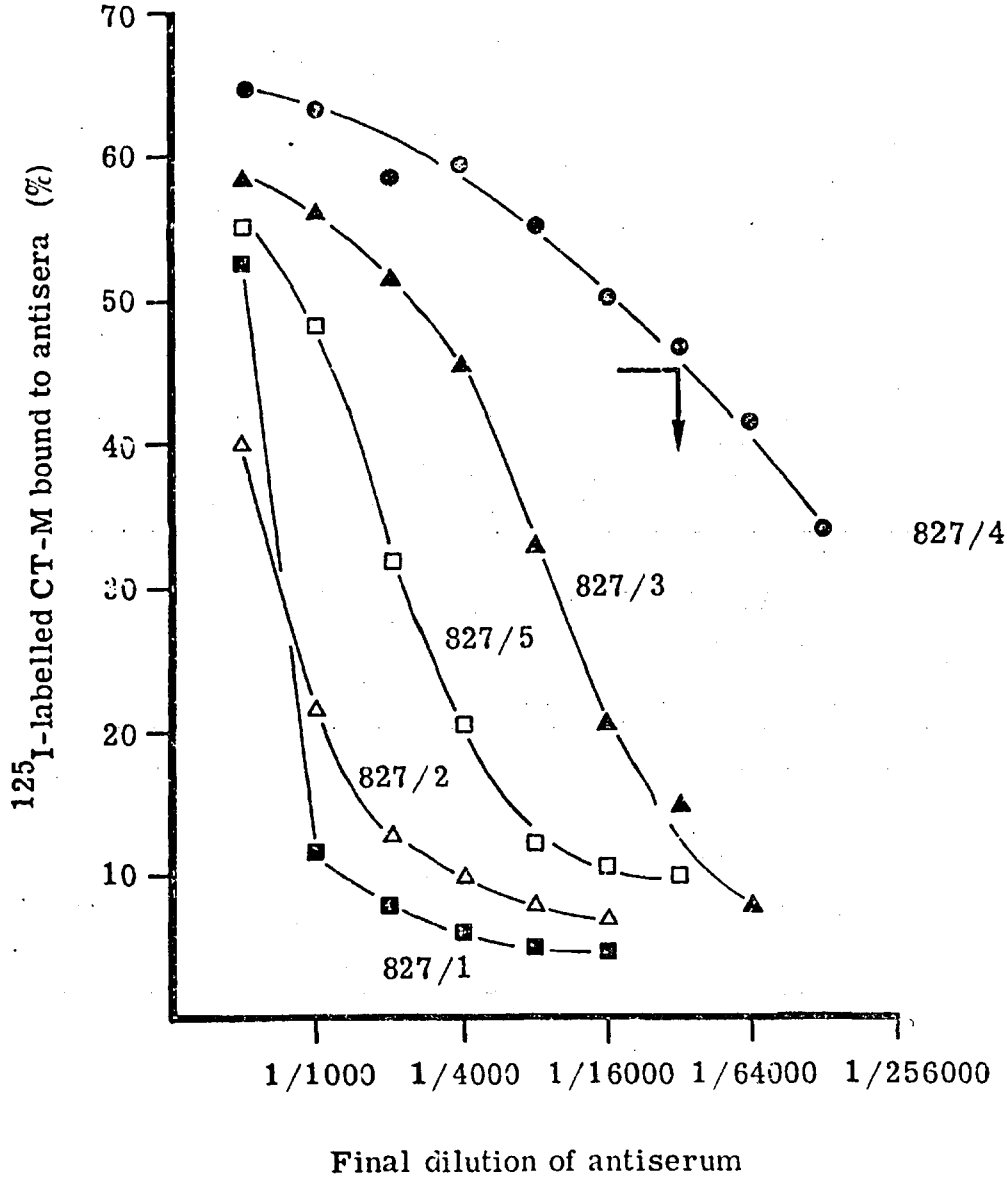


Fig. 2 Overnight antibody dilution curves for five antisera to synthetic human calcitonin. Antiserum 827/4 has a higher titre than antisera 827/1, 827/2, 827/3 and 827/5. The arrow indicates the final dilution of antiserum 827/4 that binds 45% of the ^{125}I-labelled CT-M and this is appropriate for use in a standard curve.

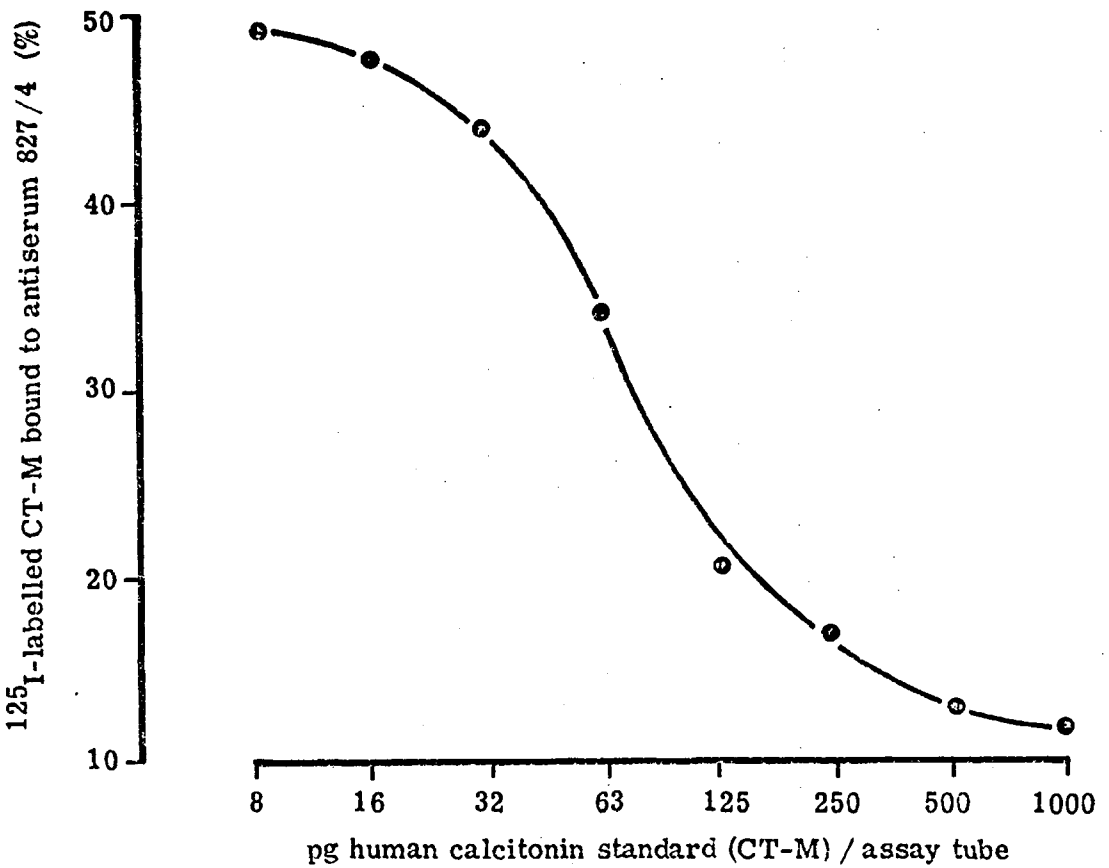


Fig. 3 An overnight standard curve for human calcitonin using antiserum 827/4. The curve is obtained by incubating serial dilutions of unlabelled standard CT-M with a fixed amount of ^{125}I-labelled CT-M and a fixed quantity of antiserum 827/4.

D) Incubation and assay procedure

All stages of the assay are carried out at approximately $4^{o}C$.

The assay buffer is 0.05 M phosphate buffer, pH 7.4 containing 0.02% sodium azide, 5% human plasma (spherosil treated), and 500 KI units of Kallikrein - trypsin inhibitor (Trasylol) (FBA Pharmaceuticals, Inc.) per millilitre. The incubation mixtures are prepared in disposable plastic tubes (Luckham LP3) in a total volume of 300 µl to contain the same concentration of ^{125}I-CT (6,000-10,000 c.p.m./50 µl) and antiserum (50 µl) but variable concentrations of standard CT or unknown sample. Total volume is made up to 300 µl by assay buffer.

i) Standard curve

Standard curves are set up in duplicates. Serial dilution of standard calcitonin is made (1,000-2 pg/tube) in 100 µl assay buffer using an automatic diluter (LKB), (ten tubes). In each assay four tubes are included containing only assay buffer (250 µl) and 50 µl labelled CT to indicate "non specific binding", as well as four tubes containing only assay buffer (200 µl), labelled CT (50 µl) and antiserum (50 µl) but no cold calcitonin for the "total binding" (zero point of standard curve). Four tubes containing 50 µl labelled CT only are set up to provide "total counts".

ii) Test samples

These are generally column fractions of tissue or plasma extract, which have been lyophilized prior to radioimmunoassay. The lyophilized material is dissolved in assay buffer (400-2,000 µl) and samples are assayed in duplicate at 2-6 dilutions (up to 100 µl in the first tube). Controls without antibody are included for each sample, when the final dilution is 1 : 20 or more. Buffer, label and antiserum are added as for standard curves.

For overnight assays, an equilibrium system is employed, the antiserum (827/4) is used at a final dilution of 1 : 48,000 which gives 40-50% binding of the labelled peptide at the end of the incubation period. The detection limit of this assay is 4 pg/tube. Sensitivity is improved in a non equilibrium assay system in which the unlabelled standard and unknown are preincubated at $4^{o}C$ with antiserum for 3-4 days and then for an additional 2-3 days with labelled calcitonin. For the non equilibrium assay the antiserum is used at a final dilution of 1 : 60,000 and the detection limit of this assay is 2 pg/tube.

E) Separation of bound from free hormone

At the end of the incubation period, free labelled calcitonin is separated from antibody-bound material by adding

200 µl of dextran (0.1%) coated charcoal (1%) to each tube (because the concentration of serum in test samples is generally less than 10-20%), mixed thoroughly on a vortex mixer and then centrifuged within 30 minutes at 2,000-3,000 r.p.m. for 5 minutes.

The supernatant solution containing the antibody-bound ^{125}I-calcitonin is aspirated by suction through a needle connected to a water suction pump, and the free labelled calcitonin is counted in an automatic gamma counter (Packard).

F) Reagents

- 0.05 M phosphate buffer pH 7.4 (for assay).

14.2 g Na_2 H PO_4 and 3.9 g Na H_2 PO_4 are dissolved in 2.5 litres of distilled water. Sodium azide is added in a concentration of 0.02%.

- 0.05 M phosphate buffer pH 7.4 (for labelling).

This is prepared by dissolving 7.5 g Na_2 H PO_4 and 1.95 Na H_2 PO_4 in 125 ml distilled water. One ml aliquots are prepared and stored frozen at -20°C.

- Amberlite for purification of label.

Amberlite CG400 (BDH) is treated first with 3 M KOH. The supernatant is decanted and the resin is washed through a Buchner funnel with the following solutions in succession: 3 M KOH (2 volumes), distilled water (5 volumes), glacial acetic

acid (1 volume) and 0.05 M acetate buffer pH 4.8. Amberlite is stored in acetate buffer at 4°C and is made fresh every six months.

- Dextran coated charcoal.

1 g Norit Sx charcoal is shaken with approximately 60 ml distilled water, allowed to settle for 30 minutes and the supernatant is aspirated off. 100 mg Dextran T70 (Pharmacia) is added and shaken vigorously to coat the charcoal. This is made up to 100 ml with 0.05 M phosphate buffer.

The charcoal suspension is freshly prepared every week.

1.2.2 Interpretation of RIA data

A) Sensitivity

The working sensitivity of the calcitonin radioimmunoassay is calculated by finding the smallest concentration of standard that produces a significantly ($p < 0.05$) lower than tracer binding at zero point of the standard curve. This is obtained either from the concentration which produces inhibition of at least two standard deviations of the percentage labelled calcitonin bound from the zero point or more commonly, the concentration that produces 10% deviation from the zero point. This latter procedure gives a working sensitivity for the overnight equilibrium assay of 4-8 pg/tube or 40-80 pg/ml and

for the non equilibrium longer incubation assay of 2-4 pg/tube of 20-40 pg/ml.

B) Specificity

This assay has a high specificity for the human peptide; at concentrations up to 10^4 times those of human calcitonin, porcine (supplied by Dr. W. Rittel) and salmon calcitonins (supplied by Armour Pharmaceuticals) show essentially no cross-reaction in this assay. On the other hand it cross-reacts with rat calcitonin because of the similarity in sequence between rat and human calcitonins.

C) Reproducibility

The interassay variation is < 20% and the intraassay variation is < 10%.

1.3 Development of region-specific radioimmunoassay for human calcitonin

Immunoassays specific for limited regions of a given peptide may be developed in several ways (Barling et al. 1975). In this study, it was planned to develop region-specific immunoassays for human calcitonin by the use of antibodies produced by immunization with several fragments of CT-M.

1.3.1 Production of antisera

A) Immunogen

i) Human calcitonin fragment

Four fragments of human calcitonin 1-10, 11-23, 17-23 and 11-32 (kindly supplied by Dr. W. Rittel, Ciba-Geigy, Basle) were used. The last three were covalently coupled to a carrier protein (ovalbumin) to make them more immunogenic.

ii) Coupling method

The coupling method using glutaraldehyde is as described by Sachs and Winn (1970) and is summarized as follows:

An aqueous solution of the peptide fragments (4 mg in 0.4 ml saline) was added to an aqueous solution of ovalbumin (24 mg in 1.6 ml of 0.1 M phosphate buffer pH 7.1) and ∿ 60,000 c.p.m. of ^{125}I-peptide.

An aqueous glutaraldehyde solution was added (200 µl of 2%), and the mixture stirred for 60 minutes at 4°C. The reaction was quenched by the addition of saturated $NaHSO_3$ solution (200 µl). The conjugate was applied to a Biogel P-30 column (100 x 2.5 cm) and run in 0.2 M ammonium sulphate to purify the fragment-carrier conjugate. The amount of radioactivity in the eluate served as a guide to the degree of incorporation of haptens into the protein (Figs. 4 and 5).

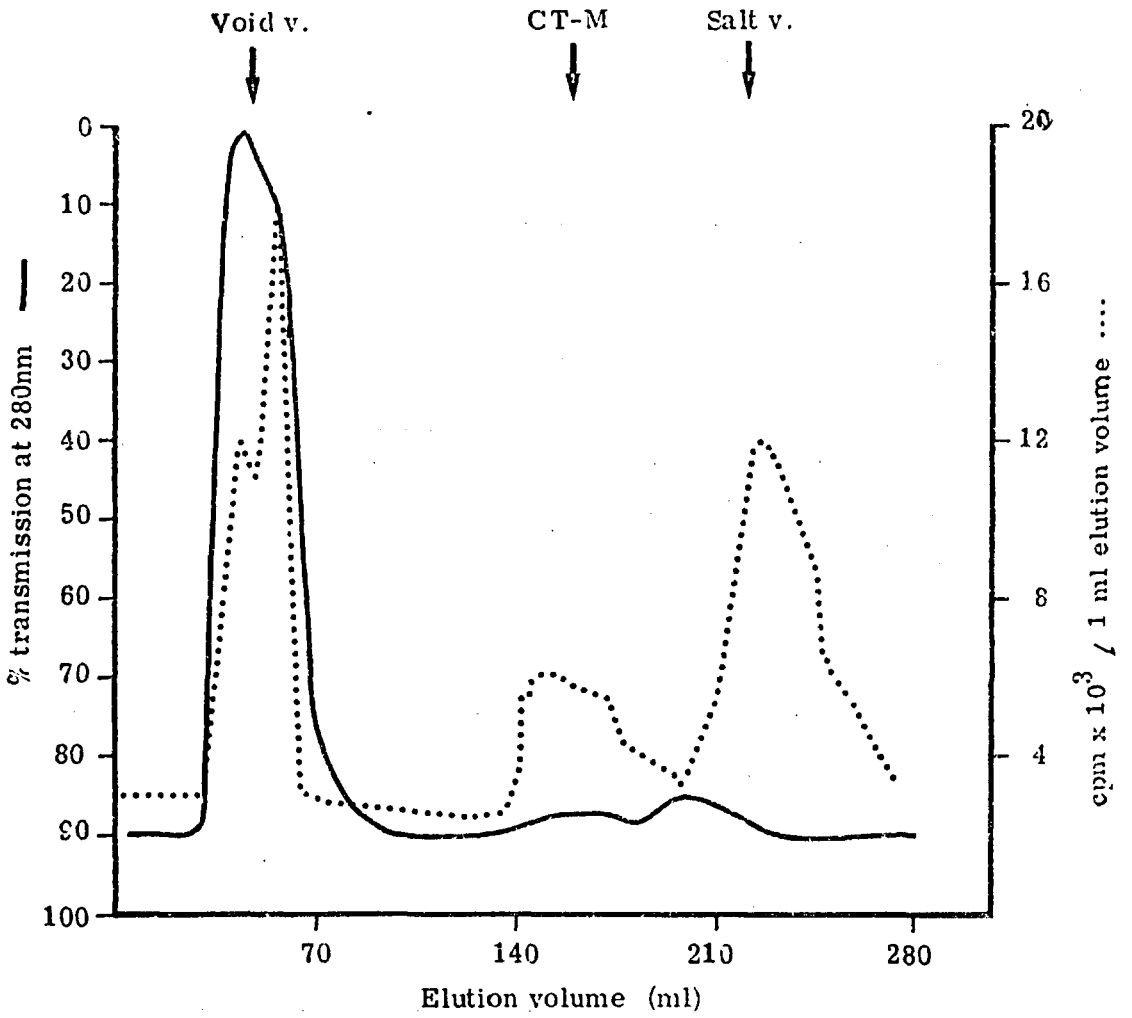


Fig. 4 *Gel filtration chromatography of* (17-32)-CT-M-ovalbumin conjugate reaction mixture. The conjugate was applied to a Biogel P-30 column (100 x 2.5 cm) and run in 0.2 M ammonium sulphate pH 5.6. Flow rate was 14.2 ml/h and fractions were collected every 30 minutes. Radioactivity was measured by counting 50 µl of each fraction for 1 minute in a gamma counter. The degree of coupling was calculated from the ratio of tracer present in the fractions co-eluting with the void volume to the total tracer count less the count present in the salt volume (indicating free iodine). Fractions co-eluting with the void volume were pooled for dialysis.

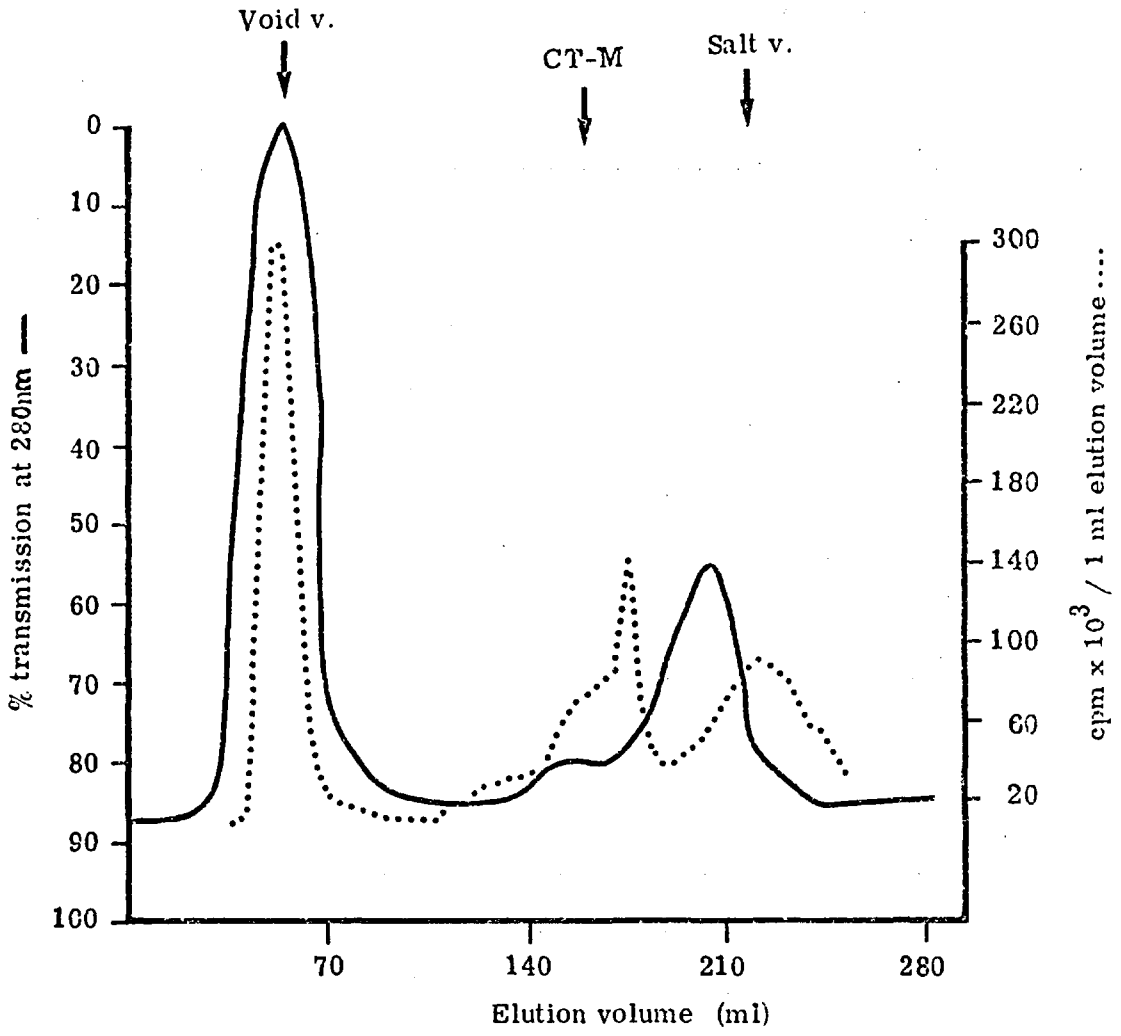


Fig. 5 Gel filtration chromatography of the (11-32)-CT-M-ovalbumin conjugate reaction mixture. The conjugate was applied to a Biogel P-30 (100 x 2.5 cm) column and run in 0.2 M ammonium sulphate. Flow rate and fraction volumes were the same as on Fig. 4.

The coupled peptides were further purified by dialysis for 24 h at $4^{o}C$ in a D-12 cellophane membrane against normal saline, then lyophilized prior to use for immunization.

On the average 60-65% of each peptide was conjugated to ovalbumin.

B) Immunization

From each conjugate an amount equivalent to 150 µg peptide was emulsified with Freunds complete adjuvant (Difco) and injected into a New Zealand white rabbit (4 rabbits for each conjugate) using the multiple intradermal technique of Vaitukaitis et al. (1971). Booster injections were given every 6 weeks for 12-18 weeks using approximately 5% of the immunizing dose. In the case of fragment 1-10 which is difficult to solubilize in aqueous solution (hence difficult to couple) the dry peptide was emulsified directly into complete Freunds adjuvant. Animals were bled from an ear vein every three weeks, usually 10 days after boosting for assessment of antisera.

1.3.2 Evaluation of antisera

A) Antibody dilution curve

Serial dilutions of the serum from each bleed were made in 0.05 M phosphate buffer and were incubated with a fixed amount of ^{125}I CT-M (10,000 c.p.m.) overnight. The final antiserum

dilution that binds 40-50% of the ^{125}I CT-M was determined (Fig. 6). Those antisera which showed a titre of 1/2,000 or more were used for setting up a standard curve. The slope of the standard curve reflects the avidity of the antisera. The effect of incubation time on binding of ^{125}I-HCT to antiserum was also tested (Fig. 7).

B) Results of immunization

No antibodies were obtained from rabbits immunized with fragment (1-10) nor with fragment (11-23) coupled to ovalbumin. The inability to produce antisera to fragment (1-10) CT may be explained by lack of "foreign-ness" as most of the amino acid residues of this fragment are conserved in all known species of calcitonin, and antigenic sites are most often found in non-conserved regions of peptides and proteins (Schwyser, 1968).

All rabbits immunized with the ovalbumin coupled fragments (17-32) and (11-32) produced antisera after 6-14 weeks of initial immunization. From these antisera four were selected because of their high titre and affinity: antisera 5/1, 7/2, 10/2 and 12/3. Details of the production and titre of these four antisera and of antisera 827/4 and 336/6 (Hillyard, 1979) are summarized in Table 2.

C) Antigenic specificity of antisera

The antigenic specificities of the six antisera listed in Table 2 were assessed by comparing the inhibition of specific

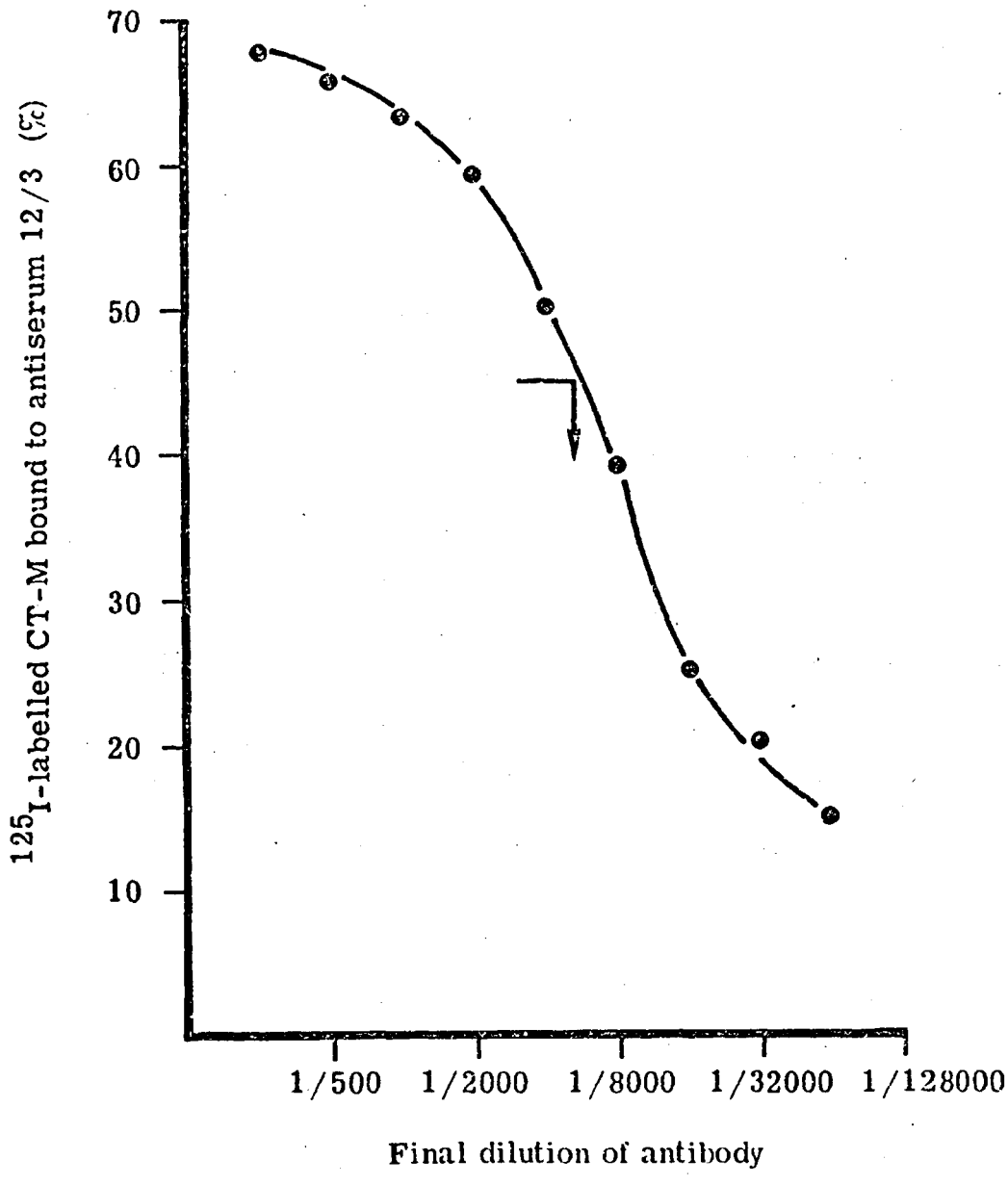


Fig. 6 Overnight antibody dilution curve for antiserum 12/3 (rabbit no. 12, immunized with (11-32)-CT-M coupled to ovalbumin) obtained by incubating serial dilutions of the antiserum with a fixed amount of ^{125}I-labelled CT-M. The arrow indicates the final dilution of the antiserum for use in standard curves.

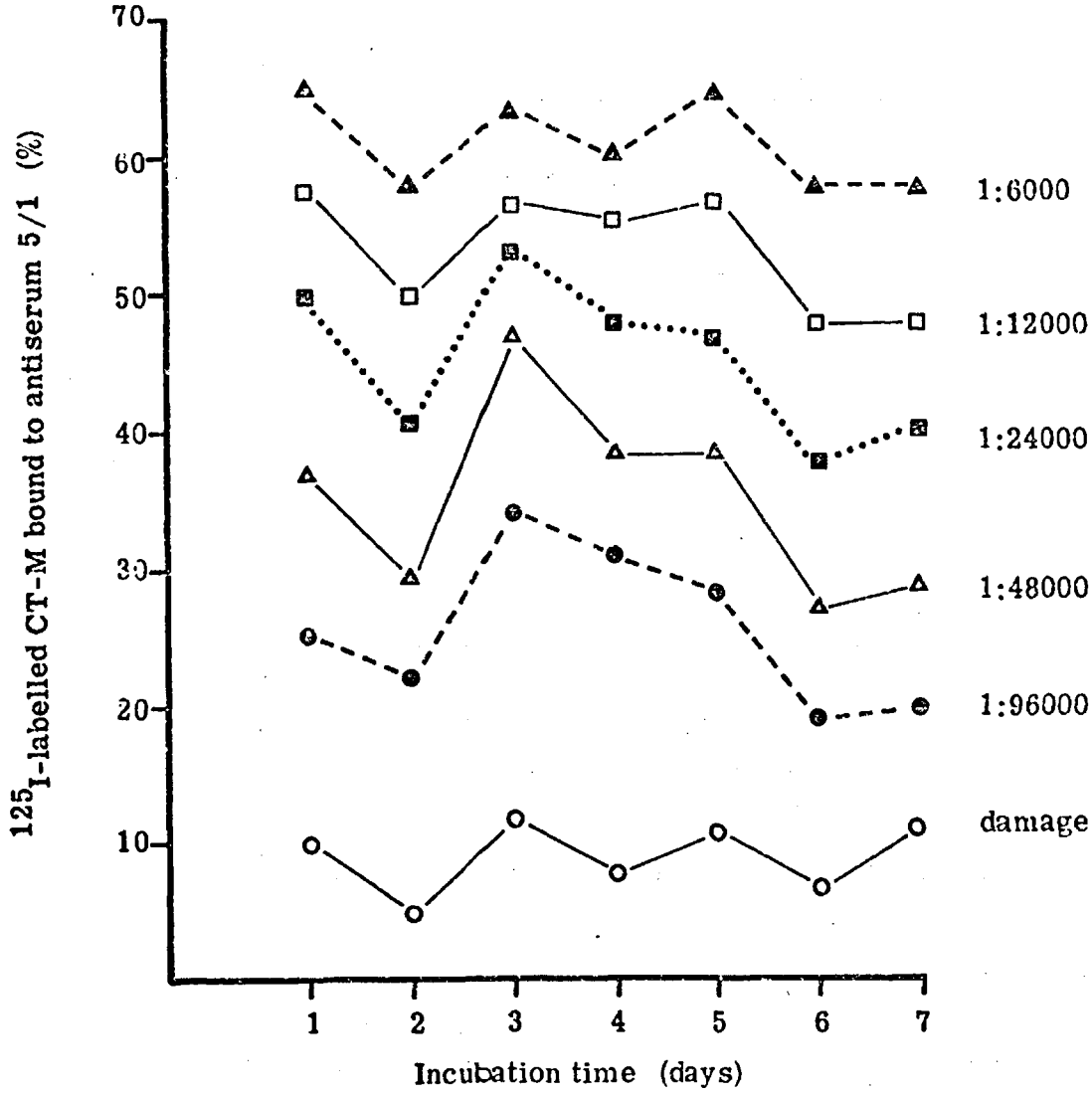


Fig. 7 Antibody dilution curves for antiserum 5/1 over seven days at various dilutions (rabbit no. 5 was immunized with fragment (17-32)-CT-M coupled to ovalbumin). Generally binding of tracer increased 3 days after incubation.

Table 2

Summary of the properties of the six antisera.

Antiserum	Immunogen	Duration of Immunization (weeks)	Titre (Total incubation volume 300 µl)	*Detection limit of CT-M (pg/assay tube) in an overnight equilibrium assay
5/1	(17-32) CT-M coupled to ovalbumin via glutaraldehyde	6	1 : 24,000	63
7/2	" "	10	1 : 12,000	16
10/2	(11-32) CT-M coupled to ovalbumin via glutaraldehyde	10	1 : 6,000	63
12/3	" "	14	1 : 6,000	8
827/4	synthetic CT-M unconjugated	27	1 : 48,000	4
336/6	" "	27	1 : 12,000	8

* Assay sensitivity could be improved by using a disequilibrium long incubation (3 + 2 days) and slightly more dilute antiserum.

binding of ^{125}I CT-M to each antiserum by equimolar concentrations of CT-M and 15 analogues of CT-M which differ from the parent molecule by one or two amino acids. Table 3 is a list of these analogues which were kindly donated by Dr. W. Rittel. In addition a comparison of the reaction of CT-M and synthetic methionine 8-sulphoxide CT-M with the six antisera was also carried out.

Table 3

List of CT-M analogues used for characterization of the six antisera.

Val^{8}-CT	Tyr^{22}-CT	Ala^{29},Val^{31}-CT
Lys^{11}-CT	Arg^{24}-CT	Ser^{29},Thr^{31}-CT
Leu^{12}-CT	Asn^{26}-CT	Lys^{11},Arg^{24}-CT
Leu^{16}-CT	Thr^{27}-CT	Asn^{26},Thr^{27}-CT
Leu^{19}-CT	CT-OH	Leu^{12},Thr^{27}-CT

Replacement of amino acids at positions 8, 11, 12, 16, 19, 22, 24, 26, 27, 31 and 32 affects the binding energy of such analogues to one or more of the antisera, hence each analogue could be distinguished from the parent molecule.

Using one or more of the six antisera, single or double amino acid substitutions at the antigenic determinant of the hormone produce either a loss or inhibition of binding by the antiserum or a change in the slope of the displacement curve (Figs. 8-22).

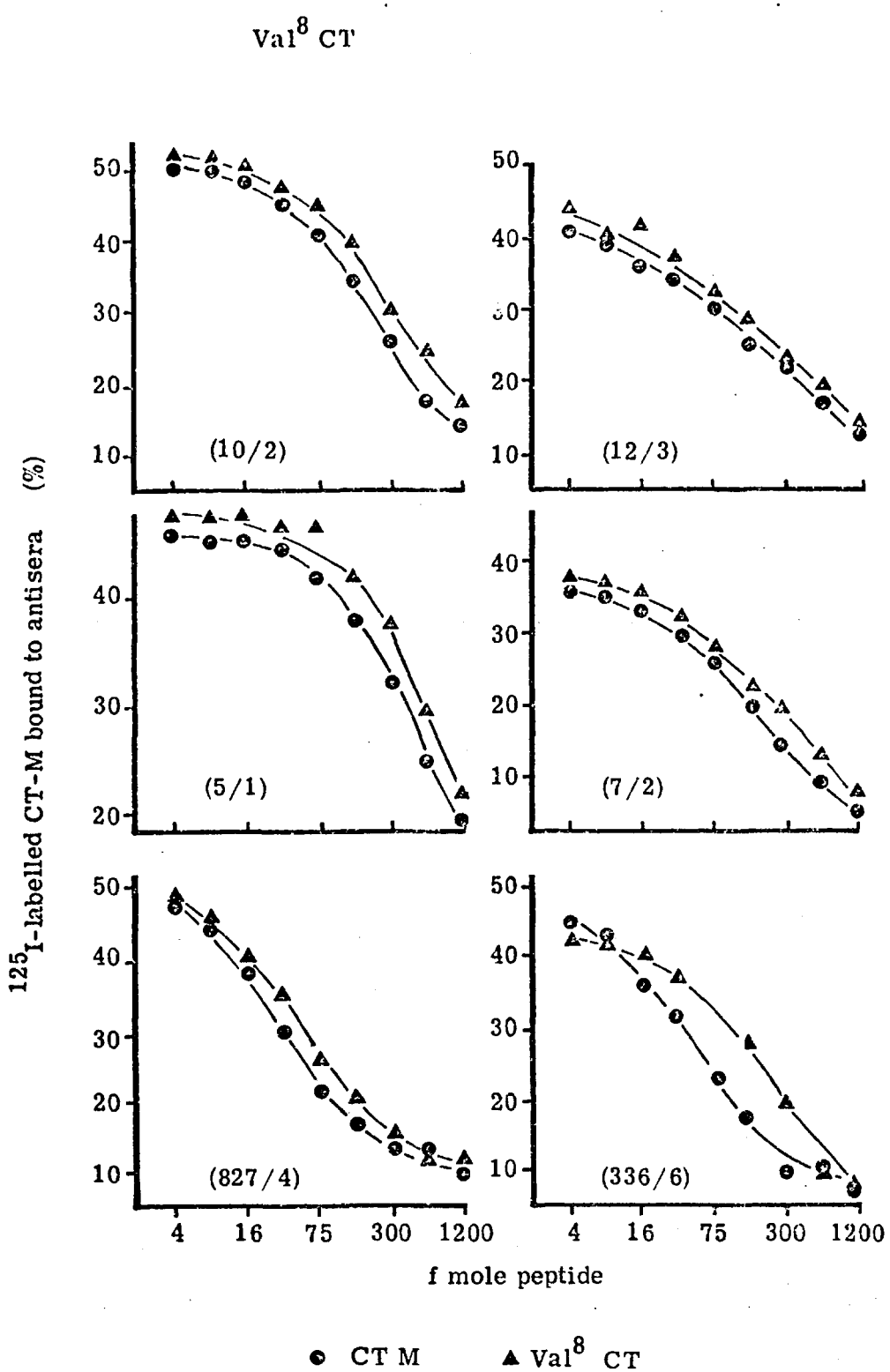


Fig. 8 Comparison on a molar basis of the reaction of Val^8-CT (▲) and CT-M (●) with the six antisera using ^{125}I-labelled CT-M as a tracer in an overnight incubation assay. Substitution at position 8 is recognized by antiserum 336/6, as shown by diminution in binding energy.

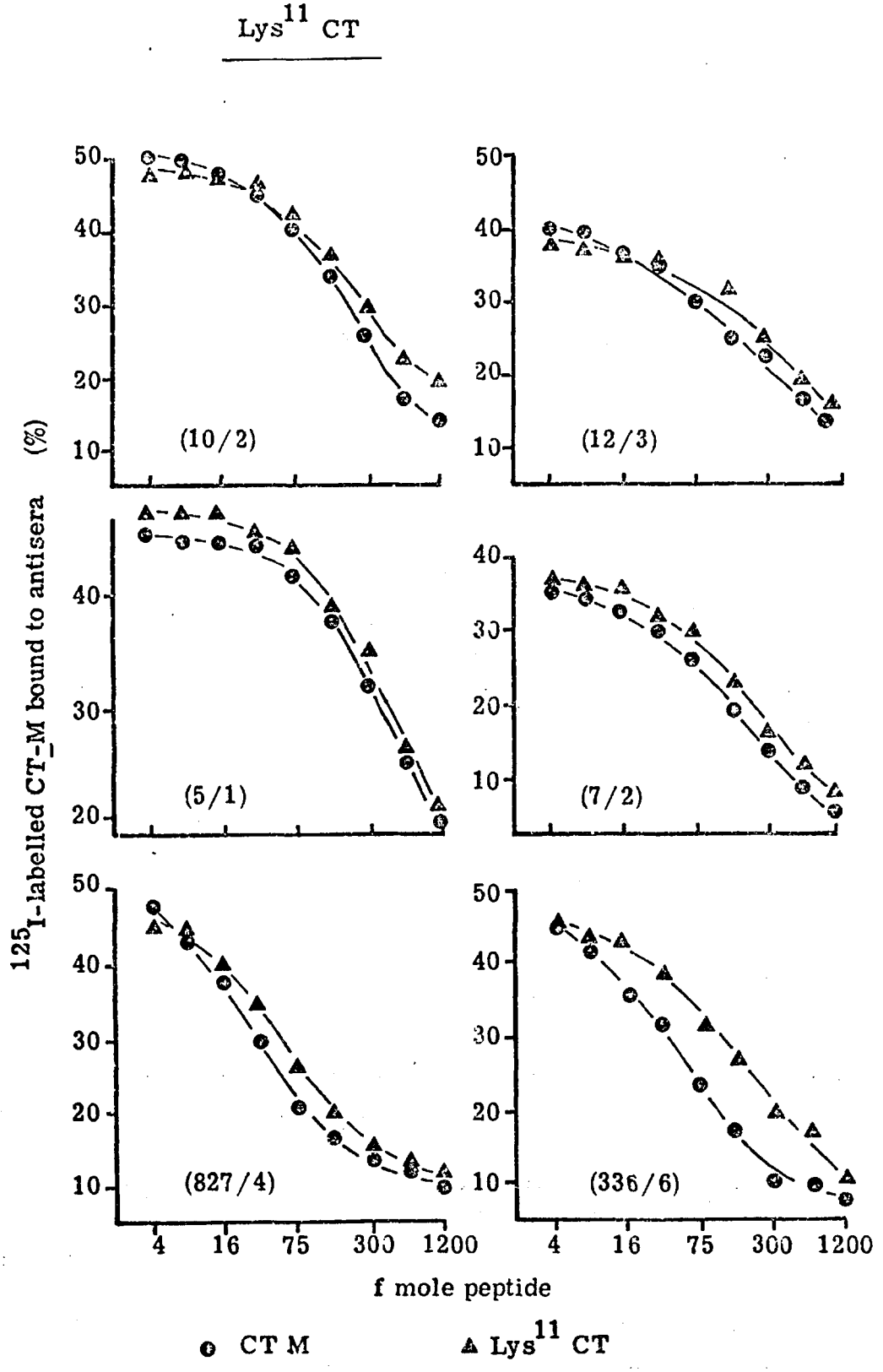


Fig. 9 Comparison on a molar basis of the reaction of Lys^{11}-CT (▲) and CT-M (●) with the six antisera. Alteration at position 11 is mainly recognized by antiserum 336/6.

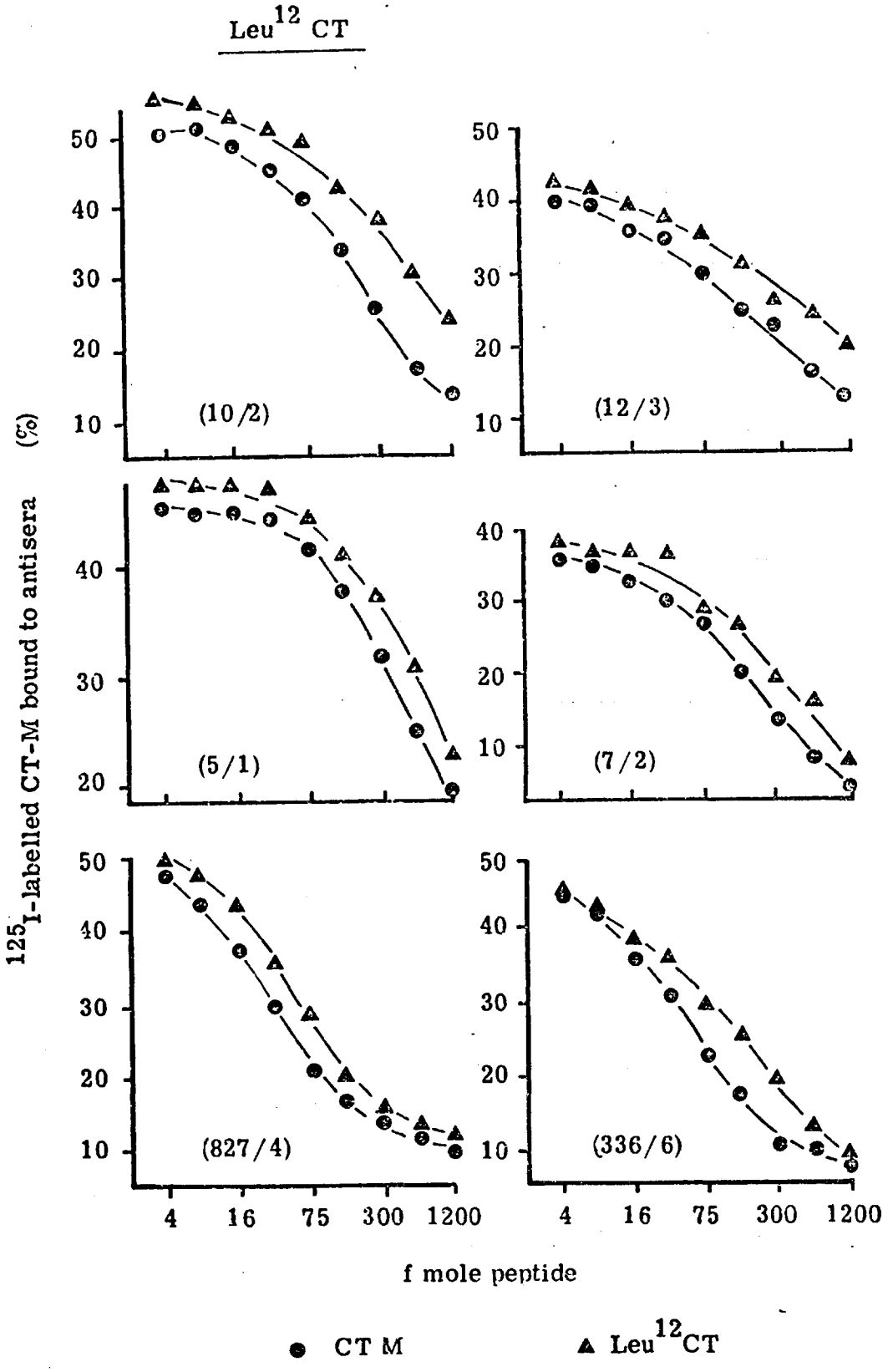


Fig. 10 Comparison on a molar basis of the reaction of Leu^{12}-CT (▲) and CT-M (●) with the six antisera. Substitution at 12 is recognized by antisera 336/6, 10/2 and 12/3.

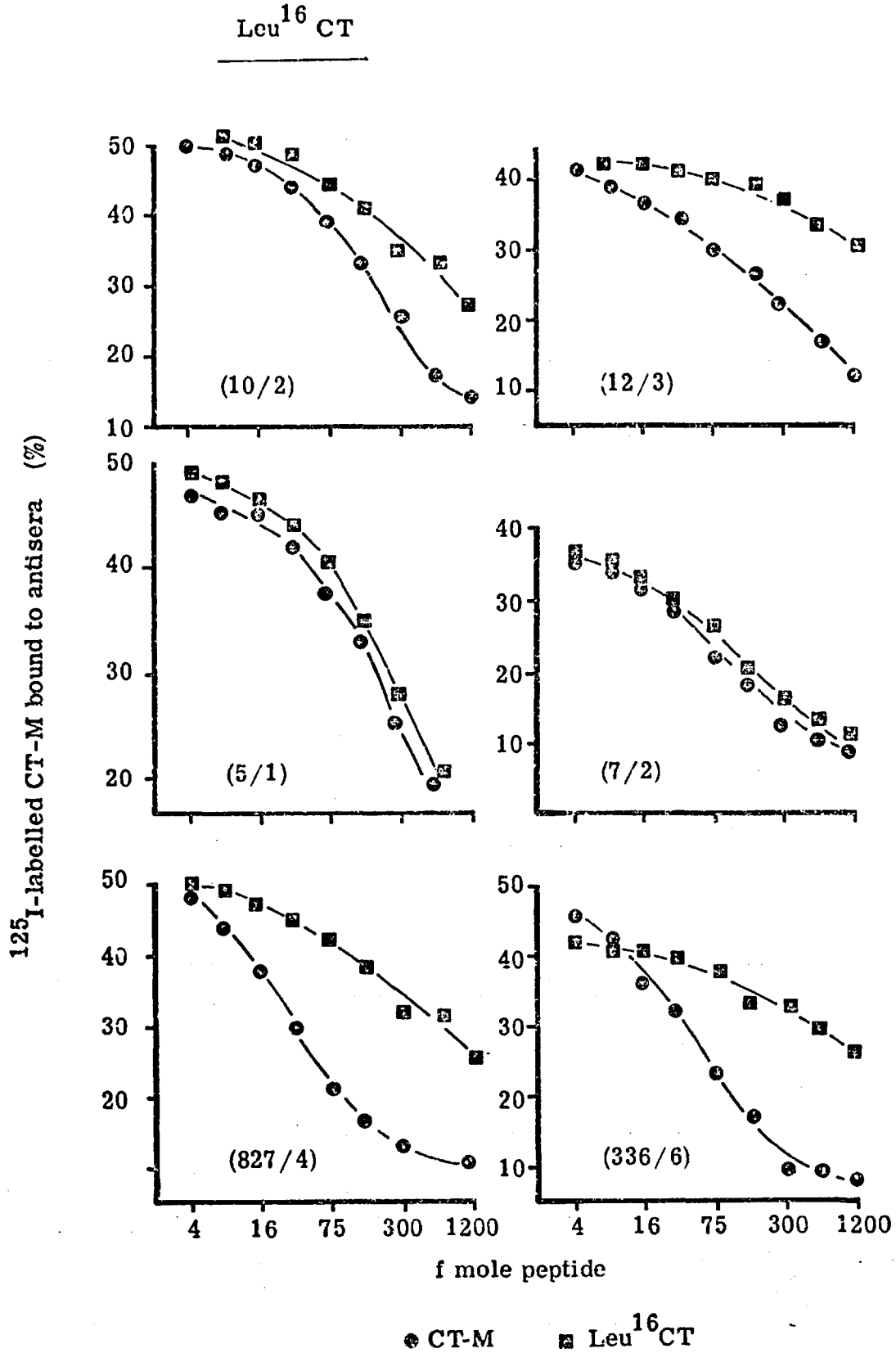


Fig. 11 Comparison on a molar basis of the reaction of Leu^{16}-CT (■) and CT-M (●) with the six antisera. Antisera 5/1 and 7/2 do not recognize the alteration at position 16 in contrast to antisera 827/4, 336/6, 10/2 and 12/3.

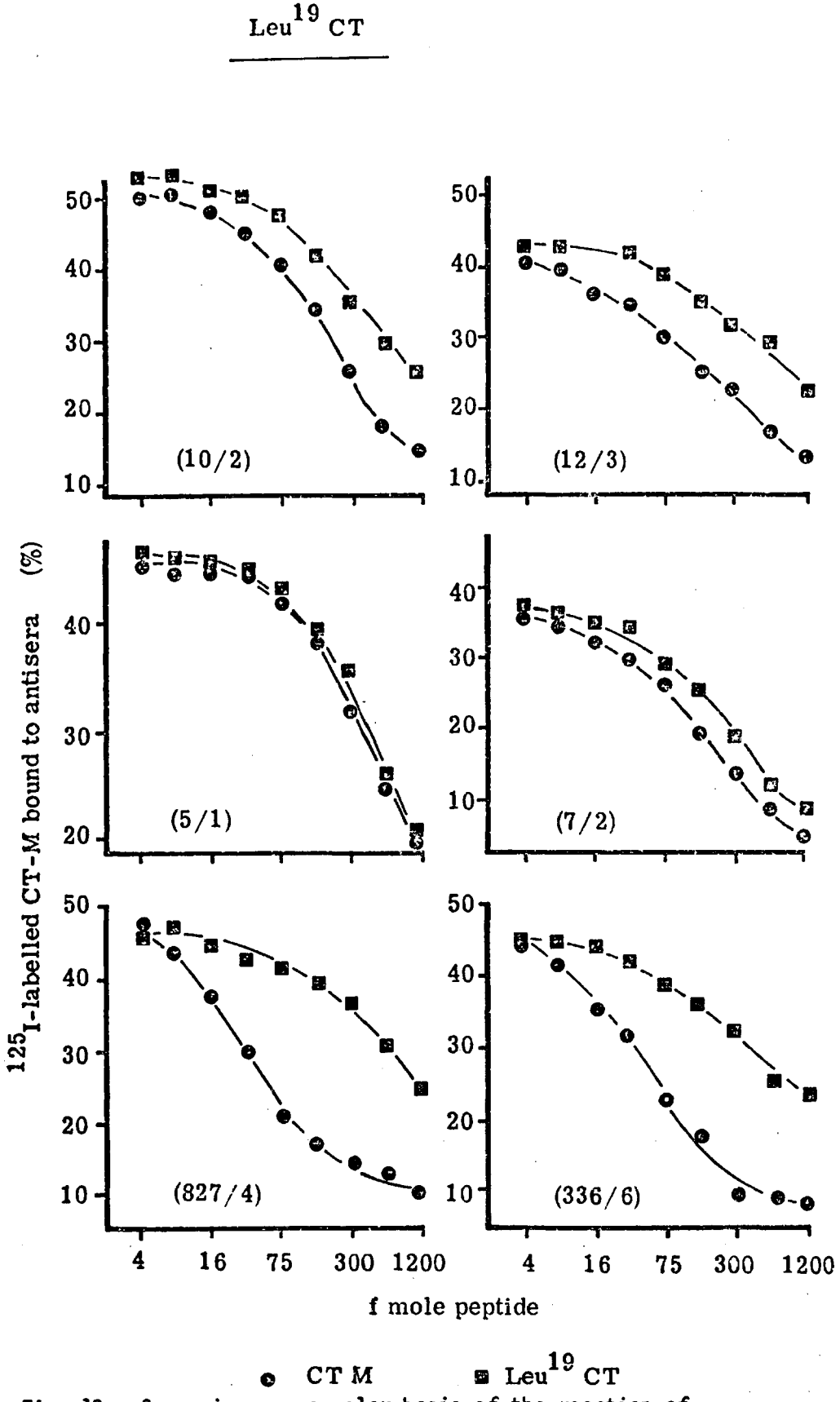


Fig. 12 Comparison on a molar basis of the reaction of Leu^{19}-CT (■) and CT-M (●) with the six antisera. Substitution at position 19 is recognized by all antisera except antiserum 5/1.

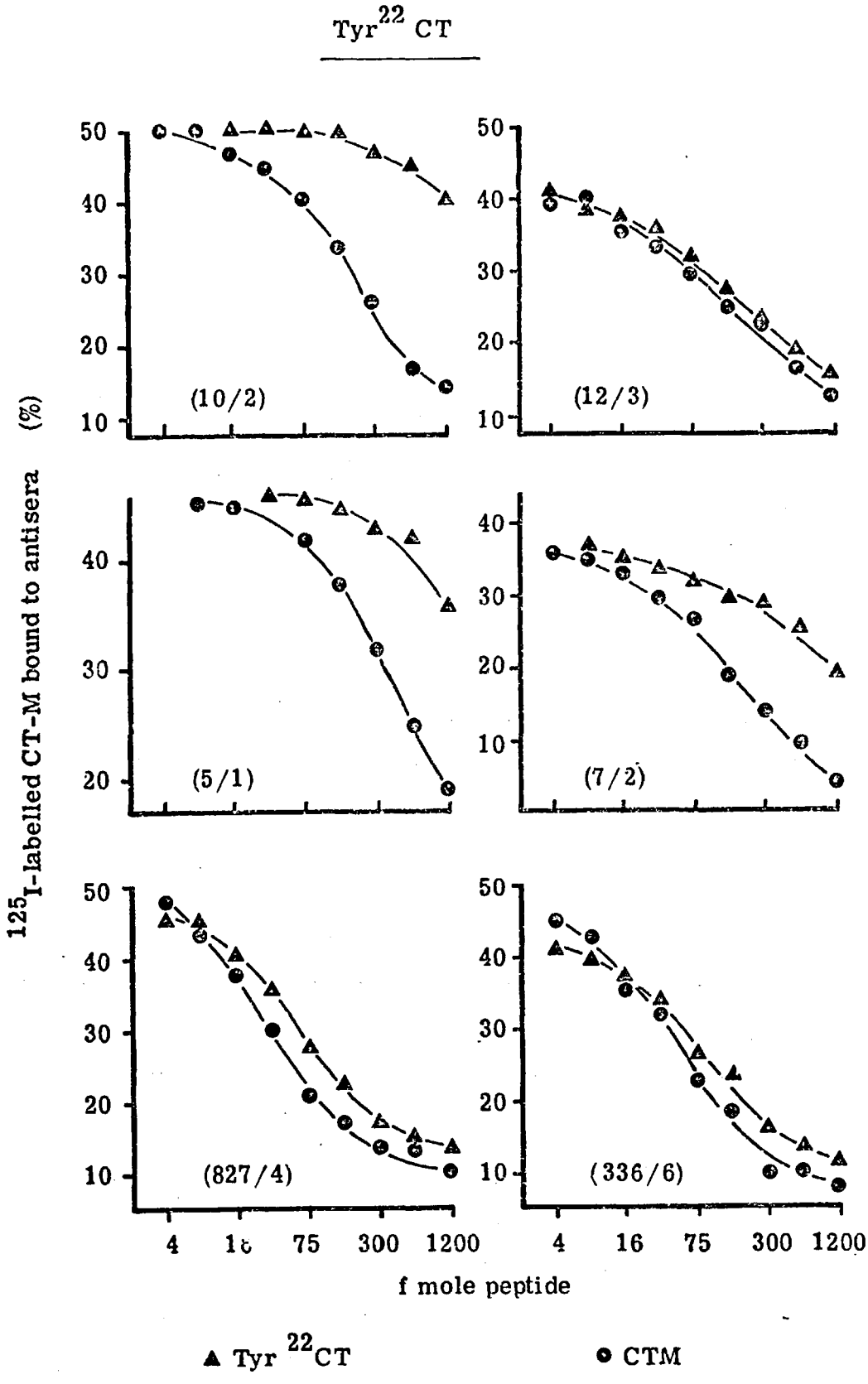


Fig. 13 Comparison on a molar basis of the reaction of the analogue Tyr^{22}-CT (▲) and CT-M (●) with the six antisera. Substitution at 22 is clearly recognized by antisera 7/2, 5/1 and 10/2.

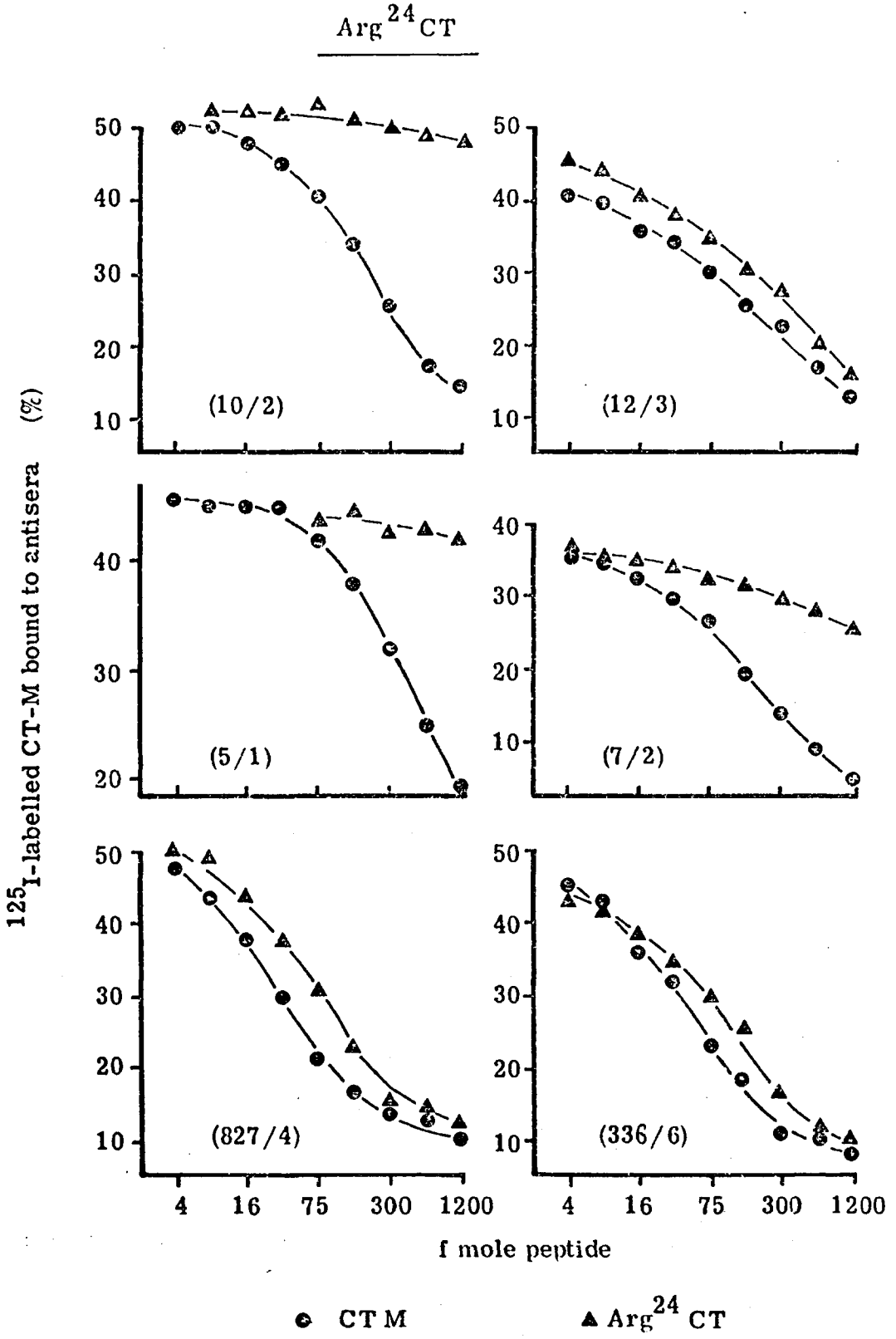


Fig. 14 Comparison on a molar basis of the reaction of Arg^{24}-CT (▲) and CT-M (●) with the six antisera. Substitution at 24 is recognized by antisera 5/1, 10/2 and 7/2.

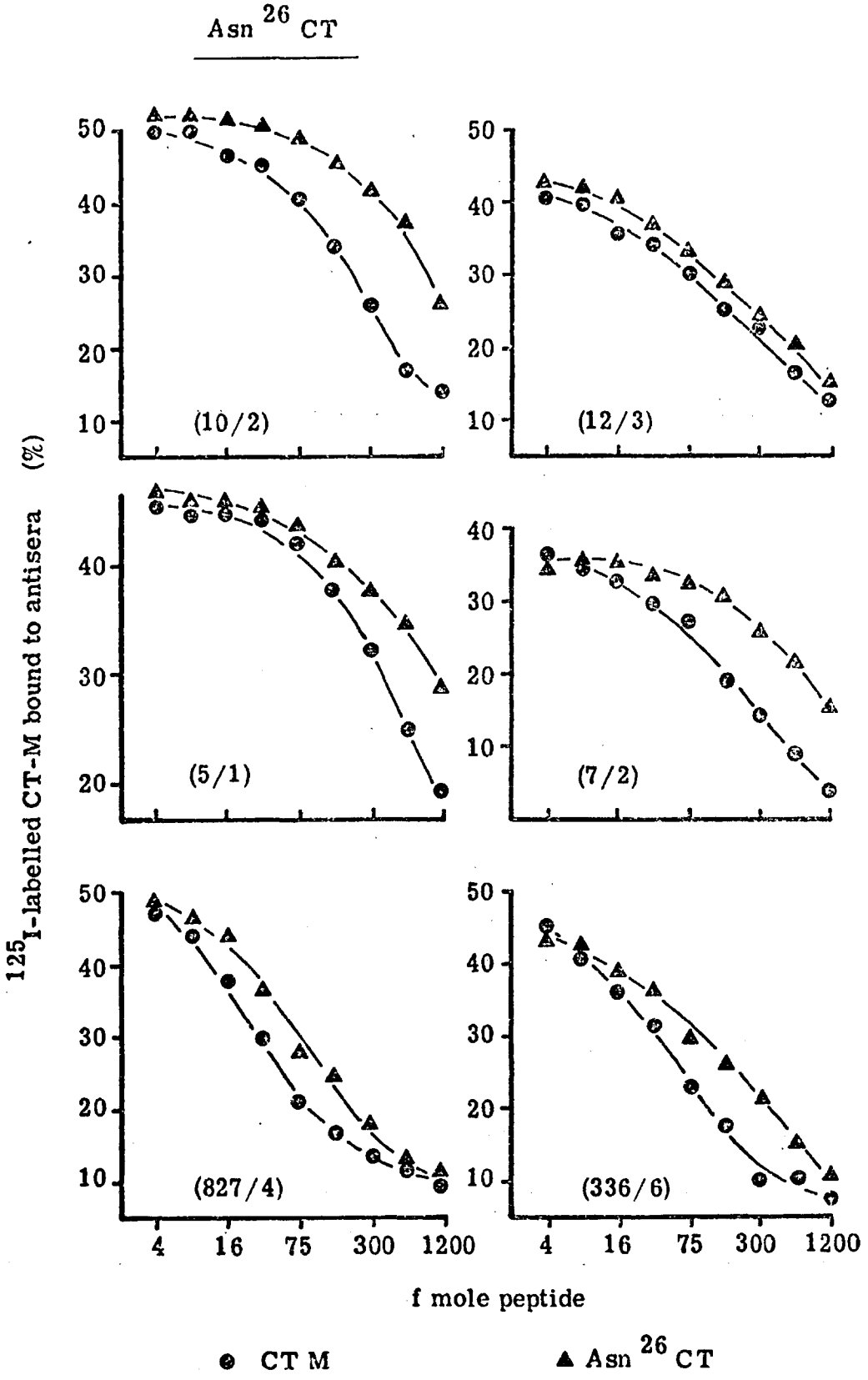


Fig. 15 Comparison on a molar basis of the reaction of Asn^{26}-CT (▲) and CT-M (●) with the six antisera. Substitution at 26 is recognized with variable degrees by the six antisera.

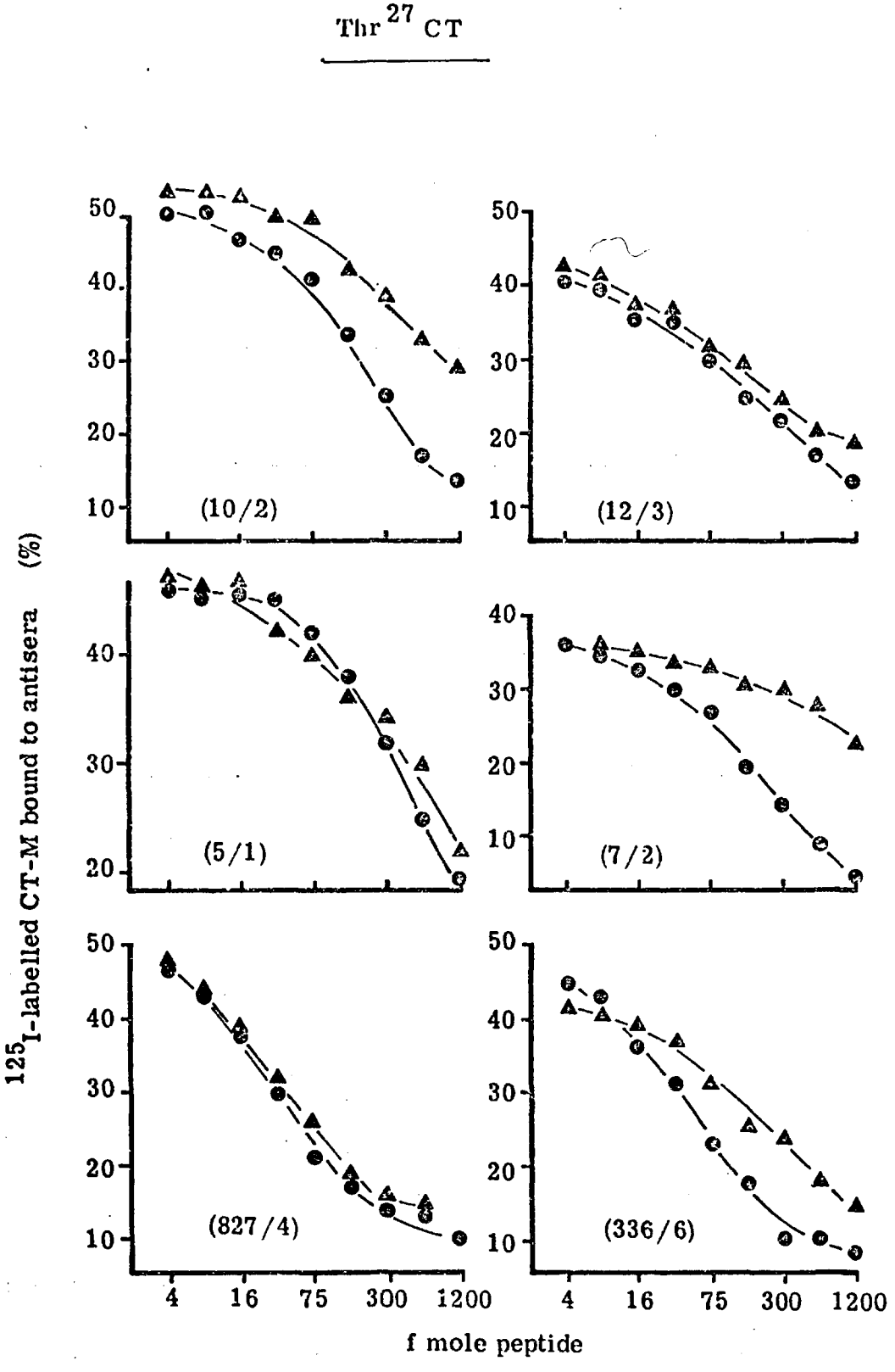


Fig. 16 Comparison on a molar basis of the reaction of Thr^{27}-CT (▲) and CT-M (●) with the six antisera. Substitution at position 27 is recognized by antisera 7/2, 336/6 and 10/2.

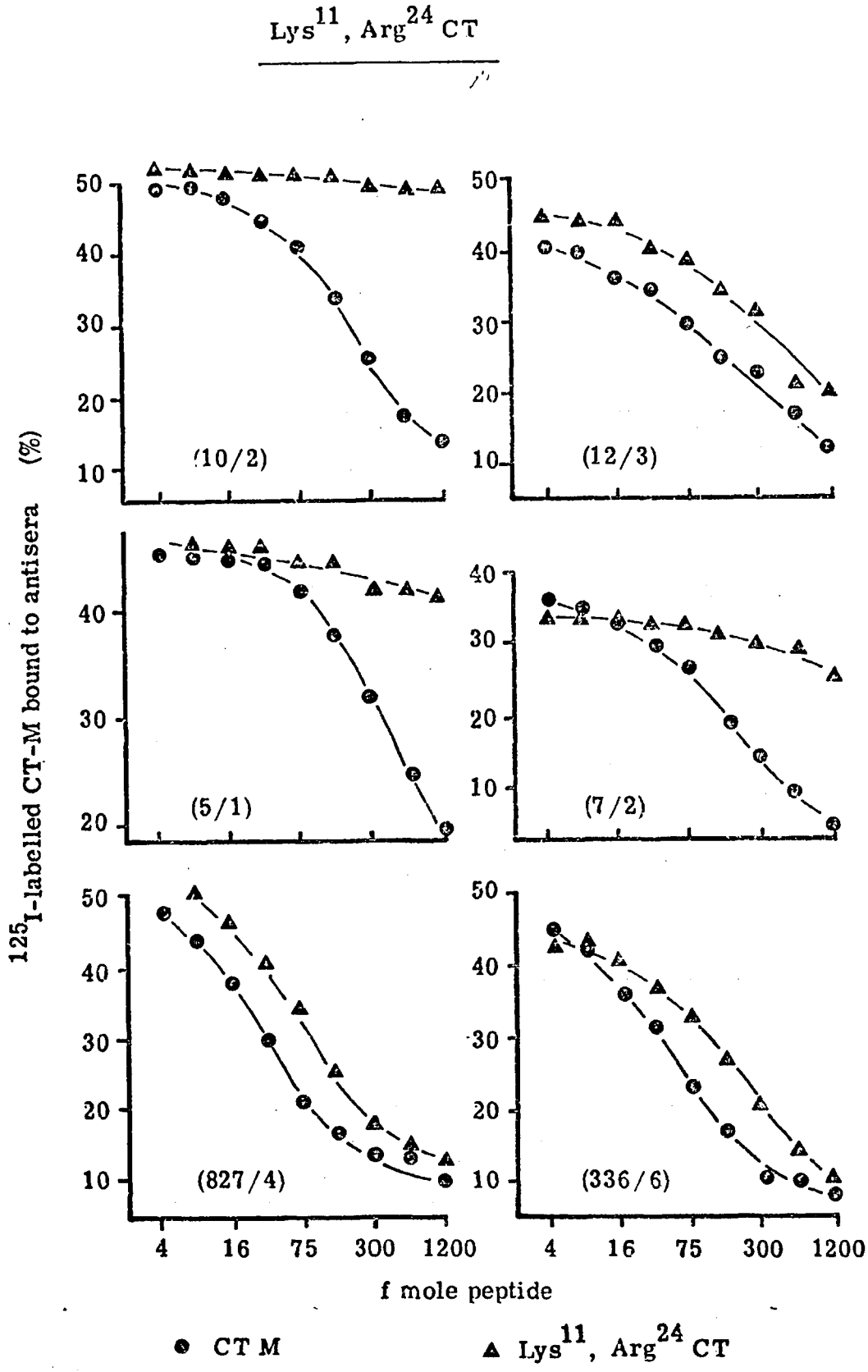


Fig. 17 Comparison on a molar basis of the reaction of the analogue Lys^{11},Arg^{24}-CT (▲) and CT-M (●) with the six antisera. This double substitution is clearly recognized by antisera 10/2, 5/1 and 7/2.

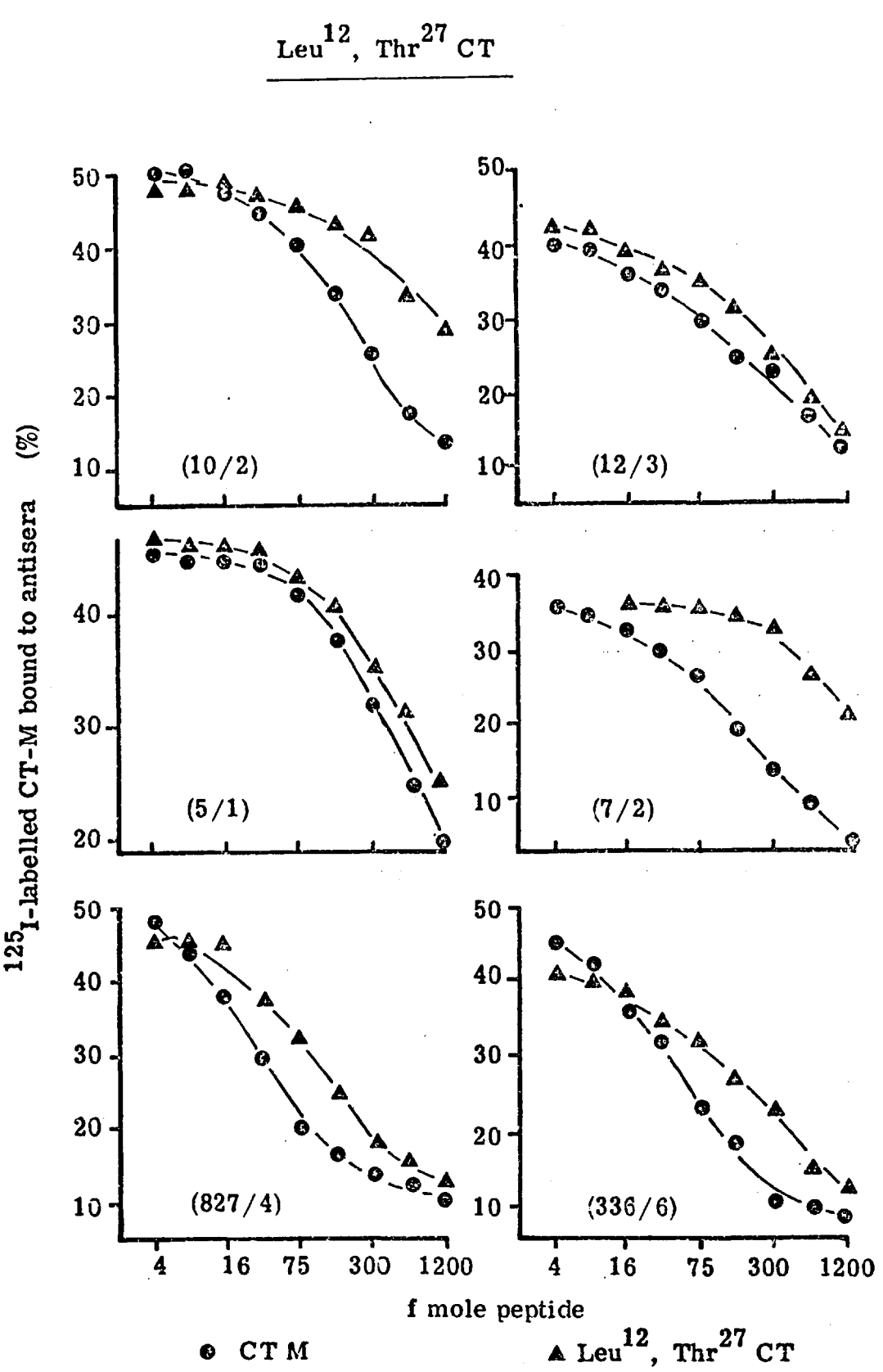


Fig. 18 Comparison on a molar basis of the reaction of Leu^{12}, Thr^{27}-CT (▲) and CT-M (●) with the six antisera. This double substitution is recognized by all the antisera except antiserum 5/1.

Asn^{26}, Thr^{27} CT

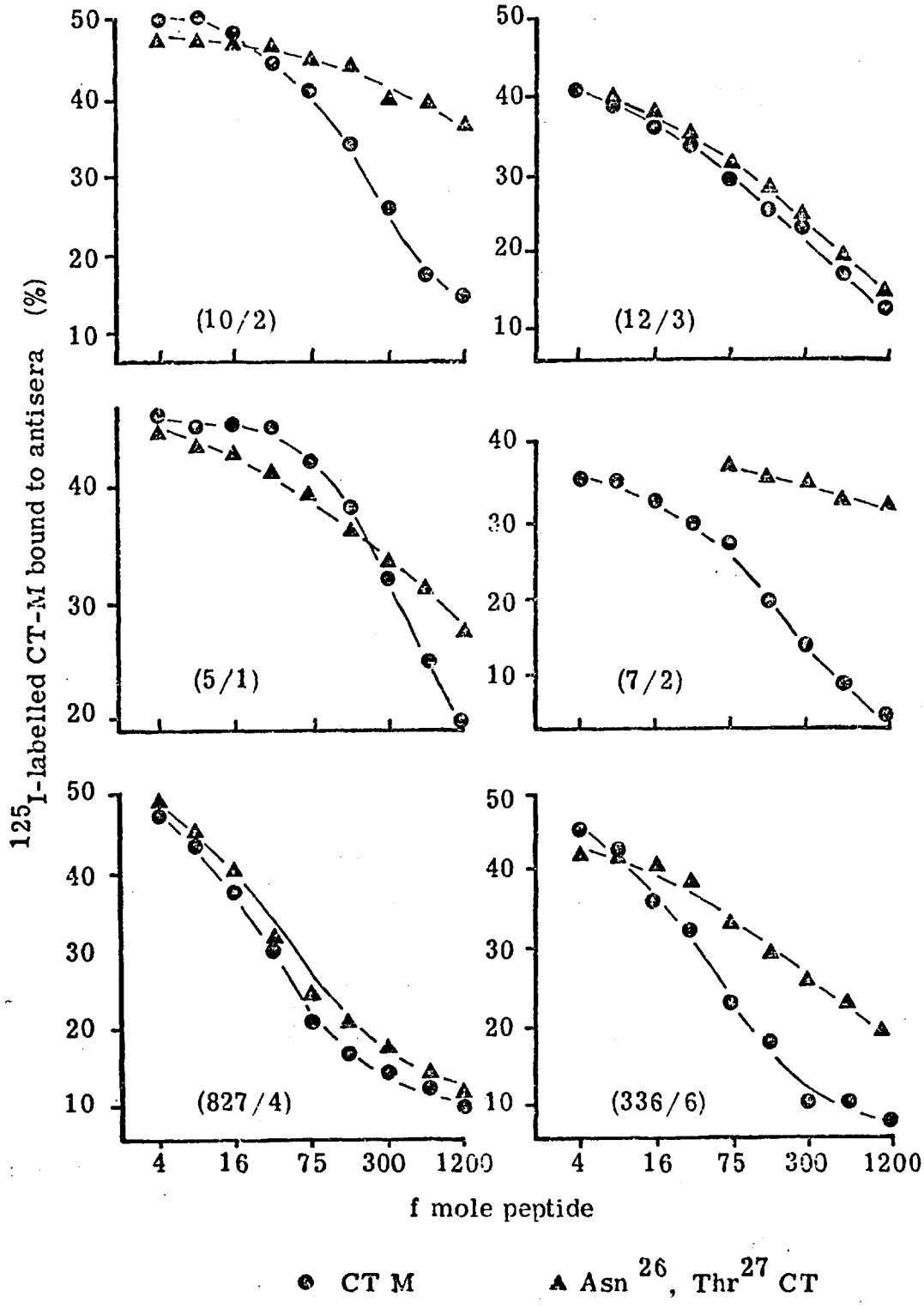


Fig. 19 Comparison on a molar basis of the reaction of Asn^{26},Thr^{27}-CT (▲) and CT-M (●) with the six antisera. This double substitution is recognized with variable degrees by all the antisera. Obvious recognition by antisera 7/2, 10/2 and 336/6.

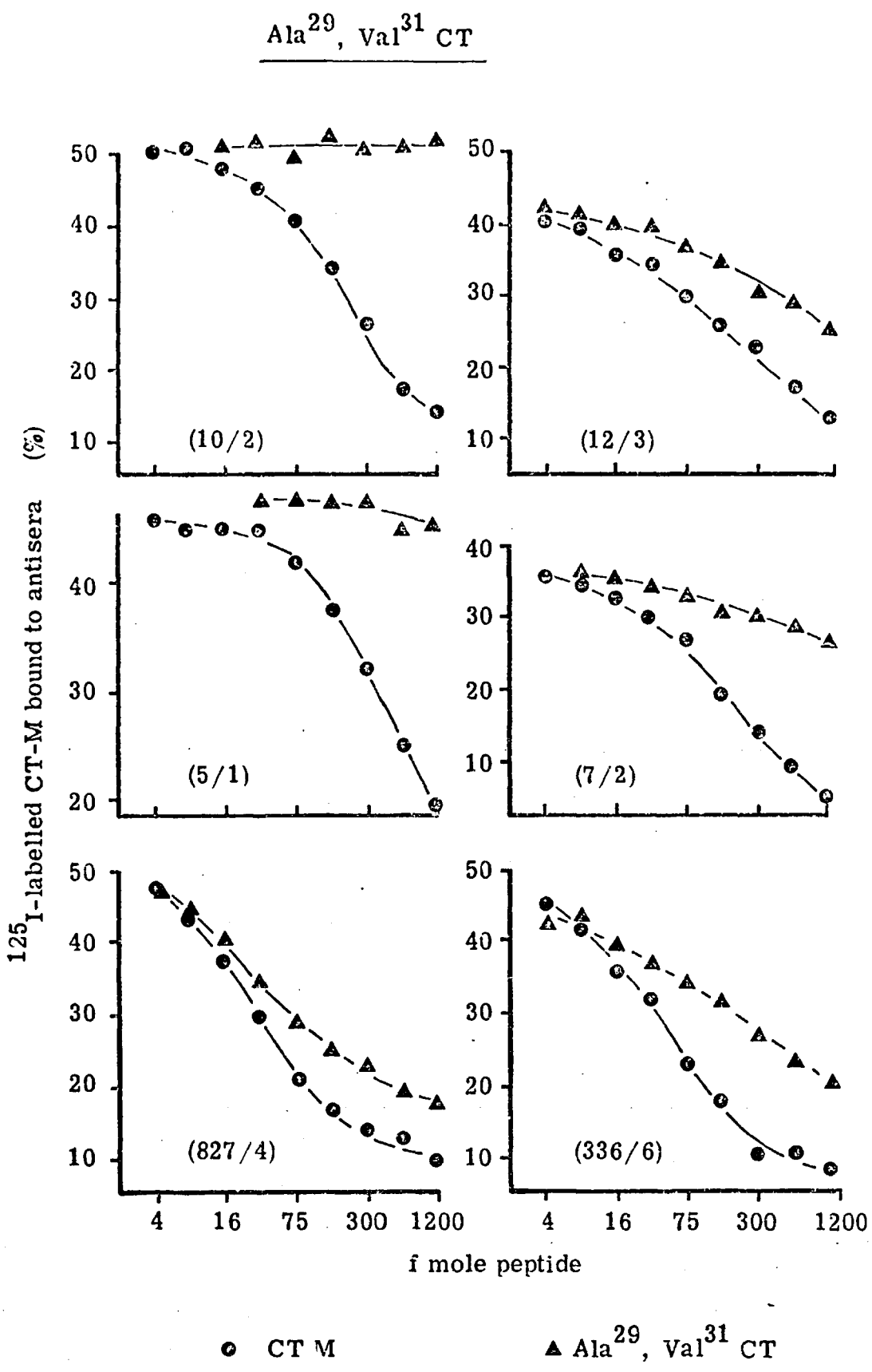


Fig. 20 Comparison on a molar basis of the reaction of Ala^{29},Val^{31}-CT (▲) and CT-M (●) with the six antisera. These substitutions are recognized by all the antisera and complete loss of immunoreactivity is seen with antisera 5/1 and 10/2.

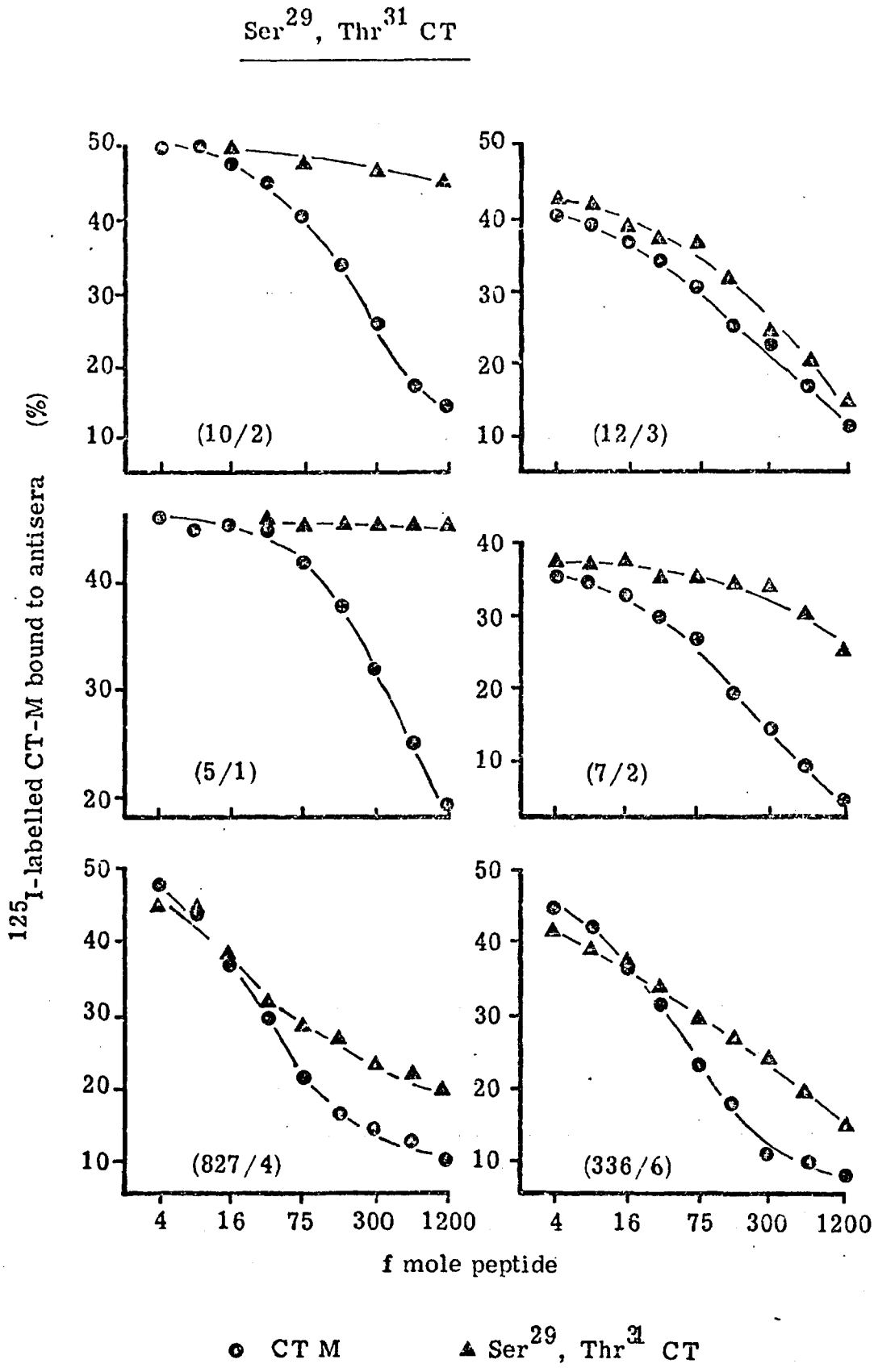


Fig. 21 Comparison on a molar basis of the reaction of Ser^{29},Thr^{31}-CT (▲) and CT-M (●) with the six antisera. These substitutions are recognized by all the antisera and complete loss of immunoreactivity is shown with antiserum 5/1.

CT-OH free acid

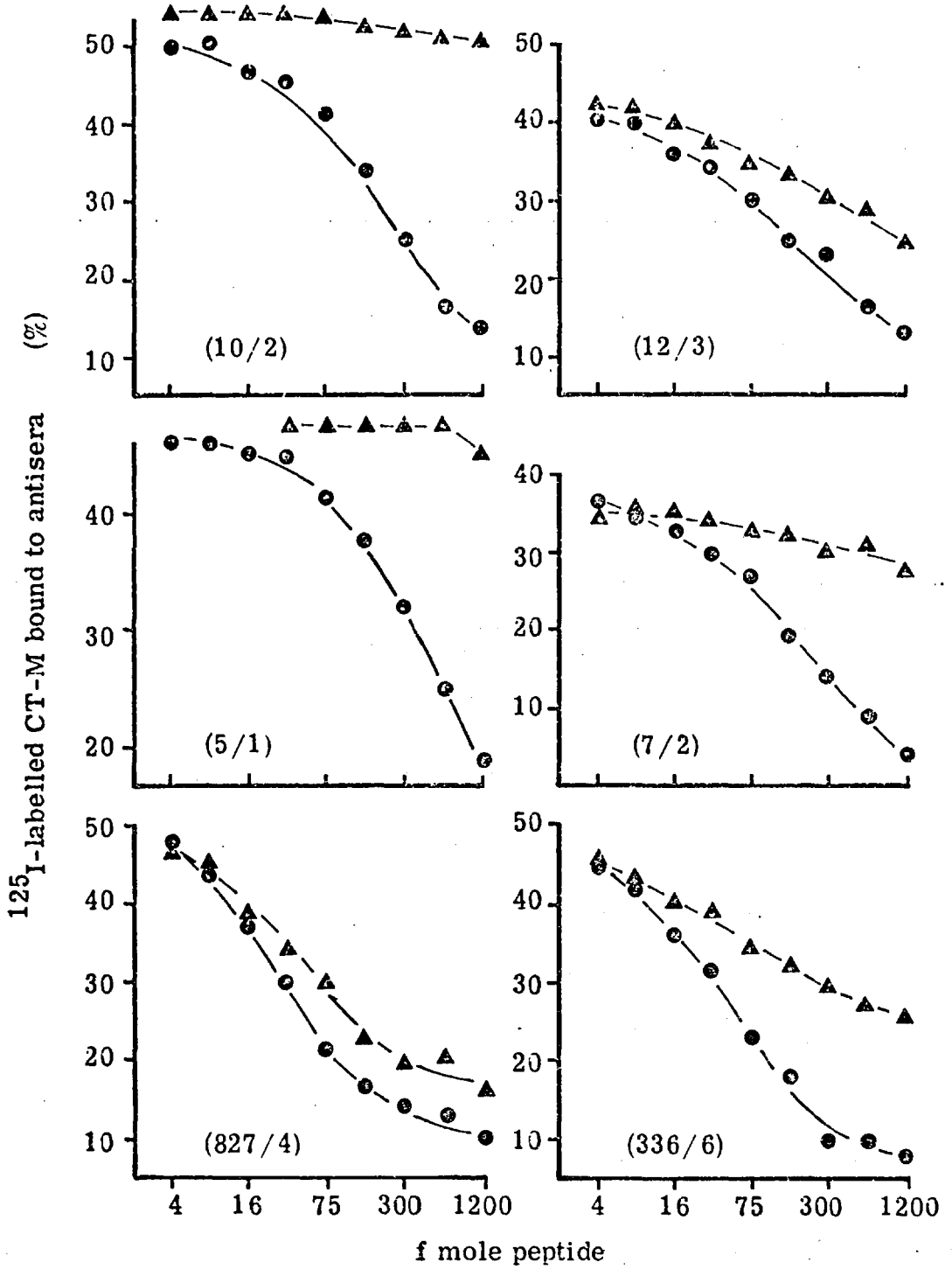


Fig. 22 Comparison on a molar basis of the reaction of CT-OH free acid (▲) and CT-M (●) with the six antisera. Alteration at the C-terminal end of calcitonin is recognized by all the antisera. Complete loss of immunoreactivity is shown with antisera 5/1 and 10/2.

The synthetic methionine 8 sulphoxide CT-M has a higher affinity to antisera 5/1, 7/2 and 10/2 than the native hormone. However, it has a lowered affinity to antisera 827/4, 336/6 and 12/3 (Fig. 23). The most likely explanation of the increased affinity of antisera 5/1, 7/2 and 10/2 for binding to methionine 8 sulphoxide CT-M is an effect of conformational changes (Sela et al. 1967; Goodman, 1969) as these antisera were raised against fragments 11-32 or 17-32. A similar finding was also reported by Byfield et al. (1972).

The molar concentration of each peptide which produces 50% inhibition of specific tracer binding is shown in Table 4. The relative potencies of the 15 analogues and of CT-M sulphoxide to CT-M with the six antisera expressed as the ratio:

$$\frac{\text{Molar concentration of analogue to produce 50\% inhibition of binding}}{\text{Molar concentration of CT-M to produce 50\% inhibition of binding}}$$

are shown in Table 5. This ratio reflects the importance of a given site for antibody binding and hence antigenic specificity.

The combined specificities of the six antisera cover most of the CT-M molecule with the exception of the 1-9 region which is highly conserved. So the use of these six antisera in the characterization of a CT-M like molecule in plasma or tissue extracts would provide sufficient information on its immunological identity or otherwise with CT-M. Furthermore, when this immunological characterization is used in combination with a refined chromatographic system, more detailed information on

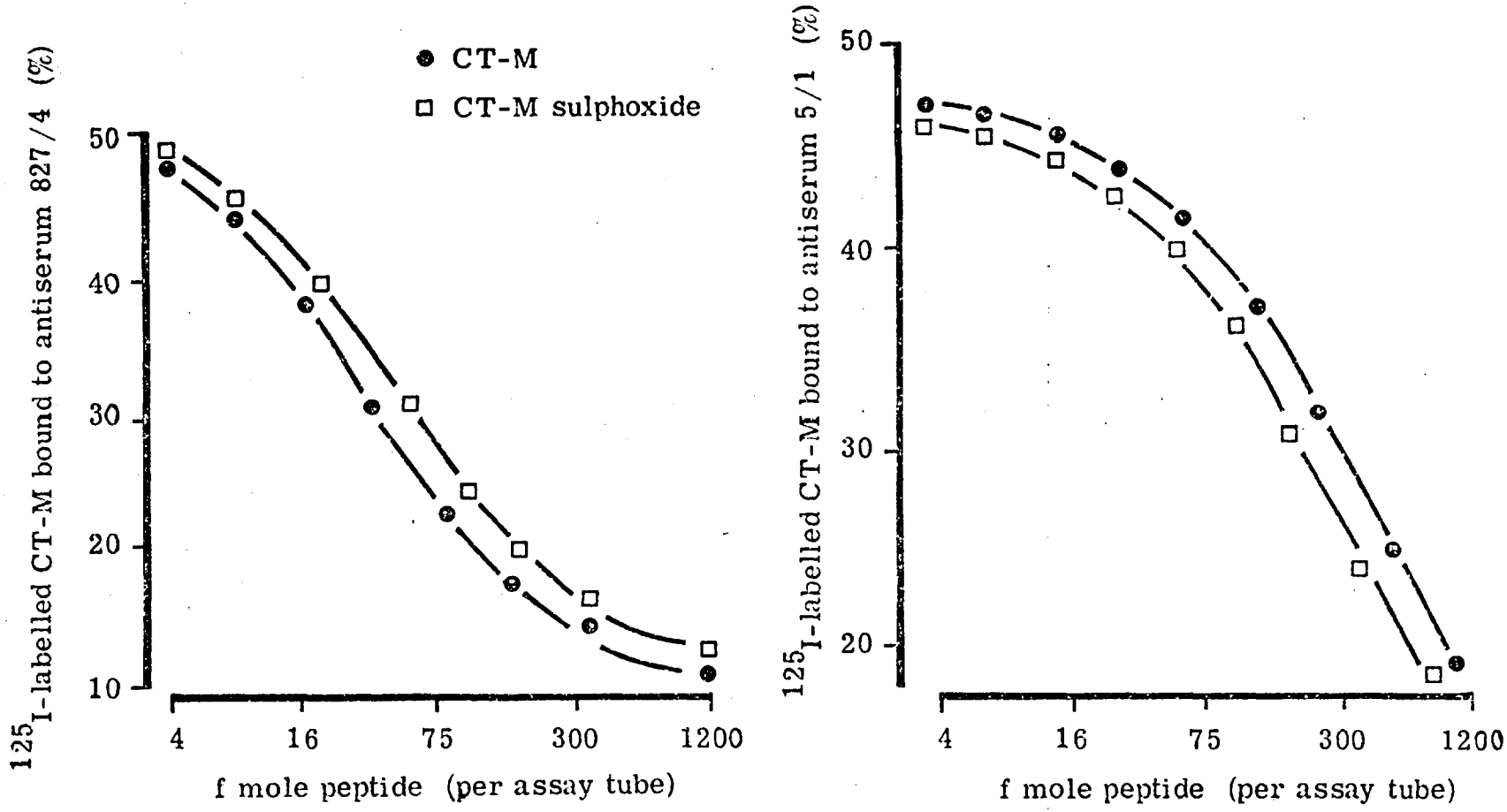


Fig. 23 Displacement curves of CT-M (●) and CT-M sulphoxide (□) in reaction with antisera 5/1 and 827/4. CT-M sulphoxide gave the same percentage of tracer bound to antiserum 5/1 at lower molar concentrations than CT-M. This means that antiserum 5/1 has a higher binding energy for CT-M sulphoxide than for CT-M.

Table 4

Molar concentrations of CT-M and its analogues that produce 50% inhibition of tracer binding to the six antisera.

Peptide	antiserum 827/4	antiserum 336/6	antiserum 5/1	antiserum 7/2	antiserum 12/3	antiserum 10/2
CT-M	4.4×10^{-14}	6.4×10^{-14}	3×10^{-13}	1.5×10^{-13}	1.2×10^{-13}	1.8×10^{-13}
Val^{8}-CT	6.7×10^{-14}	1.8×10^{-13}	3×10^{-13}	1.5×10^{-13}	1.2×10^{-13}	1.8×10^{-13}
Lys^{11}-CT	6.4×10^{-14}	2.1×10^{-13}	3×10^{-13}	1.5×10^{-13}	1.9×10^{-13}	2.6×10^{-13}
Leu^{12}-CT	5.6×10^{-14}	1.5×10^{-13}	3×10^{-13}	1.5×10^{-13}	2.3×10^{-13}	2.7×10^{-13}
Leu^{16}-CT	8.4×10^{-13}	1.2×10^{-12}	3×10^{-13}	1.5×10^{-13}	1.2×10^{-12}	5.4×10^{-13}
Leu^{19}-CT	8.4×10^{-13}	8.4×10^{-13}	3×10^{-13}	2.6×10^{-13}	6×10^{-13}	3.3×10^{-13}
Tyr^{22}-CT	7.5×10^{-14}	1.1×10^{-13}	$> 1.2 \times 10^{-12}$	1.2×10^{-12}	1.5×10^{-13}	$> 12 \times 10^{-13}$
Arg^{24}-CT	6.4×10^{-14}	1.2×10^{-13}	$> 1.2 \times 10^{-12}$	$> 1.2 \times 10^{-12}$	2.9×10^{-13}	$> 12 \times 10^{-13}$
Asn^{26}-CT	7.5×10^{-14}	1.8×10^{-13}	6×10^{-13}	7.2×10^{-13}	1.5×10^{-13}	4.8×10^{-13}
Thr^{27}-CT	5.5×10^{-14}	1.5×10^{-13}	3×10^{-13}	$> 1.2 \times 10^{-12}$	1.2×10^{-13}	5.1×10^{-13}
Lys^{11},Arg^{24}-CT	7.5×10^{-14}	2.1×10^{-13}	$> 1.2 \times 10^{-12}$	$> 1.2 \times 10^{-12}$	2.4×10^{-13}	$> 12 \times 10^{-13}$
Leu^{12},Thr^{27}-CT	1.2×10^{-13}	2.3×10^{-13}	3×10^{-13}	$> 1.2 \times 10^{-12}$	1.8×10^{-13}	9.8×10^{-13}
Asn^{26},Thr^{27}-CT	7.5×10^{-14}	4.2×10^{-13}	6×10^{-13}	$> 1.2 \times 10^{-12}$	1.5×10^{-13}	$> 12 \times 10^{-13}$
Ser^{29},Thr^{31}-CT	7.5×10^{-14}	2.6×10^{-13}	$> 1.2 \times 10^{-12}$	$> 1.2 \times 10^{-12}$	2.7×10^{-13}	$> 12 \times 10^{-13}$
Ala^{29},Val^{31}-CT	9.7×10^{-14}	4.5×10^{-13}	$> 1.2 \times 10^{-12}$	$> 1.2 \times 10^{-12}$	7.2×10^{-13}	$> 12 \times 10^{-13}$
CT-OH (free acid)	7.5×10^{-14}	1.2×10^{-12}	$> 1.2 \times 10^{-12}$	$> 1.2 \times 10^{-12}$	6×10^{-13}	$> 12 \times 10^{-13}$
CT-M sulphoxide 8-methionine	6.4×10^{-14}	8.0×10^{-14}	2.3×10^{-13}	1.2×10^{-13}	1.5×10^{-13}	2.2×10^{-13}

Table 5

The relative immunological potencies of 15 analogues to CT-M with the various antisera, expressed as the ratio:

$$\frac{\text{Molar concentration of analogue to produce 50\% inhibition of tracer binding}}{\text{Molar concentration of CT-M to produce 50\% inhibition of tracer binding.}}$$

Peptide	Antiserum					
	827/4	336/6	5/1	7/2	12/3	10/2
CT-M	1.0	1.0	1.0	1.0	1.0	1.0
Val^{8}-CT	1.5	2.8	1.0	1.0	1.0	1.0
Lys^{11}-CT	1.5	3.3	1.0	1.0	1.6	1.4
Leu^{12}-CT	1.3	2.3	1.0	1.0	1.9	1.5
Leu^{16}-CT	19.0	18.8	1.0	1.0	10.0	3.0
Leu^{19}-CT	19.0	13.0	1.0	1.7	5.0	1.8
Tyr^{22}-CT	1.7	1.7	> 4.0	8.0	1.3	> 6.7
Arg^{24}-CT	1.5	1.9	> 4.0	> 8.0	2.4	> 6.7
Asn^{26}-CT	1.7	2.8	2.0	4.8	1.3	2.7
Thr^{27}-CT	1.3	2.3	1.0	> 8.0	1.0	2.8
Lys^{11},Arg^{24}-CT	1.7	3.3	> 4.0	> 8.0	2.0	> 6.7
Leu^{12},Thr^{27}-CT	2.7	3.6	1.0	> 8.0	1.5	5.4
Asn^{26},Thr^{27}-CT	1.7	6.6	2.0	> 8.0	1.3	> 6.7
Ser^{29},Thr^{31}-CT	1.7	4.1	> 4.0	> 8.0	2.3	> 6.7
Ala^{29},Val^{31}-CT	2.2	7.0	> 4.0	> 8.0	6.0	> 6.7
CT-OH (free acid)	1.7	19.0	> 4.0	> 8.0	5.0	> 6.7

the peptide of interest could be inferred. The chromatographic systems used in this thesis are described below.

2. Chromatographic Methods

2.1 High performance liquid chromatography

2.1.1 Introduction

High performance liquid chromatography (HPLC) is one of the most rapidly expanding techniques in analytical chemistry. It offers high speed, high resolution and reproducibility. It has been used for separation and analysis of a number of biologically important compounds (Grushka, 1974; Brown, 1973), and more recently successfully used in separation of peptide mixtures (Burgus and Rivier, 1976; Gruber et al. 1976; Krummen and Frei, 1977; Bennett et al. 1977; O'Hare and Nice, 1979).

2.1.2 Principle

Separation of peptides by the HPLC system is based on the powerful adsorption of peptides from aqueous solutions onto a hydrophobic supporting matrix (e.g. octadecasilyl silica (ODS)), and their subsequent elution with an aqueous solvent mixture containing increasing proportions of a relatively hydrophobic organic solvent (e.g. methanol, acetenitrile). The elution position of a given peptide is thought to depend primarily on the total number of hydrophobic residues and their

distribution within the molecule. Elution time is extremely sensitive to solvent composition; a small decrease in organic solvent concentration increases elution time considerably. The addition of an electrolyte (e.g. trifluoroacetic acid (TFA) or NaCl) minimizes ion exchange effects which would otherwise interfere with the separation.

2.1.3 Instrumentation

The system used is basically as described by Bennett et al. (1977). A prepacked partisil ODS analytical column 0.4 cm internal diameter x 25 cm length with a 10 µm pore size (Whatmann Limited) was coupled to a small steel guard column (0.4 cm internal diameter x 4 cm length), packed with dry Co:Pell ODS using pressure steel joints. The guard column was easy to replace and was coupled in front of the high resolution column as an additional safeguard against loss of resolution due to the binding of strongly hydrophobic materials which may be present in biological samples. The column was coupled to a Milton-Roy high pressure (1,000 psi) pump and a pressure gauge.

The outlet was channelled through a 2.5 cm path length continuous flow cell in a UV spectrophotometer (Cecil Instruments) as described by Bennett et al. (1973), and the UV absorption at 280 nm was recorded by an LKB pen recorder.

Fractions of eluate were collected in an LKB fraction collector, dried in a vacuum oven and separation was monitored by radioimmunoassay as described in Chapter 2.

2.1.4 Solvent gradient

Prior to loading, the column was equilibrated with starting solvent (20% methanol, 1% TFA). For loading the column, the lyophilized sample was dissolved in 20% methanol, 1% TFA (1-5 ml) and ∿ 250 µg bovine insulin was included in each sample as an internal marker (see below), and then pumped directly into the column. The column was run with a linear gradient of aqueous methanol containing 1% TFA. A gradient of 20-80% methanol was used. The total volume was 100 ml and the gradient was made up by connecting two identical flasks containing equal volumes (50 ml each) of starting solvent (20% methanol, 1% TFA) and final solvent (80% methanol, 1% TFA) with a glass siphon containing final solvent. The two flasks were placed on a hot plate to minimize the amount of dissolved air entering the system and the solvent from the first flask (20% methanol, 1% TFA) was stirred magnetically and pumped into the column.

The flow rate was 1.1 ml/minute which gave rise to a pressure between 500 psi and 1,000 psi in the column. The complete run took 1½ hours. After each run the column was washed for 30 minutes with 80% methanol and 1% TFA. Occasionally the column was washed with a small volume of formic acid which was effective in reducing the running pressure which had a tendency to rise gradually after prolonged use of the column.

2.1.5 Internal standard and calibration of the column

Internal standards are essential in all runs to compensate for errors made in the preparation of solution and other variables. An ideal internal marker should be pure, and elute near the peak of interest, but must not interfere with the detection of this peak.

Bovine insulin (250-500 µg, Ciba-Geigy) was found to be the ideal internal marker for calcitonin separation because:

1. It does not cross-react with the CT-M antisera used for monitoring separation (Fig. 24).

2. It gives a UV detectable peak which is only 1 ml earlier than the CT-M peak (Fig. 25).

This was obtained by checking the elution position of 100 µg CT-M, as monitored by UV, against the elution position of 3 other peptides: h-ACTH (1-39) 40 µg, Synacthen 1-24 40 µg and porcine glucagon 50 µg (Fig. 26).

Calibration of column with CT-M in nanogram concentration: A fresh solution of CT-M was made just before application on the column (20 µg/2 ml 1% TFA in water) and only 2 µl + 1 ml aliquot of saline containing 250 µg bovine insulin was pumped as described above - then the fractions around the insulin peak were assayed for CT-M immunoreactivity (Fig. 27).

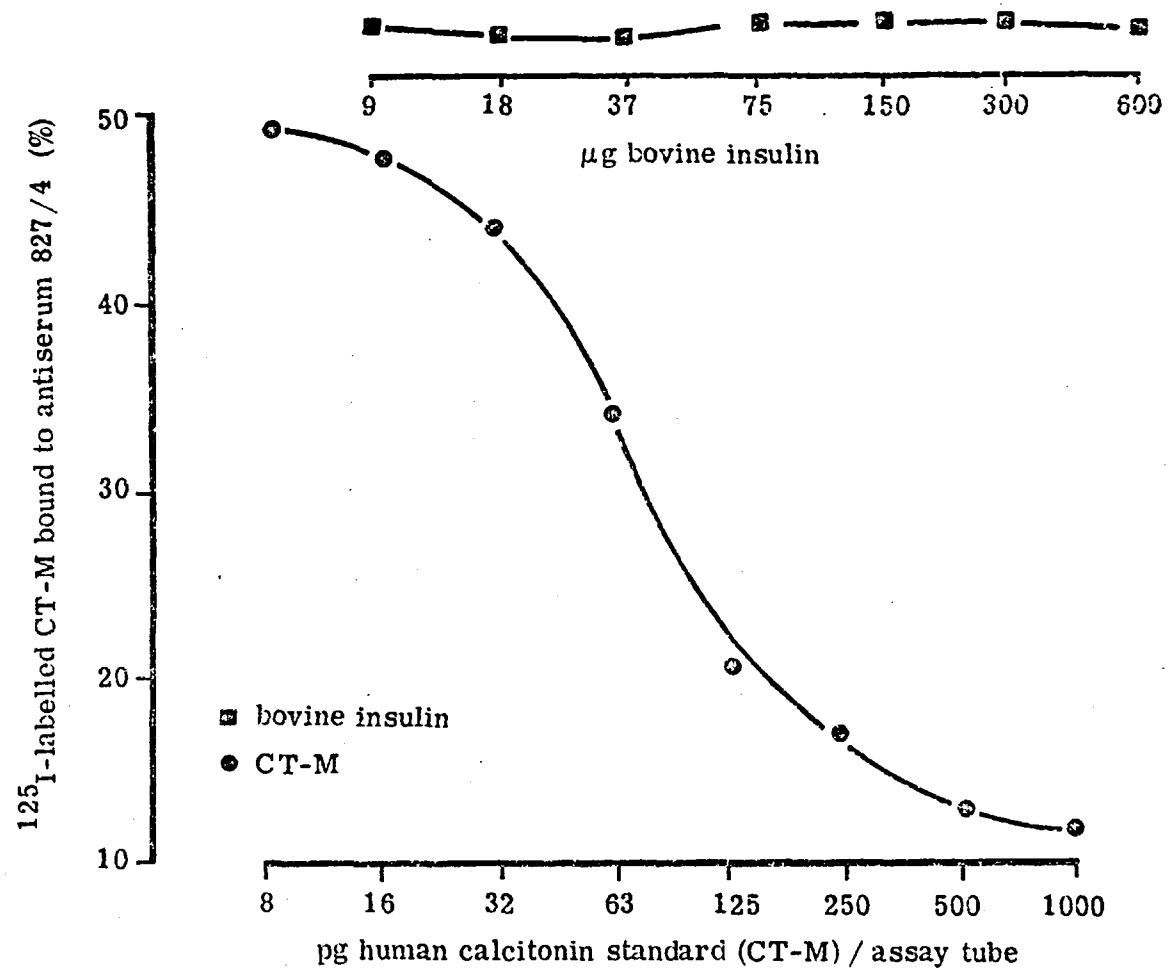


Fig. 24 *Absence of cross-reaction of bovine insulin, at various concentrations, with antiserum 827/4. The maximum concentration tested was 600 µg/tube.*

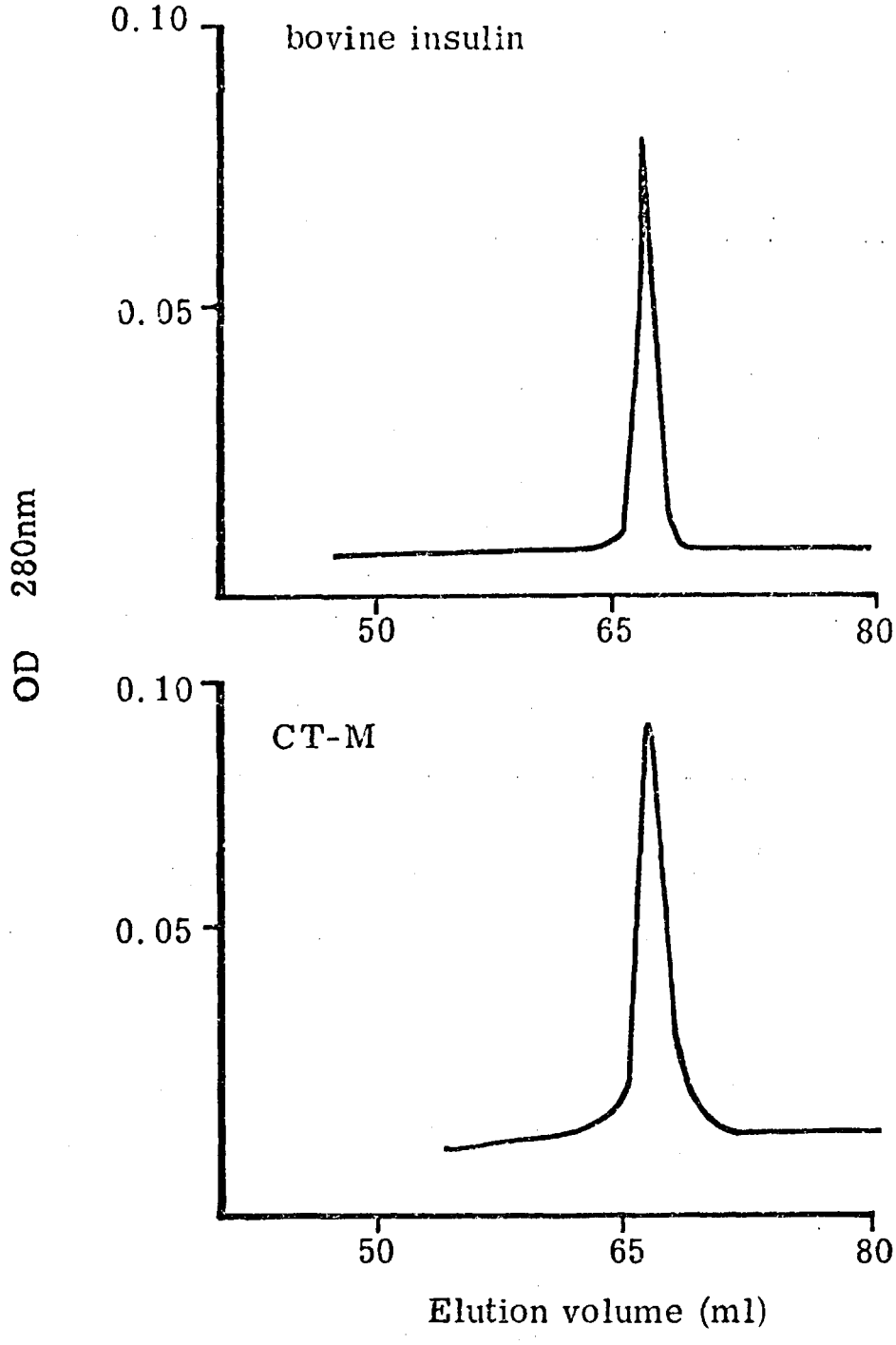


Fig. 25 High performance liquid chromatograms of bovine insulin (500 µg) and CT-M (100 µg). The elution position of both peptides was monitored by a UV spectrophotometer at 280 nm.

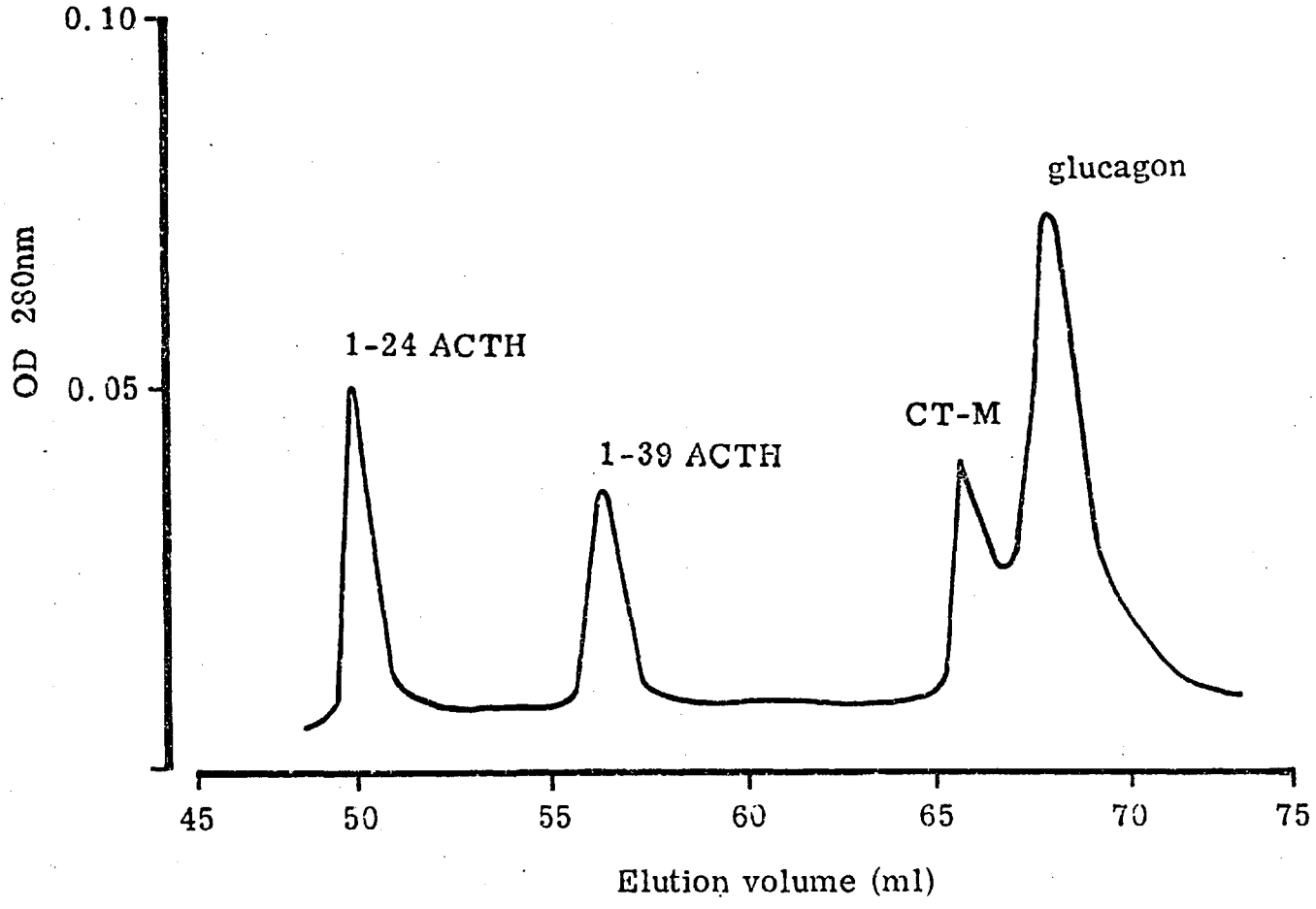


Fig. 26 High performance liquid chromatogram of a mixture of peptides. The elution position of various peptides monitored by a UV spectrophotometer: human ACTH (1-39) 40 µg, bovine insulin 250 µg, porcine glucagon 50 µg, synacthen (1-24) 50 µg.

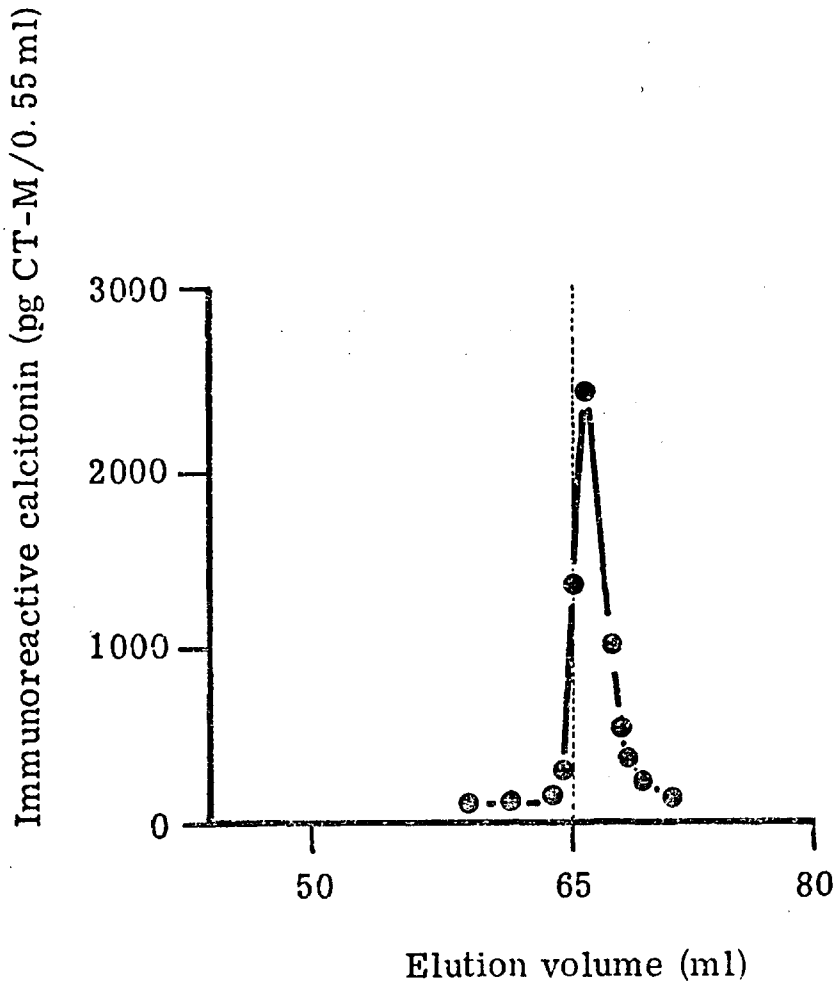


Fig. 27 High performance liquid chromatogram of a mixture of CT-M (10 ng) and bovine insulin (250 µg). The elution position of insulin, monitored by a UV spectrophotometer at 280 nm, is indicated by the dotted line. The elution position of CT-M, monitored by radioimmunoassay, is shown as solid circles.

Because of the known memory effect of reverse phase chromatography, before any sample was applied (all samples were in nanogram concentrations) the column was checked for any residual calcitonin which might give rise to a "ghost peak". This was done by collecting fractions eluted using a linear solvent gradient as before but without any samples being applied ("blank run"), and the calcitonin immunoreactivity was checked in the fractions near the insulin peak. Only when the blank run showed no calcitonin immunoreactivity were the samples applied.

Previous studies of calcitonin analogues on HPLC have shown that analogues differing by one or two amino acids can be distinguished by HPLC alone (Rittel, personal communication). These analogues are the same as those used for characterization of the antisera. Analogue Ala^{29},Val^{31} is an exception in that it is indistinguishable from CT-M on HPLC.

2.2 Gel filtration

Details of gel filtration media and elution buffers and dimensions of columns are described in Chapters 3 and 4.

CHAPTER 3

NATURE OF NORMAL HUMAN CALCITONIN IN THE CIRCULATION

IS THE NORMAL HUMAN CALCITONIN IDENTICAL WITH CT-M OF MEDULLARY THYROID CARCINOMA?

1. Introduction

The sequence of human calcitonin or CT-M was obtained from purified extracts of a medullary thyroid carcinoma (Neher et al. 1968c). Although the presence of the hypocalcaemic factor in normal human thyroid was reported by Aliapoulios, Voelkel and Munson (1966); and Kaplan et al. (1968), isolation of the peptide was not possible because of the low yield of the hypocalcaemic factor from human thyroid compared to other species (Haymovits and Rosen, 1967; Barrett and Bell, 1969). With the development of the radioimmunoassay for human calcitonin, based on antisera against synthetic CT-M or extracts of medullary thyroid carcinoma, many laboratories reported low levels of immunoreactive calcitonin in the plasma of normal subjects (Tashjian et al. 1974; Silva et al. 1974c;Parthemore et al. 1975; and Hillyard et al. 1977). Although the cross-reactivity of normal human plasma with antisera against CT-M suggests similarity in structure, this is not sufficient to prove identity. Thus, the strategy for identification of the normal hormone in plasma required the extraction of peptide from a large volume of plasma to obtain sufficient material for full, refined immunological and chromatographic characterization.

2. Materials and Methods

2.1 Normal plasma

Calcitonin secretion in normal subjects exhibits a diurnal variation with a peak at noon (Hillyard et al. 1977).

Therefore, midday blood samples (100-200 ml) were obtained from 20 normal volunteers (12 males, 8 females) aged 25-35 years and placed in heparinized chilled tubes. Plasma was separated by centrifugation within 30 minutes of collection, at 4°C for 10-20 minutes, at 2,000 r.p.m. Ten aliquots of approximately 100 ml of plasma were lyophilized and kept at -20°C until extraction.

The plan for immunochemical characterization is summarized in Fig. 28.

2.2 Extraction procedure

Each aliquot of lyophilized plasma was defatted with ether (50 ml containing 0.1% mercaptoethanol) and then extracted with a mixture of 1-butanol/acetic acid/water (75 : 7.5 : 21, by volume) as described by Byfield et al. (1976). The extract was evaporated *in vacuo*, the dried residue suspended in 0.1 M formic acid (10 ml), mixed thoroughly and centrifuged at 4°C for 30 minutes at 5,000 r.p.m. to get rid of any insoluble particles. The clear supernatant was lyophilized, dissolved in 0.1 M formic acid (4 ml) and prior to gel filtration an aliquot (100 µl) was set aside for radioimmunoassay of calcitonin using antiserum 827/4 as described in Chapter 2. As a control of the extraction method, CT-M (40 ng) was added to calcitonin free plasma (50 ml) and extracted in the same way.

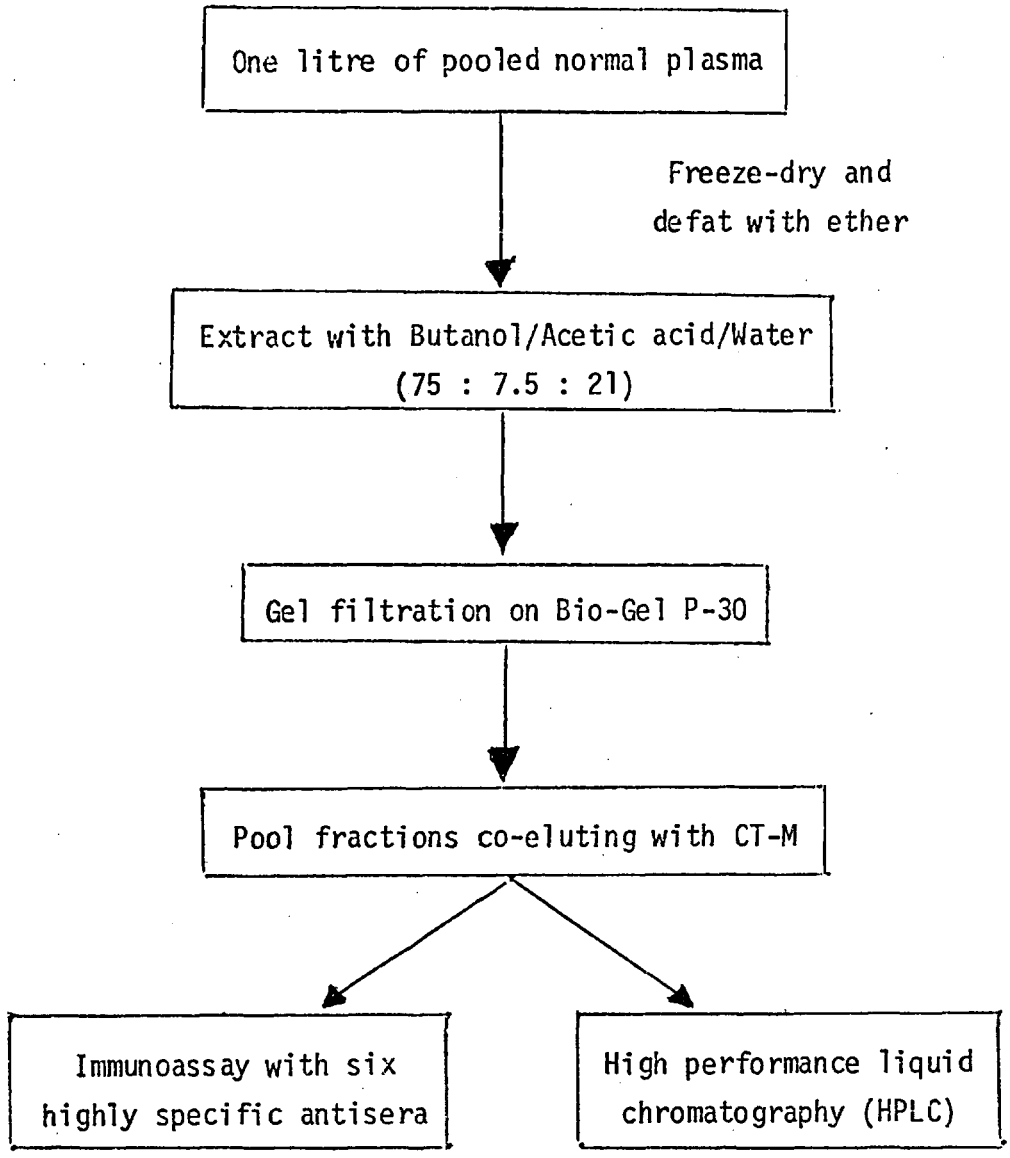


Fig. 28 Plan for immunochemical characterization of normal human calcitonin.

2.3 Gel filtration

To determine the molecular size(s) of the immunoreactive calcitonin in normal plasma, the extract from one of the ten aliquots was applied to a column (130 x 1 cm) of Bio-Gel P-30 (100-200 mesh) which was made up and eluted with 0.1 M formic acid containing 10% isopropanol (v/v) (pH 2.2). The column was calibrated with Blue Dextran 200 (2 mg), NaCl (500 mg) and ^{125}I-CT-M (100,000 c.p.m.). Flow rate was 9 ml/h and 1 ml fractions were collected, lyophilized and dissolved in assay buffer and calcitonin immunoreactivity was determined by using antiserum 827/4. Only the peak which co-eluted with ^{125}I-CT-M was chosen for further characterization. From extracts of the other nine aliquots (900 ml), the fractions co-eluting with CT-M (elution volume 102-114 ml) were pooled without prior radioimmunoassay and this sample was called "normal calcitonin". It was divided into 3 portions and lyophilized, to be used for immunochemical and chromatographic studies.

2.4 Radioimmunoassay

One sample of the "normal calcitonin" (equivalent to 16 ng CT-M, using antiserum 827/4) was dissolved in 2.0 ml 0.05 M phosphate buffer and aliquots were assayed in duplicate and in six dilutions using the six different region-specific antisera as described in Chapter 2. This was done to compare the displacement curves produced by several dilutions of this solution with that produced by CT-M and also to see if the

apparent concentrations of this solution with reference to the same standard would differ with the various antisera.

2.5 High performance liquid chromatography

The system is as described in Chapter 2. Three samples were chromatographed:

- "normal calcitonin" (equivalent to 8 ng CT-M using antiserum 827/4).
- extracted CT-M (∿ 10 ng).
- CT-M sulphoxide (∿ 14 ng).

3. Results

3.1 Extraction

Total calcitonin immunoreactivity was found to be in the range of 100-110 pg/ml in the extract(s) of normal plasma.

3.2 Gel filtration

The extractable immunoreactive calcitonin of normal human plasma was found to be heterogeneous. One peak had the same elution position as synthetic CT-M, three peaks had an apparently higher molecular size than CT-M, while two small peaks eluted later than CT-M (Fig. 29).

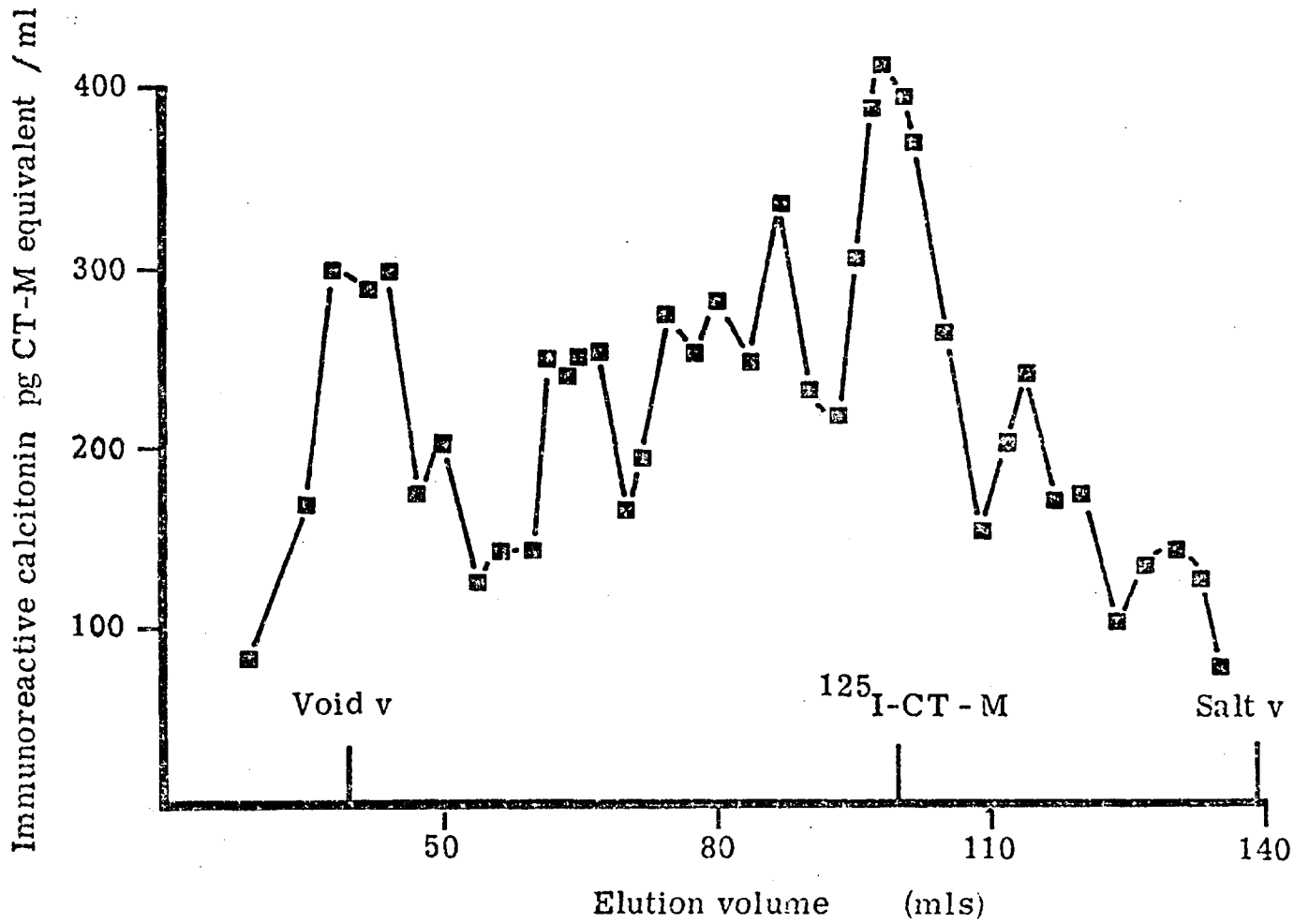


Fig. 29 Gel filtration (Biogel P-30) of an extract of pooled normal human plasma.

3.3 Radioinmunoassay

The normal calcitonin produced parallel displacement curves to that of CT-M using all six antisera (Figs. 30-35). However, the apparent concentrations using the various antisera were different (Table 6). Furthermore, similar variations in the apparent concentration were found in the case of the CT-M extraction control.

Table 6

Antiserum	Concentrations expressed as ng CT-M equivalent/ml
827/4	8.0
336/6	7.8
12/3	9.8
10/2	12.0
7/3	13.0
5/1	14.0

The apparent concentrations of "normal calcitonin" expressed as ng CT-M equivalent/ml when using the six different antisera.

3.4 High performance liquid chromatography

The elution position of "normal calcitonin" and of extracted CT-M were 6 ml earlier than the elution position of calcitonin-M. This corresponds to the elution position of CT-M sulphoxide (Figs. 36-37).

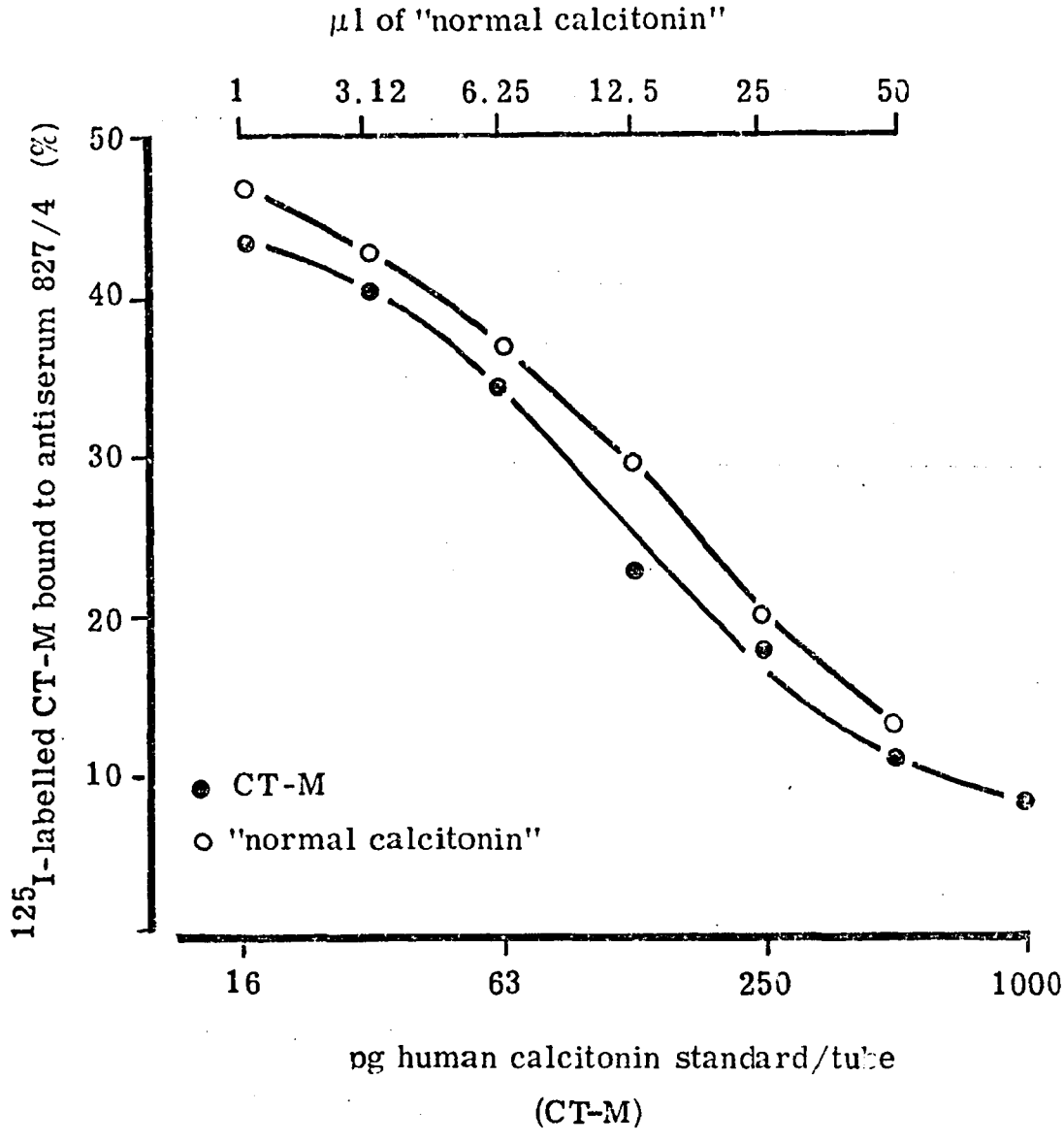


Fig. 30 Comparison of the displacement of ^{125}I-labelled calcitonin from antiserum 827/4 by normal plasma extract (after gel filtration) (○) and synthetic CT-M (●).

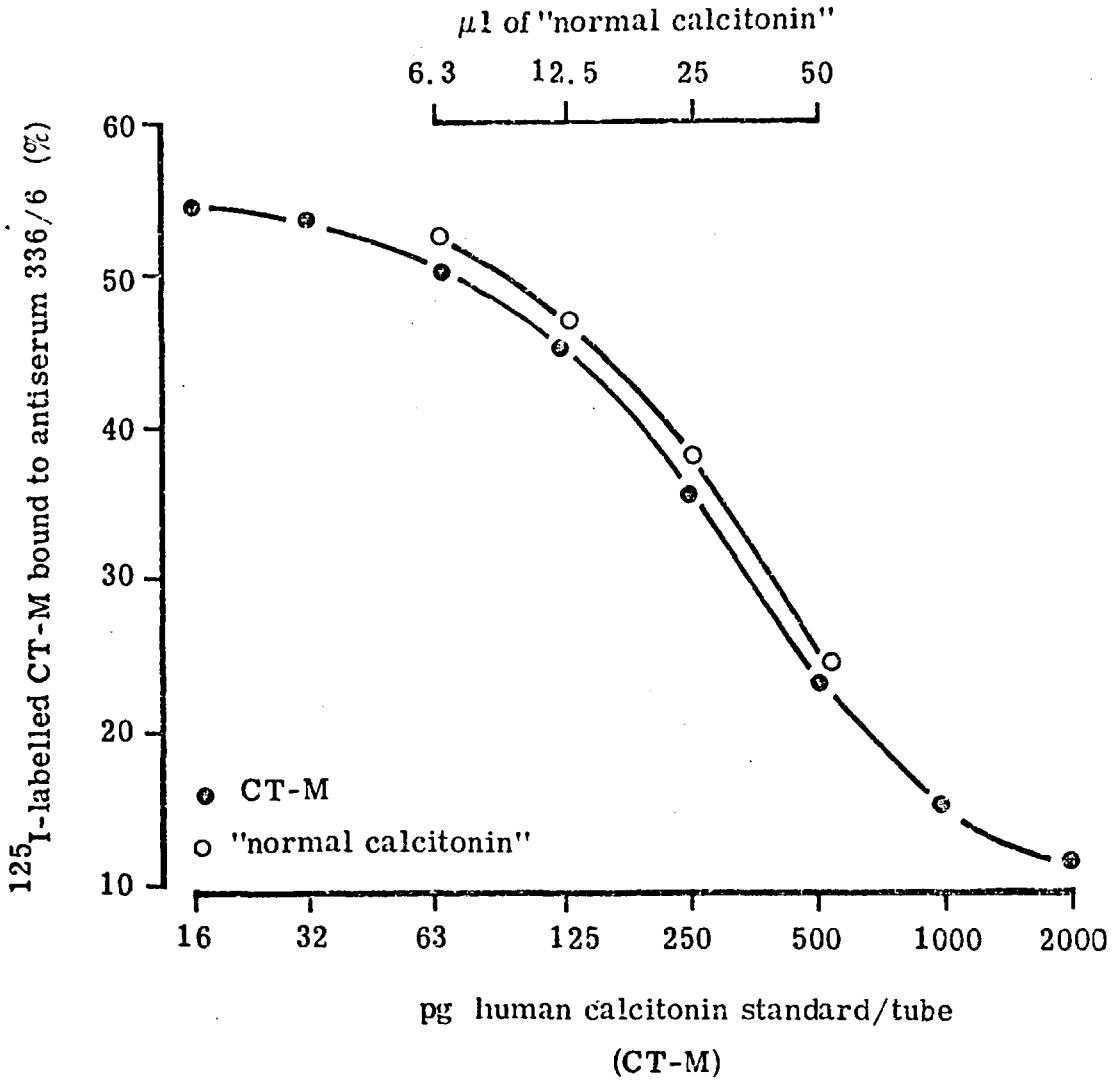


Fig. 31 Comparison of the displacement of ^{125}I-labelled calcitonin from antiserum 336/6 by normal plasma extract (O) and CT-M (●).

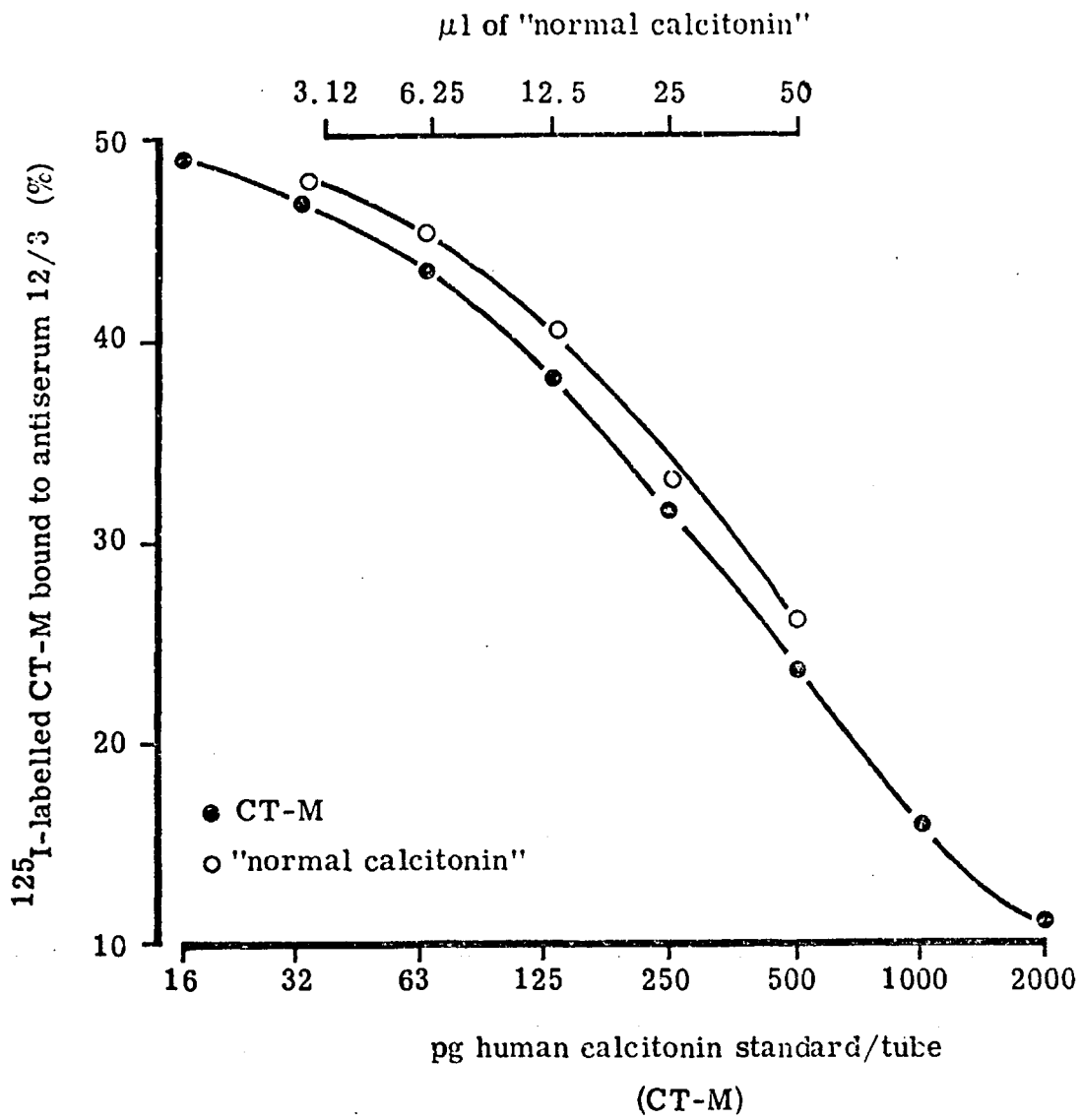


Fig. 32 Comparison of the displacement of ^{125}I-labelled calcitonin from antiserum 12/3 by normal plasma extract (O) and CT-M (●).

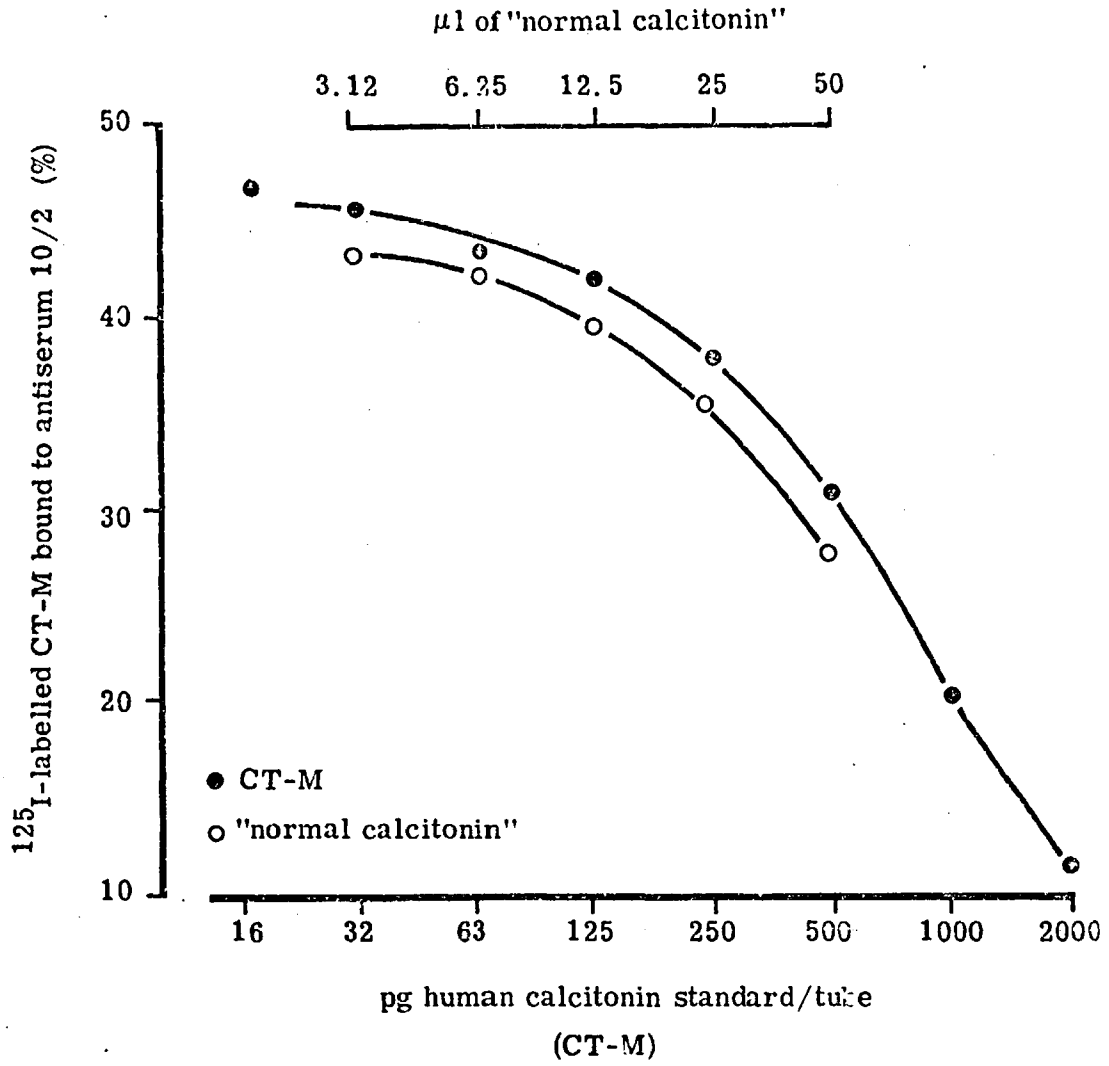


Fig. 33 Comparison of the displacement of ^{125}I-labelled calcitonin from antiserum 10/2 by normal plasma extract (○) and CT-M (●).

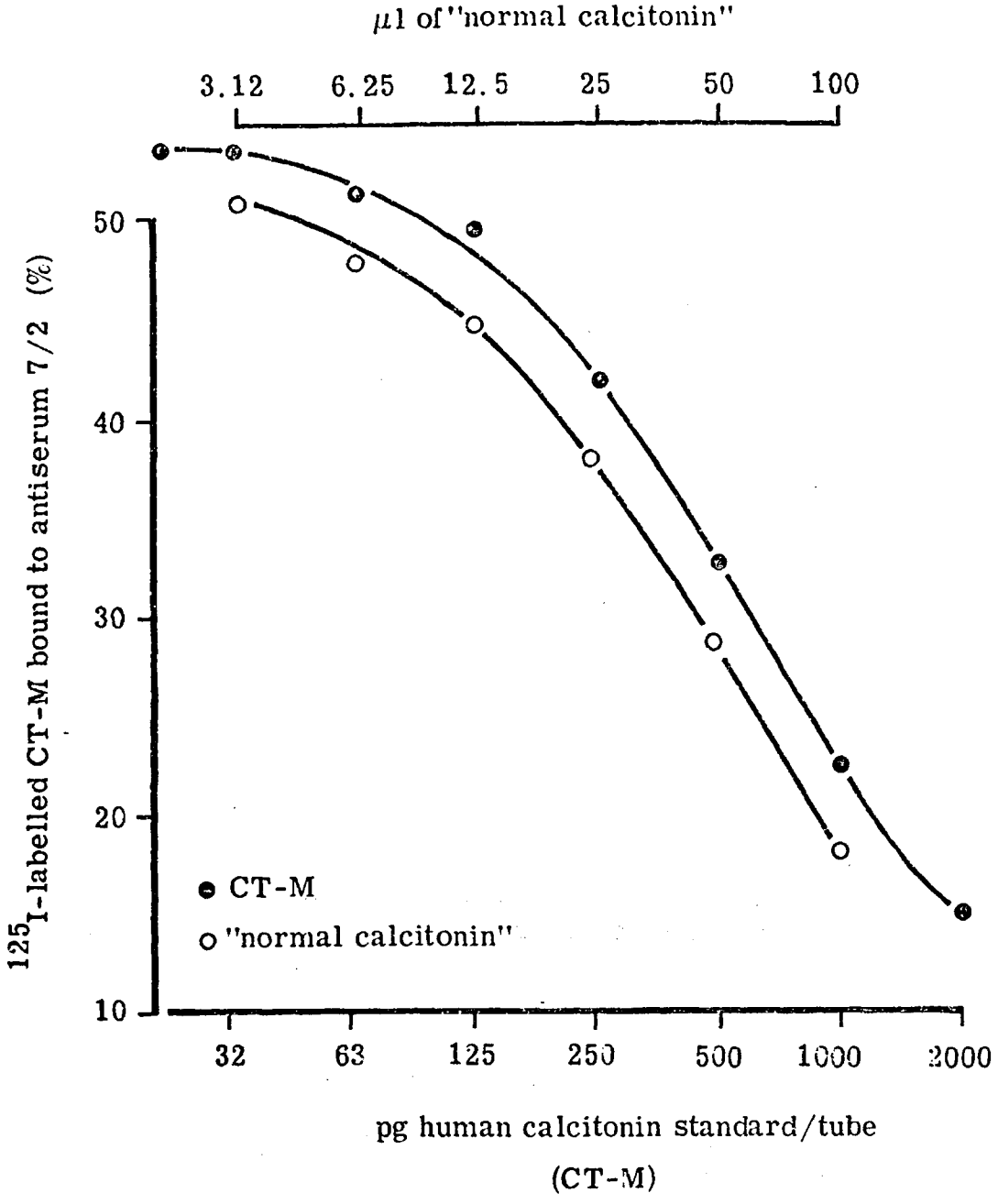


Fig. 34 Comparison of the displacement of ^{125}I-labelled calcitonin from antiserum 7/2 by normal plasma extract (○) and CT-M (●).

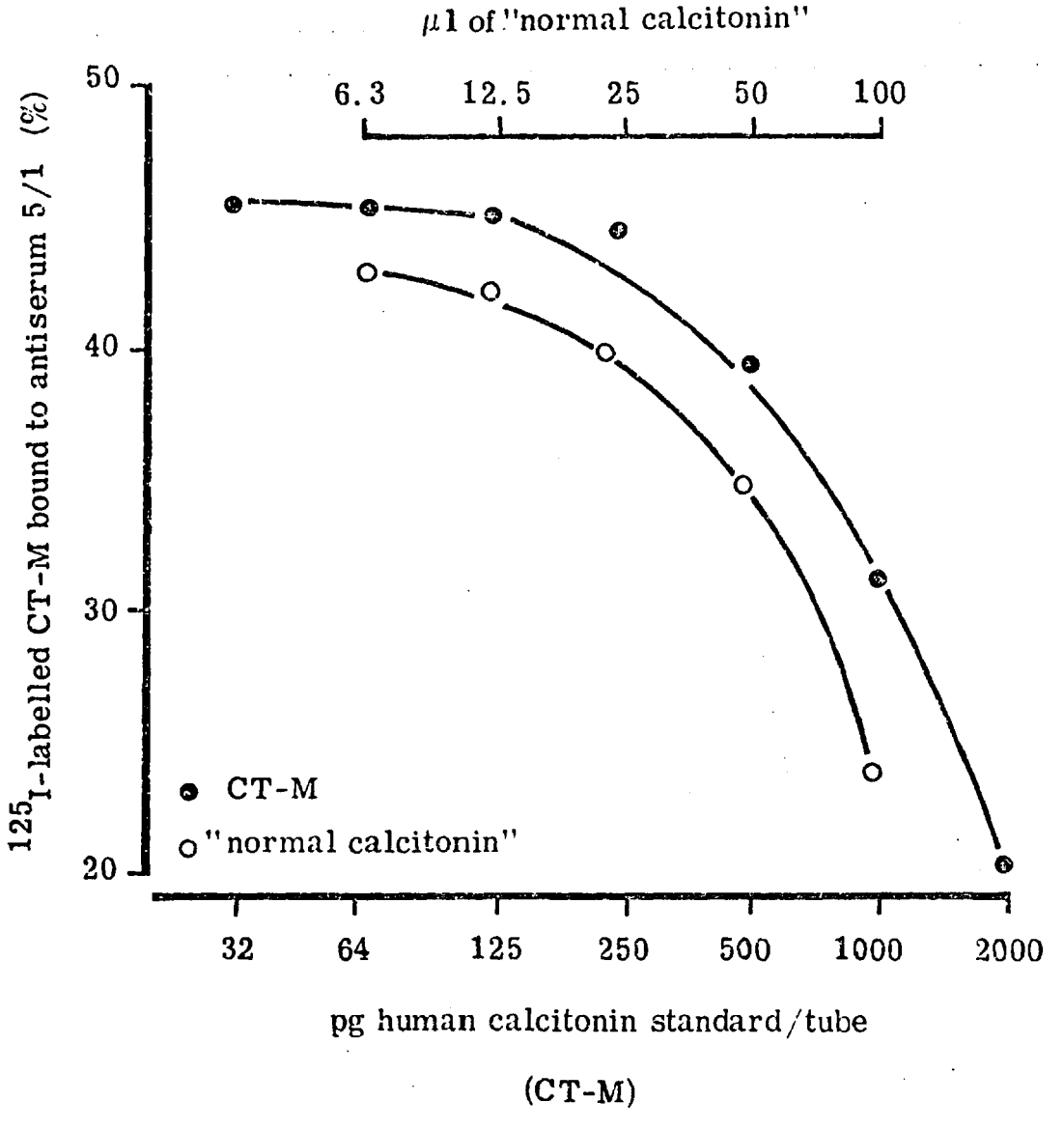


Fig. 35 Comparison of the displacement of ^{125}I-labelled calcitonin from antiserum 5/1 by normal plasma extract (○) and CT-M (●).

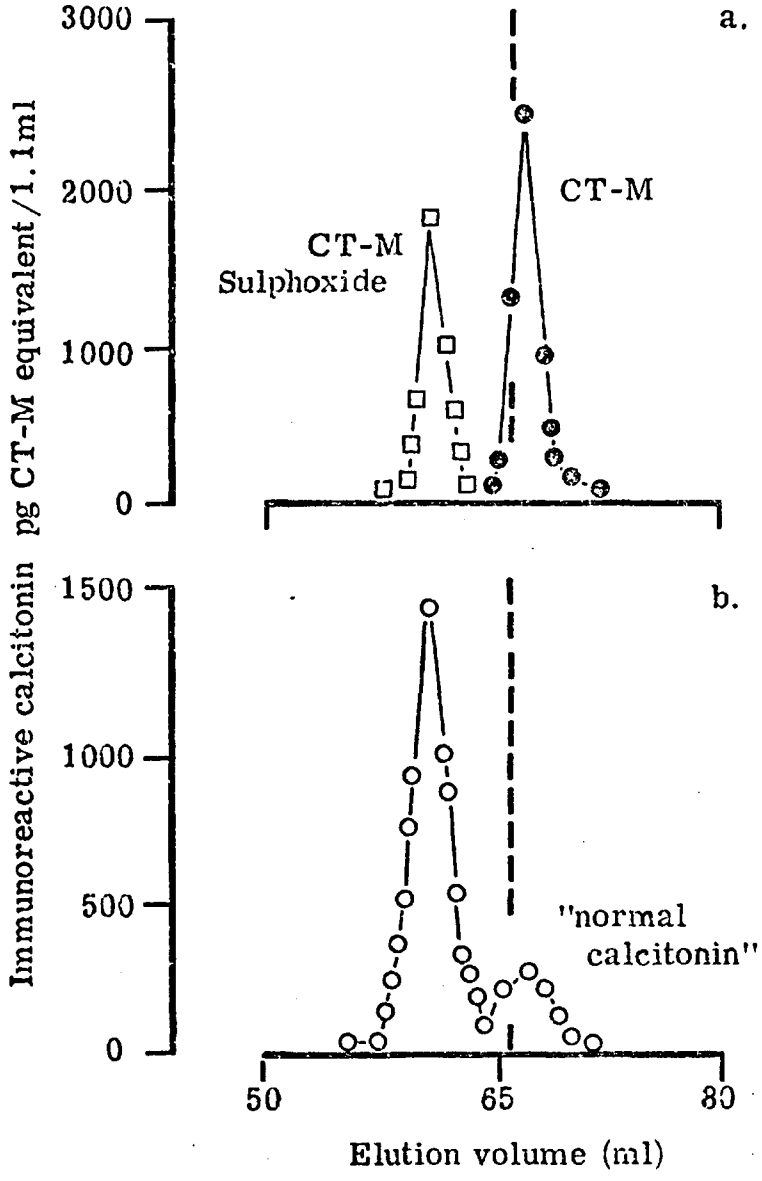


Fig. 36 High performance liquid chromatograms of CT-M and CT-M sulphoxide (a); and normal calcitonin (b). The dotted line indicates the elution position of bovine insulin which is used as an internal marker. Normal calcitonin eluted at the same position as CT-M sulphoxide.

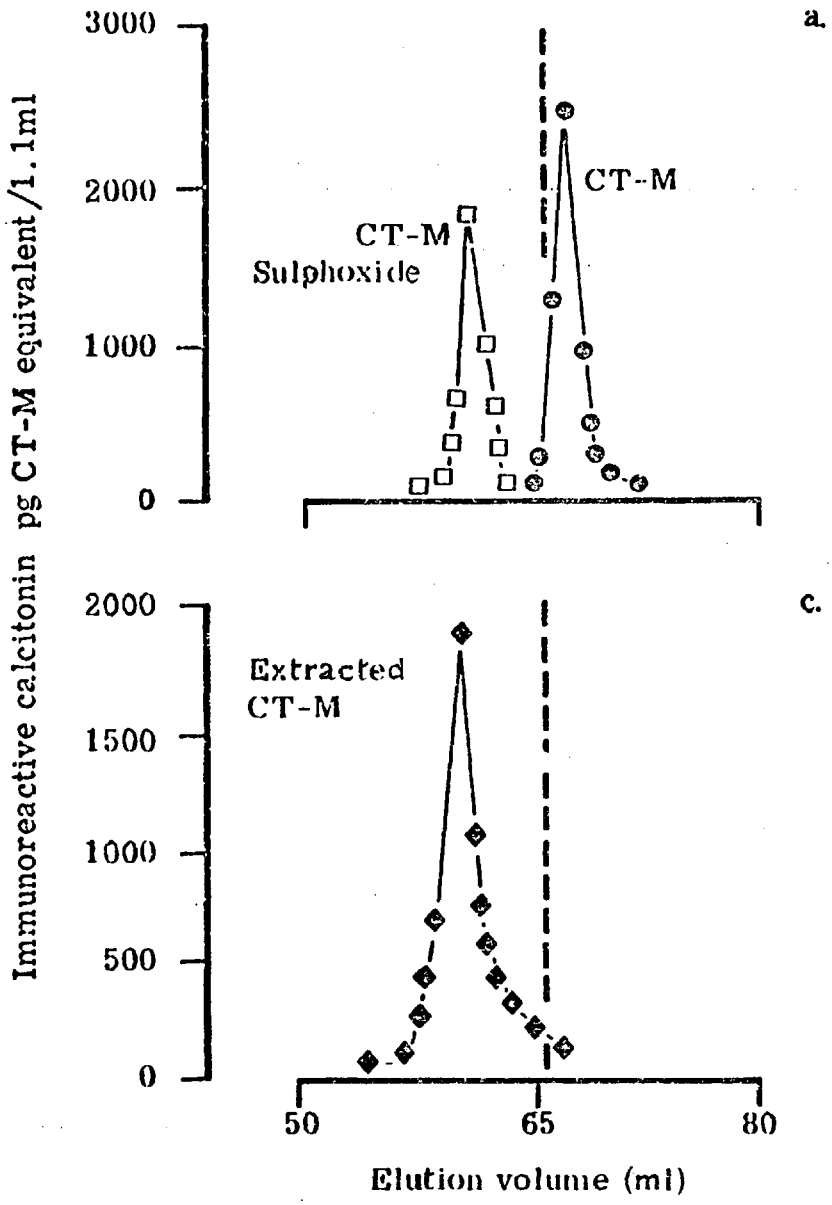


Fig. 37 High performance liquid chromatograms of CT-M and CT-M sulphoxide (a); and extracted CT-M control (c). The dotted line indicates the elution position of the internal marker (bovine insulin). Extracted CT-M control eluted at the same position as CT-M sulphoxide.

4. Discussion

These results clearly show that the immunoreactive calcitonin peak which co-eluted with CT-M on gel filtration of the normal plasma extract is chemically identical to CT-M sulphoxide as shown by the HPLC profile. Because CT-M contains methionine (at position 8) (Fig. 38), auto-oxidation to CT-M sulphoxide is inevitable during extraction or gel filtration. This was well demonstrated by the CT-M extraction control experiment, in which the extracted CT-M eluted as a single peak at the position of the less hydrophobic CT-M sulphoxide. This also rules out the suggestion that the conversion would have taken place in vivo, although it has been reported to occur in a partial sequence of adrenocorticotrophin (Hudson, 1978).

The discrepancies between the apparent concentration of "normal calcitonin" by the various antisera with reference to the same standard may be explained by differences in the affinity of the antisera to CT-M sulphoxide. Antisera 827/4, 336/6 and 12/3, which gave apparently low concentrations, have lower affinities to CT-M sulphoxide than CT-M, while antisera 10/2, 7/3 and 5/1, which gave apparently higher concentrations, have higher affinities to CT-M sulphoxide than to CT-M (see Chapter 2, Fig. 23). This also corroborates the results of HPLC showing that the immunoreactivity measured as "normal calcitonin" was due to the sulphoxide.

This study also confirms the fact that parallelism of the displacement curve of unknown samples to that of the

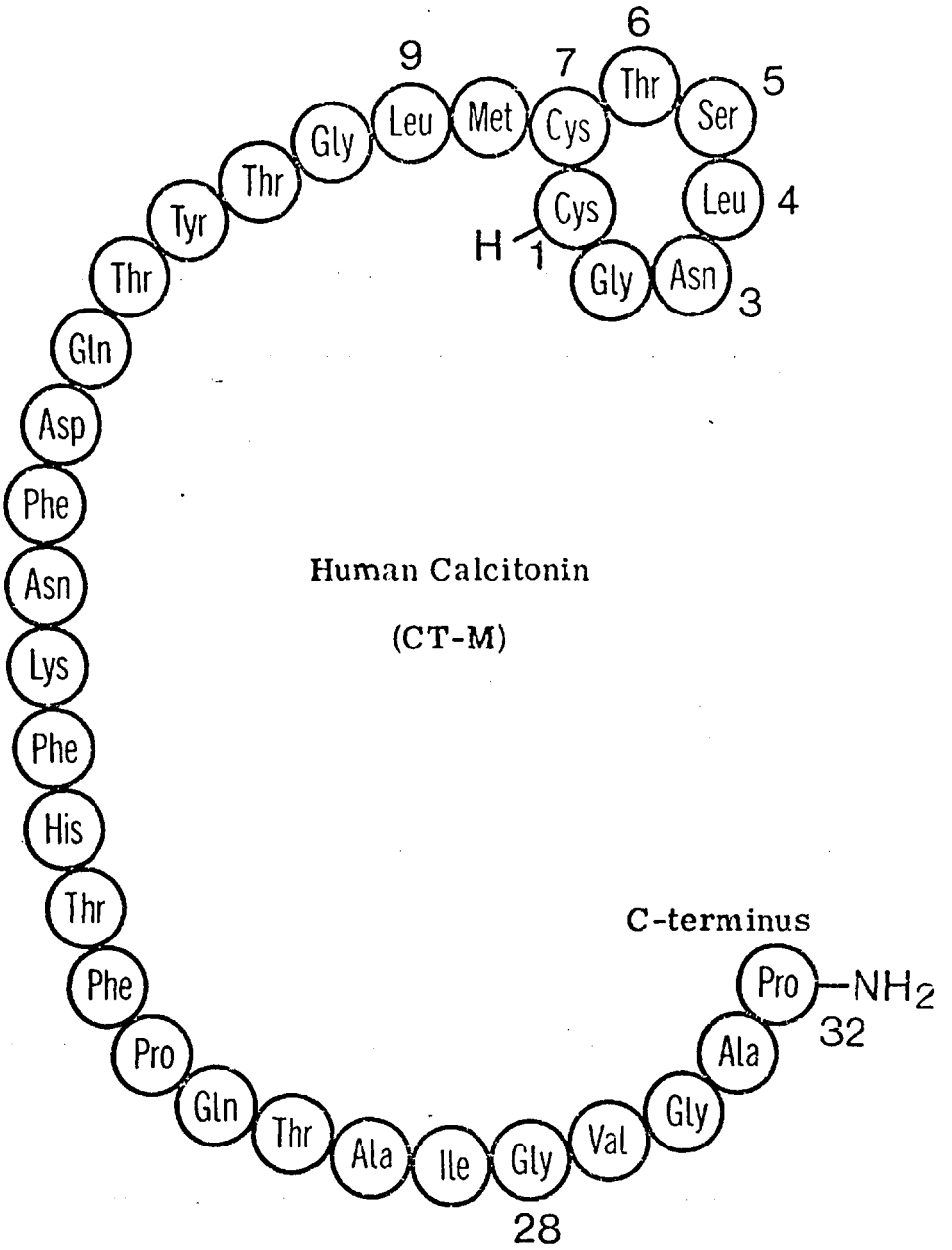


Fig. 38 Amino acid sequence of human calcitonin monomer. Notice the presence of a methionine residue (at position 8).

standard preparation is not by itself sufficient to prove chemical identity.

The other peaks of immunoreactive calcitonin in normal plasma have not been characterized in this study. However, heterogeneity in size as determined immunochemically is not uncommon in peptide hormones (Berson and Yalow, 1968) and has been observed with calcitonin in the plasma of patients with medullary thyroid carcinomas (Singer et al. 1974; Sizemore et al. 1975; and Deftos et al. 1975). This heterogeneity may explain the discrepancies in normal calcitonin levels reported by various groups (Tashjian et al. 1974; Parthemore et al. 1975; Hillyard et al. 1977; Moukhtar et al. 1973; Samaan et al. 1973; Silva et al. 1974; and Heynen and Franchimont, 1974).

Although sequence analysis is the ultimate proof of identity, it is reasonable to believe from the immunological and chromatographic results that CT-M is present in normal human plasma. Furthermore, the identity of amino acid sequences of "normal" and "tumour" derived rat calcitonin (Raulais et al. 1976; and Byfield et al. 1976) is in good agreement with this conclusion.

The demonstration of the presence of CT-M in normal plasma, apart from its theoretical interest, has a practical bearing: it explains the absence of antibody formation to synthetic human calcitonin in patients with Paget's disease of bone, even after long-term treatment (Evans et al. 1977; Ziegler et al. 1977) in contrast to those treated with salmon or porcine calcitonin (Singer et al. 1972; Haddad and Caldwell, 1972).

CHAPTER 4

IS CALCITONIN A NEUROPEPTIDE?

IDENTIFICATION OF A HUMAN CALCITONIN-LIKE MOLECULE IN THE NERVOUS SYSTEMS OF PROTOCHORDATES AND A CYCLOSTOME *MYXINE*

1. Introduction

Calcitonin is produced by C-cells of the mammalian thyroids and by cells in the ultimobranchial bodies of submammalian jawed vertebrates (Gnathostomata). An early attempt by Copp et al. (1970) to prepare an extract from the branchial region of the hagfish (Palistrotremata stoutii), did not yield any material with hypocalcaemic activity in the rat bioassay. This and the fact that cyclostomes have no ultimobranchial bodies (Watzka, 1933), led them to conclude that the phylogenic development of calcitonin and the ultimobranchial body were coincidental.

Recently Fritsch, Van Noorden and Pearse (1979) have reported the localization, by immunocytochemical techniques, of human calcitonin-like immunoreactivity in the neural ganglia of the urochordate, Ciona intestinalis. However, this method alone is not sufficient to establish identity between the immunocytochemically demonstrated material and the authentic peptide, as cross-reaction may be caused by unrelated molecules sharing a common antigenic determinant. In view of the marked sequence difference between the various species of calcitonin, this study was undertaken to characterize the material in extracts of Ciona's neural complexes by refined immunochemical methods: immunologically by using several well characterized antisera and chromatographically by gel filtration and high performance liquid chromatography. In addition, the study was extended to examine extracts of the nervous tissue of other protochordates and of hagfish brain.

2. Materials

2.1 Ciona: the following batches of animals were studied:-

- 25 animals were taken in November, 1978 by a diver in the Western Baltic at depths of up to 20 metres. The animals were 3-5 cm long and were aggregate in colonies, which were either attached to brown algae or to secondary sediment. They were kept in aerated sea water (16% O_2) in glass aquaria at 5°C for 4-5 days before dissection of the neural complexes.

- 21 animals were obtained in April, 1979 from the Norwegian trench near Sweden, these were 8-10 cm long and were dredged from a depth of 200-300 metres. They were treated in the same way as above. The lyophilized neural complexes from the above two batches (Western Baltic and the Norwegian trench) were kindly given by Dr. H. Fritsch (Anatomisches Institut der Universität Kiel).

- 10 animals were collected at Plymouth in April, 1979, these were 2-7 cm long and were sampled after some weeks living in continuous darkness in the aquarium reservoir at the Marine Biological Association of the U.K., Citadel Hill, Plymouth. The neural complexes were dissected out and snap frozen in liquid nitrogen.

- 20 animals were collected at Plymouth in August, 1979. The neural complexes were dissected into their constituent parts: neural ganglia (nervous tissue) and neural

glands (glandular tissue). The latter were used as a non-neural tissue control. The neural ganglia and neural glands were snap frozen separately.

2.2 Styela, Ascidiella, Amphioxus

Specimens of Styela (6), Ascidiella (5) and Branchiostoma (amphioxus) (7) were collected at Plymouth in April, 1979. In the case of amphioxus the head end was excised and immediately dropped in liquid nitrogen.

2.3 Myxine

Myxine (the hagfish) (5) were kindly donated by Dr. G. Howard (Department of Agriculture and Fisheries for Scotland, Aberdeen), they were collected at Blyth on the Northumberland coast in May, 1979 and were kept in sea water for 24 hours and the complete head removed with a sharp knife and the brain removed and frozen in liquid nitrogen.

The external appearance of the animals used in this study is shown in Fig. 39.

3. Methods

3.1 Extraction

One extract was obtained from the pooled nervous tissues of each group of the above species. Extracts were

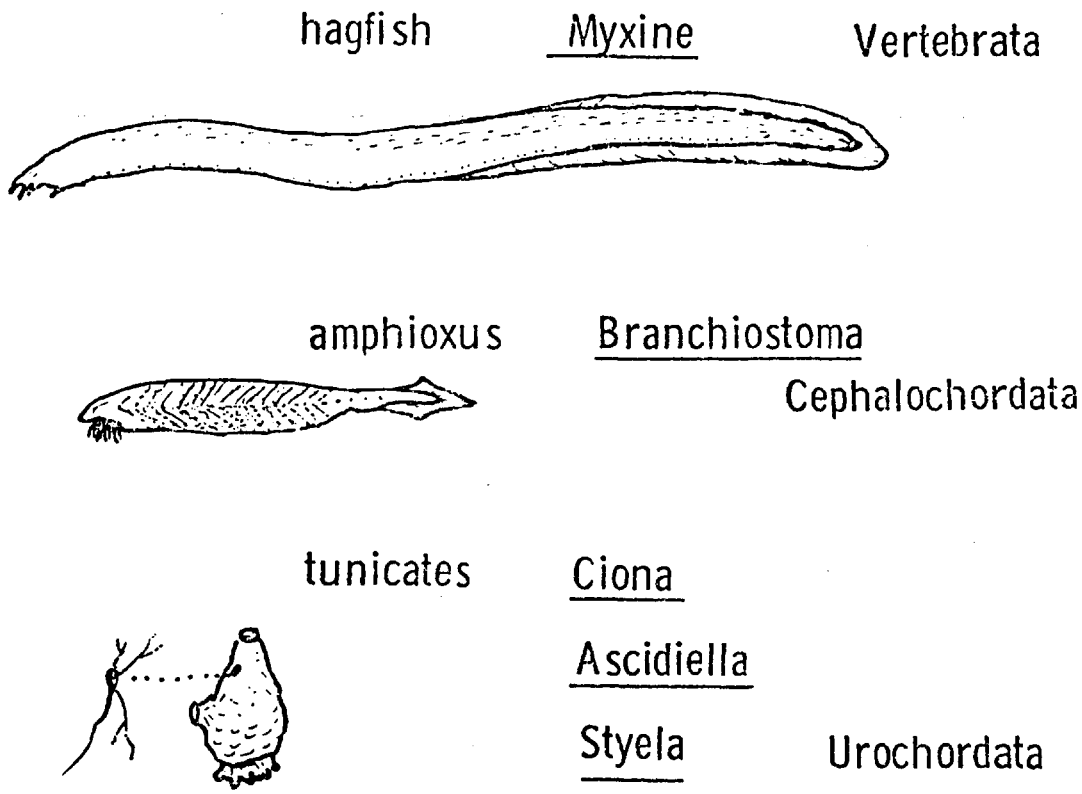


Fig. 39 External appearance of Myxine, amphioxus and Ciona.

prepared as described by Kelly et al. (1978). The finely divided tissue was homogenized in a mixture of 5% formic acid (v/v), 15% trifluoroacetic acid (TFA) (v/v), 1% sodium chloride (w/v) and 1 M hydrochloric acid using 10 volumes/g wet weight or 100 mg dry weight, followed by centrifugation at 5,000 r.p.m. for 10 minutes at $4^{o}C$. The clear supernatants were applied twice to small bed volume columns of octadecsilyl-treated silica (Porasil, Water Associates, England) (1 ml bed volume/2 g of wet weight tissue). The adsorbed peptides were washed twice with 1% TFA (twice the bed volume) and subsequently eluted with 80% methanol, 1% TFA, as described by Bennett et al. (1977). The eluates were vacuum dried and suspended into 2 or 3 ml 0.05 M phosphate buffer, prior to radioimmunoassay.

3.2 Radioimmunoassay

3.2.1 Human calcitonin assay

Aliquots of each extract were first assayed using antiserum 827/4, whose detection limit is 4 pg/tube and it was planned that only samples with appropriate levels would be assayed using antisera 336/6, 12/2, 10/2, 5/1 and 7/2 as described in Chapter 2.

3.2.2 Salmon calcitonin assay

Aliquots of each extract were also assayed in a salmon calcitonin assay system as described by Deftos et al. (1974).

The antiserum used was kindly supplied by Dr. L. Deftos (University of California, La Jolla, U.S.A.) and used in a final dilution of 1/9,000 in a disequilibrium system.

3.3 Gel filtration

Sephadex G-50 superfine (60 x 1 cm) column was made up and eluted with 0.1 M formic acid and 10% isopropanol (v/v). The column was calibrated using Dextran blue, sodium chloride and 5 ng synthetic human calcitonin. The flow rate was 6 ml/h and fractions were collected every 5 minutes. Gel filtration was carried out only on an extract of neural complexes from Ciona (Western Baltic).

3.4 High performance liquid chromatography

HPLC was carried out as in Chapter 2 except that the flow rate was 1 ml/minute instead of 1.1 ml/minute on extracts from the nervous tissues of Ciona, Ascidiella and hagfish.

4. Results

4.1 Radioimmunoassay

Human calcitonin-like immunoreactivity was found in all the extracts studied. The concentrations expressed in terms of CT-M standard using antiserum 827/4 are listed in Table 7.

Nervous tissue extracts of amphioxus, Ciona, Styela, Ascidiella and Myxine gave displacement curves parallel to that

Table 7

Concentrations of human calcitonin-like immunoreactivity in extracts of nervous tissue from the various animals expressed as pg CT-M standard/neural complex, ganglion, or brain when using antiserum 827/4. An extract was made from pooled neural tissues of each species and assayed in duplicate in 6-10 dilutions.

Animals	No. of animals	Concentrations pg CT-M equivalent/ brain, neural complex or neural ganglion
Myxine (brain)	5	1,300
Amphioxus (brain)	7	160
Ascidiella (neural complex)	5	320
Styela (neural complex)	6	33
Ciona neural complex		
(April/Plymouth)	10	22
(April/Norwegian trench)	21	630
(November/W. Baltic)	25	50,000
Ciona neural ganglion		
(Plymouth/August)	20	125
Ciona neural gland		
(Plymouth/August)	20	< 4

of the reaction of synthetic human calcitonin using antiserum 827/4 (Fig. 40). There was no cross-reaction with salmon calcitonin antiserum (Fig. 41).

There was one striking finding: the three samples of Ciona neural complex studied contained a widely different range of concentrations. The sample containing the least amount came from a laboratory stock living in an aquarium reservoir in continuous darkness. Extract of the neural complexes from Ciona obtained in November from the Western Baltic contained the highest concentration of CT-M-like immunoreactivity. This extract gave displacement curves parallel to that of synthetic CT-M using five other antisera (Figs. 42, 43). However, the apparent concentrations of CT-M-like immunoreactivity were not the same using all the antisera. The concentrations expressed as ng CT-M equivalent per one neural complex were as follows: 50, 40, 41, 20, 25, 34 when using antisera 827/4, 336/6, 12/2, 7/3, 10/2 and 5/1 respectively. The concentrations of immunoreactive calcitonin in other species were not sufficient to carry out further immunological characterization. In the control experiment no immunoreactivity was found in the extract of pooled neural glands of Ciona; while the extract from the neural ganglia alone contained calcitonin immunoreactivity equivalent to 125 pg CT-M per neural ganglion.

4.2 Gel filtration

The human calcitonin-like immunoreactivity of the

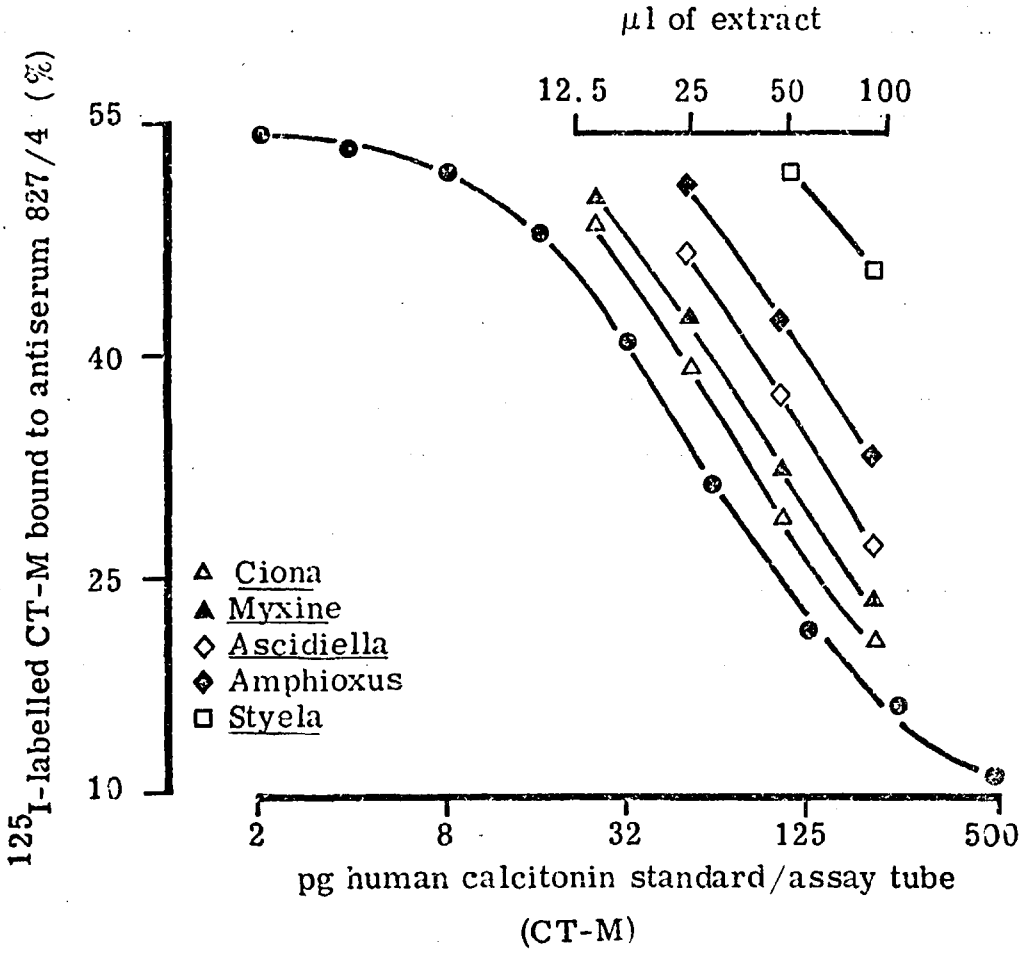


Fig. 40 Displacement curves of neural tissue extracts of Ciona (△), hagfish (▲), amphioxus (◆), Ascidiella (◇), Styela (□) and CT-M (●), using antiserum 827/4.

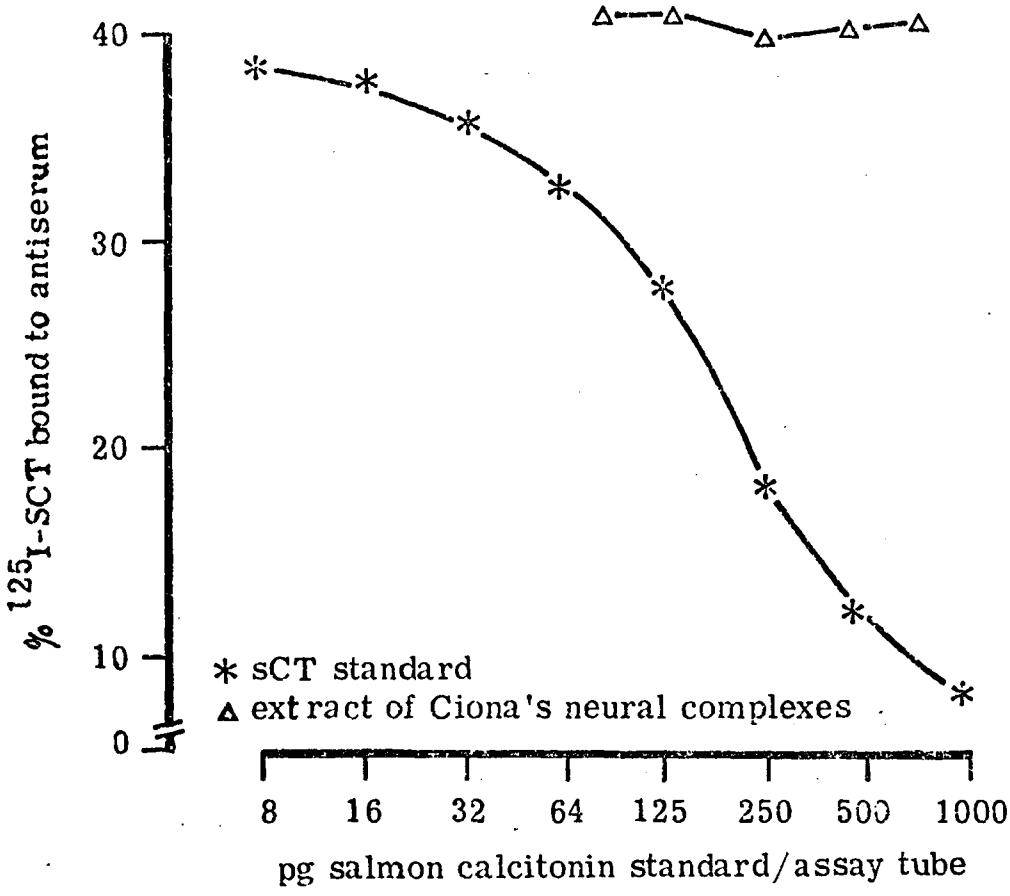


Fig. 41 Salmon calcitonin radioimmunoassay of neural complex extract of Ciona - no cross-reaction was observed.

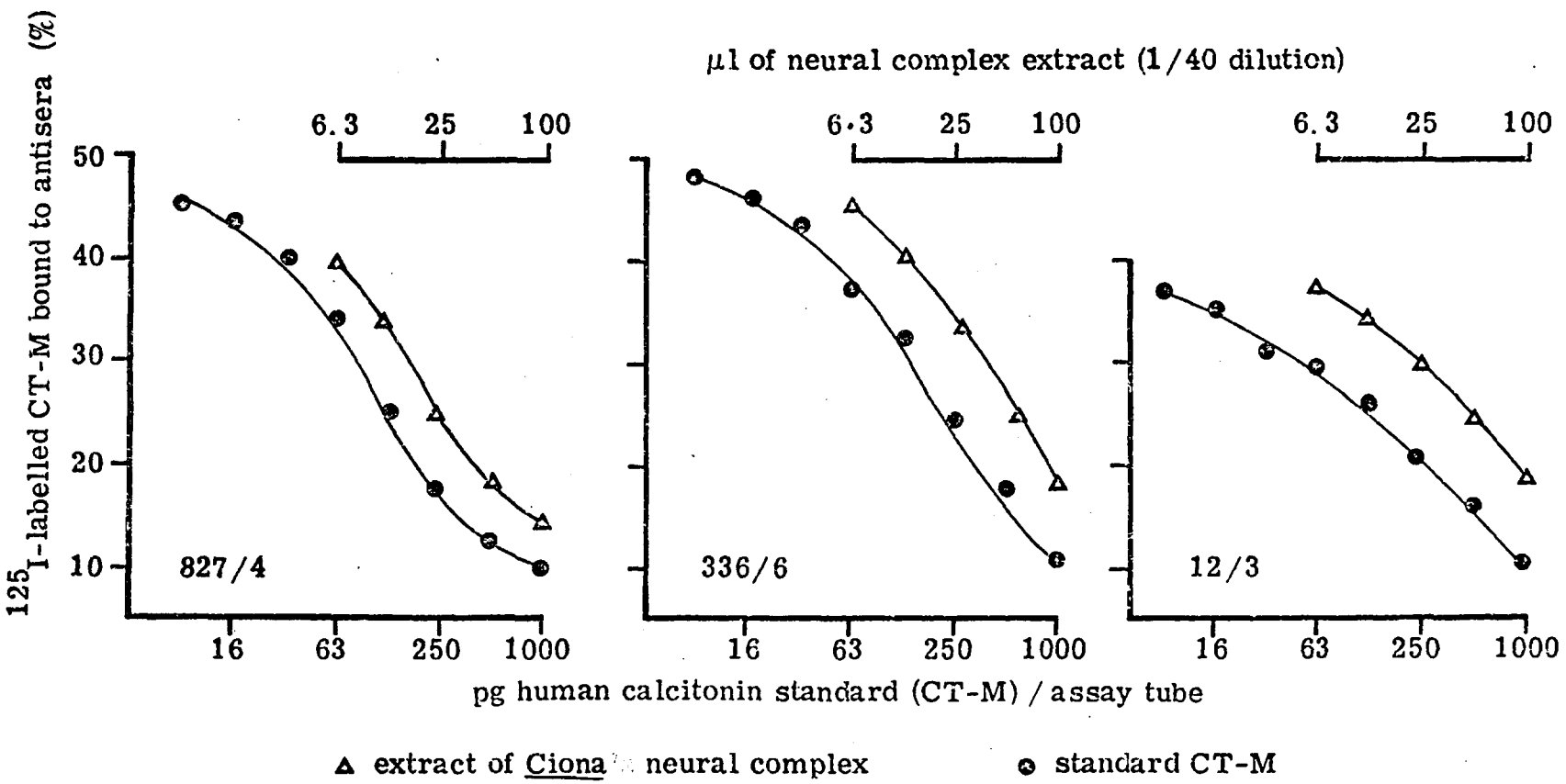


Fig. 42 Displacement curves of neural complex extract of Ciona (Western Baltic batch) (▲) and of CT-M (●) with antisera 827/4, 336/6 and 12/3.

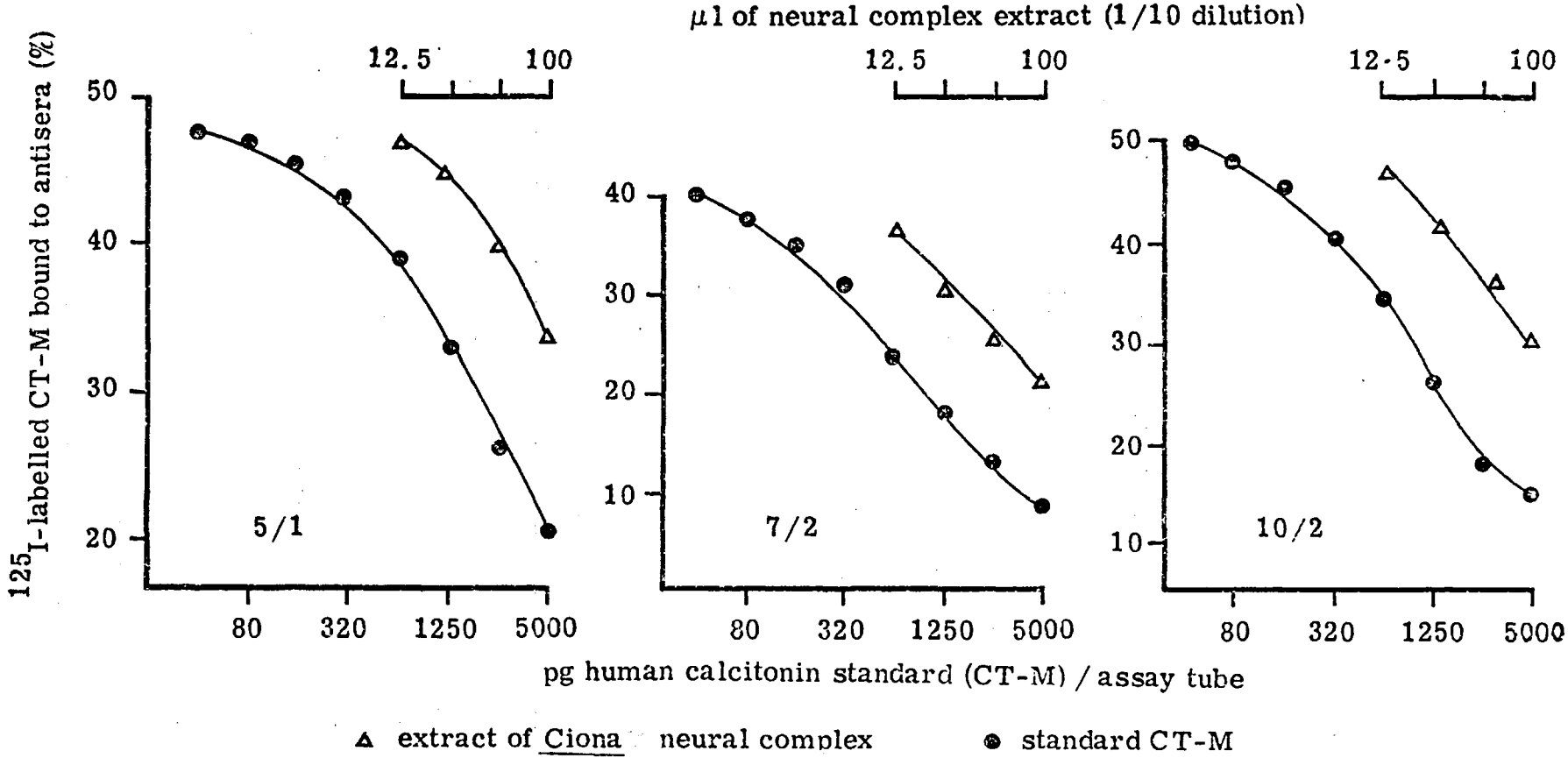


Fig. 43 Displacement curves of neural complex extract of Ciona (Western Baltic batch) (△) and CT-M (●) with antisera 5/1, 7/2 and 10/2.

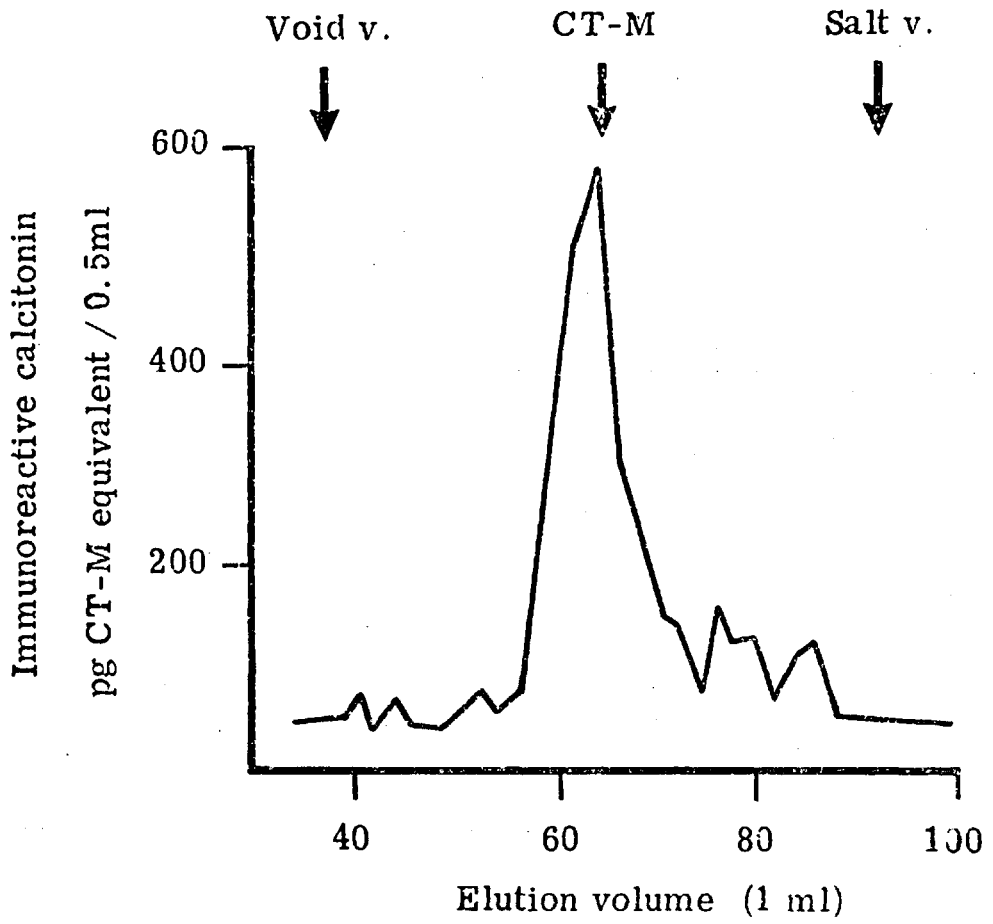


Fig. 44 Gel filtration of neural complex extract of Ciona in a Sephadex G-50 superfine column: the major immunoreactive peak co-elutes with CT-M.

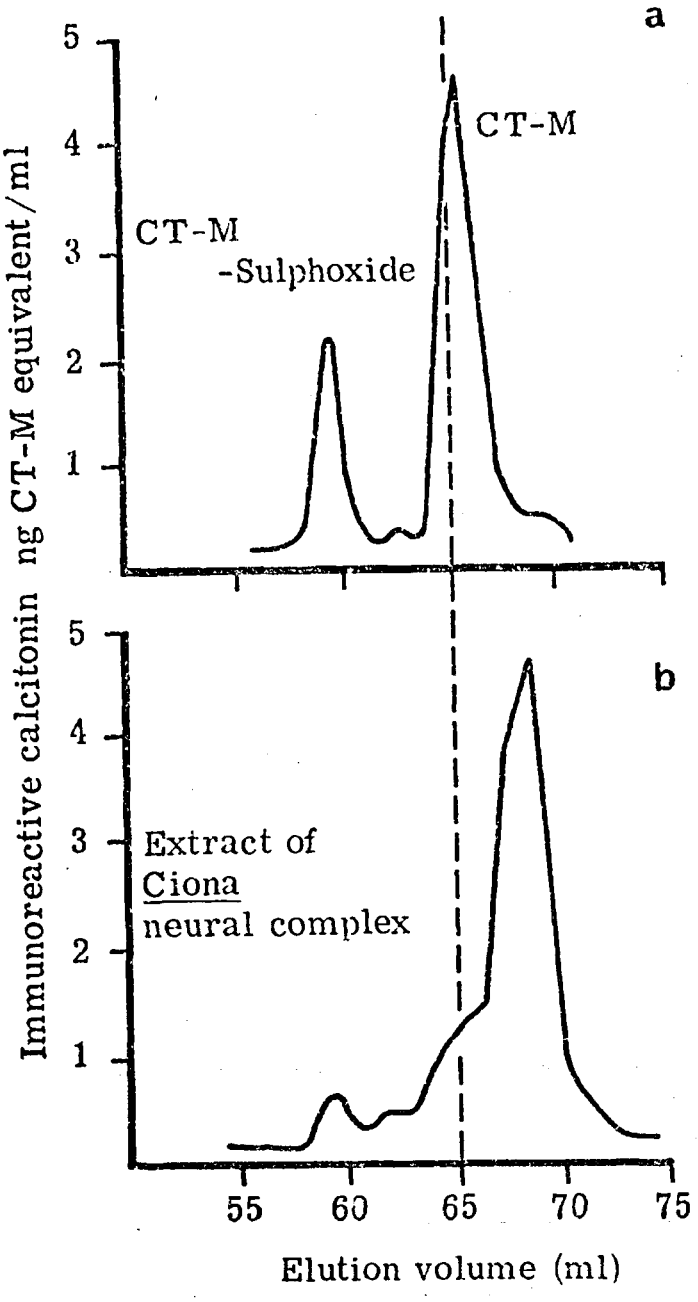


Fig. 45 Elution profile of Ciona neural complex extract (b) and CT-M and CT-M sulphoxide (a) on high performance liquid chromatography. The elution position of the internal marker is indicated by the dotted line. The major immunoreactive peak of Ciona's extract eluted after CT-M indicating a more hydrophobic molecule.

extract of Ciona neural complex appeared as a major peak at the same elution position as CT-M (Fig. 44).

4.3 High performance liquid chromatography (HPLC)

More than one immunoreactive peak was seen in the case of extracts of Ciona, Ascidiella, amphioxus and Myxine. In all samples the major peak was more retarded than CT-M indicating a more hydrophobic molecule (Fig. 45).

5. Discussion

These studies clearly show that the molecule under investigation is similar to human calcitonin, although not identical. It is slightly more hydrophobic and gives a significantly lower apparent concentration with antisera of C-terminal specificity, compared to antisera of both mid-portion and C-terminal specificities. The difference in hydrophobicity may be due to a single substitution of one hydrophilic amino acid, (e.g. serine, threonine or asparagine) for a hydrophobic amino acid, (e.g. alanine, leucine, methionine, valine or proline). The increased hydrophobicity of the molecule may lead to conformational changes with a resultant effect on immunoreactivity. From the sequence of CT-M one would predict the substitution of threonine at position 21 or 25 by methionine or alanine. Such a substitution is possible during evolution and could be explained by a change of a single base pair (Staehelin, 1971).

The presence of a molecule which is closely similar to CT-M and not to salmon calcitonin in the nervous tissue of primitive chordates and hagfish is of evolutionary interest. It suggests that a human calcitonin-like molecule is the parent brain peptide from which the later calcitonins found in the ultimobranchial bodies of teleosts and mammalian thyroids are derived. It also suggests that the common ancestor of calcitonin must have had a different function from the modern calcitonins.

Speculation about the function of this CT-M-like molecule in protochordates is perhaps premature, but the presence of calcitonin-like immunoreactivity in the neural ganglia of Ciona and also its localization in nerve fibres situated in the medullary zone (Fritsch, van Noorden and Pearse, 1979), suggests the possibility that this molecule may act as a neurotransmitter or neuromodulator. This view is strengthened by the demonstration of adenylate cyclase in the ascidian brain (Osborne et al. 1979), through which calcitonin may act, as it does in bone and kidney (Martin et al. 1977). However, the large variations in concentration found in different samples of Ciona raise the possibility that calcitonin may have another role.

The implications of this study will be discussed in Chapter 5.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

In this thesis I have described how an immunochemical approach can be applied in the characterization of femtomolar concentrations of a CT-M like molecule in plasma or tissue extracts. This approach is based on six region specific radioimmunoassays and high performance liquid chromatography of high resolving power for peptide hormones. Using these techniques the nature of normal calcitonin in plasma of normal human subjects has been studied and a CT-M like molecule has been identified in the nervous system of Ciona, a primitive chordate.

In investigating the nature of normal human calcitonin, evidence has been presented in Chapter 3 which strongly indicates that CT-M circulates in the plasma of normal subjects. This finding implies that CT-M is not a foreign peptide and it is produced by normal C-cells. Thus, it explains the lack of antibody formation to synthetic human calcitonin in patients with Paget's disease of bone, even after long term treatment (Dietrich, 1977), in contrast to treatment with salmon or porcine calcitonin (Singer et al. 1972; Haddad and Caldwell, 1972). Previous reports of identical molecules produced by normal and tumour derived cells in the case of rat calcitonin (Byfield et al. 1976; Raulais et al. 1976) and in the case of human gastrin (Gregory et al. 1967) and insulin (Steiner and Oyer, 1967) are in good agreement with this finding.

Studies on the characteristics of the immunoreactive CT-M like molecule in the nervous system of Ciona have clearly shown that this molecule is very similar, although not identical to CT-M. It is more hydrophobic and shows an apparent lower immunoreactivity with antisera specific to the carboxy-terminal region only, compared to antisera having both mid-molecule and carboxy-terminal specificities. The possibility of substitution of threonine at position 21 or 25 in human calcitonin by methionine or alanine in the ancestor peptide was raised.

The finding of a human calcitonin-like molecule in the nervous system of primitive chordates is of great evolutionary interest. First, it refutes the previous belief that the appearance of calcitonin is coincidental with the appearance of ultimobranchial bodies in jawed vertebrates (Copp et al. 1970). Second, it suggests that a molecule closely similar to CT-M is the common ancestor from which the later calcitonins found in the ultimobranchial bodies of teleosts and the thyroid gland of mammals are derived. Third, it suggests that the common ancestor of calcitonin must have had a different function from the modern calcitonins. Although it is believed that calcitonin interacts with target cells in bone and kidney to modulate the homoeostasis of calcium and other ions, phylogenetically it preceded the appearance of any calcified tissues. Consequently differences in amino acid sequence have occurred during evolution and it is also logical to believe that target tissues for the hormone's action have also changed.

In higher vertebrates, C-cells are derived from the neural crest and it would not be surprising to find calcitonin in the nervous system. Recently, Galan Galan et al. (1980) have found calcitonin immunoreactivity in the brain of pigeons and lizards. Furthermore, specific binding sites for calcitonins in the rat external median eminence have been reported (van Howen et al. 1980) and it is possible that calcitonin may act as a neurotransmitter in these species. This view is supported by previous studies which have shown that calcitonin given intravenously in rats produces an increase in cerebral 5-hydroxytryptamine content (Nakhla and Nandi Majundar, 1978) and when injected i.c.v. in rabbits produces a central analgesic effect (Braga et al. 1978). Furthermore, calcitonin was shown to produce a mild stimulating effect on prolactin release from pituitary cells in culture (Iwasaki et al. 1979) and it would be interesting to look for calcitonin specific receptors on pituitary.

In the future, attention should be directed towards clarifying the significance and role of calcitonin in the nervous system of higher vertebrates, and there are many outstanding questions. Further research should provide answers to a number of problems:

* What is the nature of this calcitonin-like molecule?

* Is it produced by neurones or just bound to receptors, or both?

* Is it a vestigial hormone or does it possess an important function, e.g. as a neurotransmitter or a neuromodulator?

REFERENCES

Aer, J. (1968).
Effect of thyrocalcitonin on urinary hydroxyproline and calcium in rats.
Endocrinology 83: 379-380.

Aliapoulios, M.A., Savery, A. and Munson, P.L. (1965).
New experiments with thyrocalcitonin.
Fed. Proc. 24: 322.

Aliapoulios, M.A., Goldhaber, P. and Munson, P.L. (1966).
Thyrocalcitonin inhibition of bone resorption induced by parathyroid hormone in tissue culture.
Science 151: 330-331.

Aliapoulios, M.A., Voelkel, E.F. and Munson, P.L. (1966).
Assay of human thyroid glands for thyrocalcitonin activity.
J. Clin. Endocrinol. 26: 897-901.

Axen, R., Porath, J. and Ernback, S. (1967).
Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides.
Nature (Lond.) 214: 1302-1304.

Baber, E.C. (1876).
Contributions to the minute anatomy of the thyroid gland of the dog.
Proc. Roy. Soc. London 24: 240-241.

Baghadiantz, A., Foster, G.V., Edwards, A., Kumar, M.A., Slack, E., Soliman, H.A. and MacIntyre, I. (1964).
Extraction and purification of calcitonin.
Nature (Lond.) 203: 1027-1028.

Ballard, H.S., Frame, B. and Hartsock, R.S. (1964).
Familial multiple endocrine adenoma peptic ulcer syndrome.
Medicine 43: 481-516.

Barling, P.M., Hendy, G.N., Evans, M.C. and O'Riordan, J.L. (1975).
Region-specific immunoassays for parathyroid hormone.
J. Endocrinol. 66: 307-318.

Barrett, R.J. and Bell, N.H. (1969).
Partial purification of porcine and human thyrocalcitonin.
Endocrinology 84: 647-651.

Becker, H.D., Konturek, S.J., Reeder, D.D. and Thompson, J.C. (1973).
Effect of calcium and calcitonin on gastrin and gastric secretion in cats.
Amer. J. Physiol. 225: 277-280.

Becker, H.D., Reeder, D.D., Scurry, M.T. and Thompson, J.C. (1974).
Inhibition of gastrin release and gastric secretion by calcitonin in patients with peptic ulcer.
Amer. J. Surg. 127: 71-75.

Bell, P.H., Barg, W.R., Jr., Colucci, D.F., Davies, C.M., Dziobkowski, C., Englert, M.E., Heyder, E., Paul, R. and Snedeker, E.H. (1968).
Purification and structure of porcine calcitonin-I.
J. Amer. Chem. Soc. 90: 2704-2706.

Bell, N.H., Avery, S. and Johnston, C.C., Jr. (1970).
Effects of calcitonin in Paget's disease and polyostotic fibrous dysplasia.
J. Clin. Endocrinol. Metab. 31: 283-290.

Bennett, H.P.J., Lowry, P.J. and McMartin, C. (1973).
Confirmation of the 1-20 amino acid sequence of human adrenocorticotrophin.
Biochem. J. 133: 11-13.

Bennett, H.P.J., Hudson, A.M., McMartin, C. and Purdon, G.E. (1977).
Use of octadecasilyl silica for extraction and purification of peptides in biological samples.
Biochem. J. 168: 9-13.

Berson, S.A. and Yalow, R.S. (1957).
Kinetics of reaction between insulin and insulin binding antibody.
J. Clin. Invest. 36: 873-874.

Berson, S.A. and Yalow, R.S. (1959).
Species-specificity of human anti-beef, pork insulin serum.
J. Clin. Invest. 38: 2017-2025.

Berson, S.A. and Yalow, R.S. (1966).
Parathyroid hormone in plasma in adenomatous hyperparathyroidism, ureamia, and bronchogenic carcinoma.
Science 154: 907-909.

Berson, S.A. and Yalow, R.S. (1968).
Immunochemical heterogeneity of parathyroid hormone in plasma.
J. Clin. Endocrinol. Metab. 28: 1037-1047.

Bijvoet, O.L., Van der Sluys Veer, J. and Jansen, A.P. (1968).
Effects of calcitonin on patients with Paget's disease, thyrotoxicosis, or hypercalcaemia.
Lancet i: 876-881.

Binoux, M.A. and Odell, W.D. (1973).
Use of dextran-coated charcoal to separate antibody-bound from free hormone: a critique.
J. Clin. Endocrinol. Metab. 36: 303-310.

Bolton, A.E. and Hunter, W.M. (1973).
The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent.
Biochem. J. 133: 529-539.

Braga, P., Ferri, S., Santagostino, A., Olgiati, V.R. and Pecile, A. (1978).
Lack of opiate receptor involvement in centrally induced calcitonin analgesia.
Life Sci. 22: 971-978.

Brewer, H.B., Jr. and Ronan, R. (1969).
Amino acid sequence of bovine thyrocalcitonin.
Proc. Natl. Acad. Sci. U.S.A. 63: 940-947.

Brown, P.R. (1973).
High Pressure Liquid Chromatography, Biochemical and Biomedical Applications.
New York - London: Academic Press.

Burckhardt, P., Ducommun, J. and Hessler, C. (1977).
Treatment of Paget's disease with human calcitonin.
In: Human Calcitonin and Paget's Disease, pp. 155-166.
Ed. I. MacIntyre. Bern: Hans Huber Publishers.

Burgus, R. and Rivier, J. (1976).
Use of high pressure liquid chromatography in the purification of peptides.
In: Peptides, Proceedings of the 14th European Peptide Symposium, pp. 85-94. Ed. A. Loffet. University of Brussels, Belgium.

Bussolati, G. and Pearse, A.G.E. (1967).
Immunofluorescent localisation of calcitonin in the 'C' cells of pig and dog thyroid.
J. Endocr. 37: 205-209.

Bussolati, G., Carvalheira, A.F. and Pearse, A.G.E. (1968).
Immunofluorescent studies on the source of calcitonin and the effect of immunisation with calcitonin on the thyroid gland of guinea pig.
In: Calcitonin, Proceedings of the Symposium on Thyrocalcitonin and the C Cells, pp. 133-142. Ed. S. Taylor. London: Heinemann.

Byfield, P.G.H., Clark, M.B., Turner, K., Foster, G.V. and MacIntyre, I. (1972).
Immunochemical studies on human calcitonin M leading to information on the shape of the molecule.
Biochem. J. 127: 199-206.

Byfield, P.G.H., Matthews, E.W., Heersche, J.N.M., Boorman, G.A., Girgis, S.I. and MacIntyre, I. (1976).
Isolation of calcitonin from rat thyroid medullary carcinoma.
FEBS Letts. 65: 238-241.

Byfield, P.G.H., McLoughlin, J.L., Matthews, E.W. and MacIntyre, I. (1976).
A proposed structure for rat calcitonin.
FEBS Letts. 65: 242-245.

Canale, D.D. and Donabedian, R.K. (1975).
Hypercalcitoninemia in acute pancreatitis.
J. Clin. Endocrinol. Metab. 40: 738-741.

Care, A.D. and Keynes, W.M. (1965).
The secretion of calcitonin by the parathyroid glands of the sheep.
J. Endocr. 31: xxxi-xxxii.

Carvalheira, A.F. and Pearse, A.G.E. (1968).
Cytochemical evidence for the ultimobranchial origin of the C cells in rodent thyroid.
In: Calcitonin, Proceedings of the Symposium on Thyrocalcitonin and the C Cells, pp. 122-126. Ed. S. Taylor. London: Heinemann Medical Books.

Catt, K.J. and Tregear, G.W. (1967).
Solid-phase radioimmunoassay in antibody-coated tubes.
Science (N.Y.) 158: 1570-1572.

Clark, M.B., Byfield, P.G.H., Boyd, G.W. and Foster, G.V. (1969).
A radioimmunoassay for human calcitonin.
Lancet ii: 74-77.

Clark, J.D. and Kenny, A.D. (1969).
Hog thyrocalcitonin: urinary calcium, phosphorus, magnesium and sodium responses.
Endocrinology 84: 1199-1205.

Cohen, S.L., MacIntyre, I., Grahame-Smith, D. and Walker, J.G. (1973).
Alcohol-stimulated calcitonin release in medullary carcinoma of the thyroid.
Lancet ii: 1172-1174.

Coombes, R.C., Hillyard, C.J., Greenberg, P.B. and MacIntyre, I. (1974).
Plasma immunoreactive calcitonin in patients with non-thyroid tumours.
Lancet i: 1080-1083.

Coombes, R.C., Ellison, M.L., Easty, G.C., Hillyard, C.J., James, R., Galante, L., Girgis, S., Heywood, L., MacIntyre, I. and Neville, A.M. (1976).
The ectopic secretion of calcitonin by lung and breast carcinomas.
Clin. Endocr. 5: Suppl., 387s-396s.

Copp, D.H. and Cheney, B.A. (1962).
Calcitonin - a hormone from the parathyroid which lowers the calcium level of blood.
Nature (Lond.) 193: 381-382.

Copp, D.H. and Henze, K.G. (1964).
Parathyroid origin of calcitonin - evidence from perfusion of sheep glands.
Endocrinology 75: 49-55.

Copp, D.H., Cockcroft, D.W. and Kueh, Y. (1967).
Calcitonin from the ultimobranchial glands of dogfish and chickens.
Science 158: 924-925.

Cunliffe, W.J., Black, M.M., Hall, R., Johnstone, I.D.A., Hudgson, P., Shuster, S., Gudmundsson, T.V., Joplin, G.F., Williams, E.D., Woodhouse, N.J.Y., Galante, L. and MacIntyre, I. (1968).
A calcitonin-secreting thyroid carcinoma.
Lancet ii: 63-66.

Cutler, G.B., Jr., Habener, J.F. and Potts, J.T., Jr. (1977).
Biosynthesis and secretion of calcitonin by avian ultimobranchial glands.
Endocrinology 100: 537-548.

Deftos, L.J. (1971).
Immunoassay for human calcitonin I. Method.
Metabolism 20: 1122-1128.

Deftos, L.J., Powell, D., Parthemore, J.G. and Potts, J.T., Jr. (1973).
Secretion of calcitonin in hypocalcaemic states in man.
J. Clin. Invest. 52: 3109-3114.

Deftos, L.J. (1974).
Radioimmunoassay for calcitonin in medullary thyroid carcinoma.
JAMA 227: 403-406.

Deftos, L.J., Watts, E.G., Copp, D.H. and Potts, J.T. (1974).
A radioimmunoassay for salmon calcitonin.
Endocrinology 94: 155-160.

Deftos, L.J., Roos, B.A., Bronzert, D. and Parthemore, J.G. (1975).
Immunochemical heterogeneity of calcitonin in plasma.
J. Clin. Endocrinol. Metab. 40: 409-412.

Deftos, L.J. (1978).
Calcitonin in clinical medicine.
Adv. Intern. Med. 23: 159-193.

Deftos, L.J., Burton, D., Catherwood, B.D., Bone, H.G., Parthemore, J.G., Guillemin, R., Watkins, W.B. and Moore, R.Y. (1978) (a).
Demonstration by immunoperoxidase histochemistry of calcitonin in the anterior lobe of the rat pituitary.
J. Clin. Endocrinol. Metab. 47: 457-460.

Deftos, L.J., Burton, D., Bone, H.G., Catherwood, B.D., Parthemore, J.G., Moore, R.Y., Minick, S. and Guillemin, R. (1978) (b).
Immunoreactive calcitonin in the intermediate lobe of the pituitary gland.
Life Sci. 23: 743-748.

Desbuquois, B. and Aurbach, G.D. (1971).
Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassays.
J. Clin. Endocrinol. Metab. 33: 732-738.

Dietrich, F.M. and Rittel, W. (1970).
Antigenic site(s) of synthetic human calcitonin M.
Nature (Lond.) 225: 75-76.

Dietrich, F.M., Hunziker, W.H. and Fischer, J.A. (1975).
Synthetic human calcitonin: analysis of antibodies obtained from various animal species and determination of immunoreactive hormone in human sera.
Acta Endocrinol. 80: 465-486.

Dietrich, F.M. (1977).
Immunological studies with synthetic human calcitonin.
In: Human Calcitonin and Paget's Disease, pp. 179-194.
Ed. I. MacIntyre. Bern: Hans Huber.

Dubé, W.J., Goldsmith, R.S., Arnaud, S.B. and Arnaud, C.D. (1973).
Development of antibodies to porcine calcitonin during treatment of Paget's disease of bone.
Mayo Clinic Proc. 48: 43-46.

Dymling, J.F., Ljungberg, O., Hillyard, C.J., Greenberg, P.B., Evans, I.M.A. and MacIntyre, I. (1976).
Whisky: a new provocative test for calcitonin secretion.
Acta Endocrinol. 82: 500-509.

Ekins, R.P. (1969).
In: Protein and Polypeptide Hormones, pp. 633-635 (Discussion).
Ed. M. Margoulies. Excerpta Medica International Congress
Series No. 161. Amsterdam: Excerpta Medica Foundation.

Evans, I.M.A. (1977).
Human calcitonin in the treatment of Paget's disease: long
term trials.
In: Human Calcitonin and Paget's Disease, pp. 111-121.
Ed. I. MacIntyre. Bern: Hans Huber Publishers.

Foster, G.V., Baghdiantz, A., Kumar, M.A., Slack, E.,
Soliman, H.A. and MacIntyre, I. (1964).
Thyroid origin of calcitonin.
Nature (Lond.) 202: 1303-1305.

Foster, G.V., MacIntyre, I. and Pearse, A.G.E. (1964).
Calcitonin production and the mitochondrion-rich cells of
the dog thyroid.
Nature (Lond.) 203: 1029-1030.

Foster, G.V., Joplin, G.F., MacIntyre, I., Melvin, K.E.W. and
Slack, E. (1966).
Effect of thyrocalcitonin in man.
Lancet i: 107-109.

Freed, W.J., Perlow, M.J. and Wyatt, R.J. (1979).
Calcitonin: inhibitory effect on eating in rats.
Science 206: 850-852.

Friedman, J. and Raisz, L.G. (1965).
Thyrocalcitonin: inhibitor of bone resorption in tissue culture.
Science 150: 1465-1467.

Fritsch, H.A.R., Van Noorden, S. and Pearse, A.G.E. (1979).
Localization of somatostatin, substance P and calcitonin-like
immunoreactivity in the neural ganglion of Ciona intestinalis L.
(Ascidiaceae).
Cell Tiss. Res. 202: 263-274.

Galan Galan, F. (1980).
Personal communication.

Gellhorn, A. (1963).
The unifying thread.
Cancer Res. 23: 961-970.

Genuth, S., Frohman, L.A. and Lebovitz, H.E. (1965).
A radioimmunological assay method for insulin using insulin-^{125}I and gel filtration.
J. Clin. Endocrinol. Metab. 25: 1043-1049.

Godwin, M.C. (1937).
Complex IV in the dog with special emphasis on the relation of the ultimobranchial body to interfollicular cells in the post-natal thyroid gland.
Amer. J. Anat. 60: 299-330.

Goodfriend, T.L. (1968).
Radioimmunoassay of bradykinin and angiotensin.
In: Proteins and Polypeptide Hormones, pp. 91-92.
Ed. M. Margoulies. Amsterdam: Excerpta Medica.

Goodman, J.W. (1969).
Immunochemical specificity: recent conceptual advances.
Immunochemistry 6: 139-149.

Goodman, R.H., Jacobs, J.W. and Habener, J.F. (1979).
Cell-free translation of messenger RNA coding for a precursor of human calcitonin.
Biochem. Biophys. Res. Commun. 91: 932-939.

Gorling, R.J., Sendano, H.O., Vickers, R.A. and Corvaniko, S. (1968).
Multiple mucosal neuromas, pheochromocytoma and medullary thyroid carcinoma: a syndrome.
Cancer 22: 293-299.

Gray, T.K., Bieberdorf, F.A. and Fordtran, J.S. (1973).
Thyrocalcitonin and the jejunal absorption of calcium, water and electrolytes in normal subjects.
J. Clin. Invest. 52: 3084-3088.

Gray, T.K. and Munson, P.L. (1969).
Thyrocalcitonin: evidence for physiological function.
Science 166: 512-513.

Gregory, R.A., Grossman, M.T., Tracy, H.T. and Bentley, P.H. (1967).
Nature of gastric secretagogue in Zolinger-Ellison tumours.
Lancet ii: 543-544.

Greenberg, P.B., Doyle, F.H., Fisher, M.T., Hillyard, C.J., Joplin, G.F., Pennock, J. and MacIntyre, I. (1974).
Treatment of Paget's disease of bone with synthetic human calcitonin.
Amer. J. Med. 56: 867-870.

Grodsky, G.M. and Forsham, P.H. (1960).
An immunochemical assay of total extractable insulin in man.
J. Clin. Invest. 39: 1070-1079.

Gruber, K.A., Stein, S., Brink, L., Radhakrishnan, A. and Udenfriend, S. (1976).
Fluorometric assay of vasopressin and oxytocin: a general approach to the assay of peptides in tissues.
Proc. Natl. Acad. Sci. U.S.A. 73: 1314-1318.

Grushka, E. (1974).
Bonded stationary phases in chromatography.
Ann Arbor Sciences. Michigan: Ann Arbor.
Quoted by Burgus and Rivier, 1976.

Gudmundsson, T.V., MacIntyre, I. and Soliman, H.A. (1966).
The isolation of thyrocalcitonin and a study of its effects in the rat.
Proc. Roy. Soc. B 164: 460-477.

Guttmann, St., Pless, J., Sandrin, E., Jaquenoud, P.A., Bossert, H. and Williems, H. (1968).
Synthese des thyrocalcitonins.
Helv. Chim. Acta 51: 1155-1158.

Guttmann, St., Pless, J., Huguenin, R.L., Sandrin, E., Bossert, H. and Zehnder, K. (1969).
Synthese von salm calcitonin.
Helv. Chim. Acta 52: 1789-1795.

Habener, J.F., Singer, F.R., Deftos, L.J., Neer, R.M. and Potts, J.T., Jr. (1971).
Explanation for unusual potency of salmon calcitonin.
Nature (Lond.) 232: 91-92.

Haddad, J.G., Birge, S.J. and Avioli, L.V. (1970).
Effects of prolonged thyrocalcitonin administration on Paget's disease of bone.
New Engl. J. Med. 283: 549-555.

Haddad, J.G. and Caldwell, J.G. (1972).
Calcitonin resistance: clinical and immunologic studies in subjects with Paget's disease of bone treated with porcine and salmon calcitonins.
J. Clin. Invest. 51: 3133-3141.

Hales, C.N. and Randle, P.J. (1963).
Immunoassay of insulin with insulin-antibody precipitate.
Biochem. J. 88: 137-146.

Haymovits, A. and Rosen, J.P. (1967).
Human thyrocalcitonin.
Endocrinology 81: 993-1000.

Hazard, J.B., Hawk, W.A. and Crile, G. (1959).
Medullary (solid) carcinoma of the thyroid - a clinicopathologic entity.
J. Clin. Endocrinol. Metab. 19: 152-161.

Heding, L.G. (1966).
Labelled proteins in tracer studies.
In: Proceedings of the Conference held at Pisa, 17-19 January, 1966, pp. 345-350. Eds. L. Donata, G. Milhaud and J. Sanchis. Brussels: European Atomic Energy Community.

Heersche, J., Marcus, R. and Aurbach, G.D. (1974).
Calcitonin and the formation of 3',5'-AMP in bone and kidney.
Endocrinology 94: 241-247.

Hennessy, J.F., Gray, T.K., Cooper, C.W. and Ontjes, D.A. (1973).
Stimulation of thyrocalcitonin secretion by pentagastrin and calcium in 2 patients with medullary carcinoma of the thyroid.
J. Clin. Endocrinol. Metab. 36: 200-203.

Hennessy, J.F., Wells, S.A., Jr., Ontjes, D.A. and Cooper, C.W. (1974).
A comparison of pentagastrin injection and calcium infusion as provocative agents for detection of medullary thyroid carcinoma.
J. Clin. Endocrinol. Metab. 39: 487-495.

Herbert, V., Lau, K.S., Gottlieb, C.W. and Bleicher, S.J. (1965).
Coated charcoal immunoassay of insulin.
J. Clin. Endocrinol. Metab. 25: 1375-1384.

Heynen, G. and Franchimont, P. (1974).
Human calcitonin radioimmunoassay in normal and pathological conditions.
Europ. J. Clin. Invest. 4: 213-222.

Hillyard, C.J., Cooke, T.J.C., Coombes, R.C., Evans, I.M.A. and MacIntyre, I. (1977).
Normal plasma calcitonin: circadian variation and response to stimuli.
Clin. Endocrinol. 6: 291-298.

Hillyard, C.J. (1979).
Ph.D. Thesis. University of London.

Hirsch, P.F., Gauthier, G.F. and Munson, P.L. (1963).
Thyroid hypocalcaemic principle and recurrent laryngeal nerve injury as factors affecting the response of parathyroidectomy in rats.
Endocrinology 73: 244-252.

Holtrop, M.E., Raisz, L.G. and Simmons, H.A. (1974).
The effects of parathyroid hormone, colchicine, and calcitonin on the ultrastructure and the activity of osteoclasts in organ culture.
J. Cell. Biol. 60: 346-355.

Howard, J.E. (1957).
Calcium metabolism: bones and calcium homeostasis. A review of certain current concepts.
J. Clin. Endocrinol. Metab. 17: 1105-1123.

Hudson, A.M. (1978).
Ph.D. Thesis. Brunel University.

Hunter, W.M. and Greenwood, F.C. (1962).
Preparation of iodine 131 labelled human growth hormone of high specific activity.
Nature (Lond.) 194: 495-496.

Iwasaki, Y., Chihara, K., Iwasaki, J., Abe, H. and Fujita, T. (1979).
Effect of calcitonin on prolactin release in rats.
Life Sci. 25: 1243-1248.

Jacobs, J.W., Potts, J.T., Jr., Bell, N.H. and Habener, J. (1979).
Calcitonin precursor identified by cell-free translation of m-RNA.
J. Biol. Chem. 254: 10600-10603.

Kalina, M., Foster, G.V., Clark, M.B. and Pearse, A.G.E. (1970).
C-cells in man.
In: Calcitonin 1969, Proceedings of 2nd International Symposium, pp. 268-273. Ed. S. Taylor. London: Heinemann Medical Books.

Kallio, D.M., Garant, P.R. and Minkin, C. (1972).
Ultrastructural effects of calcitonin on osteoclasts.
J. Ultrastruct. Res. 39: 205-216.

Kaplan, E.L., Staroscik, R., Peskin, G.W. and Arnaud, C.D. (1968).
Calcitonin-like responses in man during thyroid surgery.
J. Clin. Endocrinol. Metab. 28: 740-745.

Kelly, L., Bennett, H.P.J., Hudson, A.M., McMartin, C. and Purdon, G.E. (1978).
An efficient method for the extraction of undamaged peptides from the various tissues.
J. Endocr. 77: 24P-25P.

Kenny, A.D. and Heiskell, C.A. (1965).
Effect of crude thyrocalcitonin on calcium and phosphorus metabolism in rats.
Proc. Soc. Exp. Biol. (N.Y.) 120: 269-270.

Keutmann, H.T., Lequin, R.M., Habener, J.F., Singer, F.R., Niall, H.D. and Potts, J.T., Jr. (1972).
Chemistry and physiology of the calcitonins: some recent advances.
In: Endocrinology 1971, Proceedings of the 3rd International Symposium, pp. 316-323. Ed. S. Taylor. London: Heinemann Medical Books.

Krummen, K. and Frei, R.W. (1977).
The separation of nonapeptides by reversed-phase high performance liquid chromatography.
J. Chromat. 132: 27-36.

Kumar, M.A., Foster, G.V. and MacIntyre, I. (1963).
Further evidence for calcitonin: rapid acting hormone which lowers plasma calcium.
Lancet ii: 480-482.

Kumar, M.A., Slack, E., Edwards, A., Soliman, H.A., Baghdiantz, A., Foster, G.V. and MacIntyre, I. (1965).
A biological assay for calcitonin.
J. Endocr. 33: 469-475.

Kurokawa, K., Nagata, N., Sasaki, M. and Nakane, K. (1974).
Effects of calcitonin on the concentration of cyclic adenosine 3',5' monophosphate in rat kidney *in vitro* and *in vivo*.
Endocrinology 94: 1514-1518.

Lips, C.J.M., Van der Sluys Veer, J., Van der Dork, J.A., Van Dam, R.H. and Hackeng, W.H.L. (1978).
Common precursor molecule as origin for the ectopic-hormone-producing tumour syndrome.
Lancet i: 16-18.

Ljungberg, O. (1972).
On medullary carcinoma of the thyroid.
Acta Pathol. Microbiol. Scand. (A) (Suppl.) 231: 1-57.

Luben, R.A., Wong, G.L. and Cohn, D.U. (1976).
Biochemical characterisation with parathormone and calcitonin of isolated bone cells: provisional identification of osteoclasts and osteoblasts.
Endocrinology 99: 526-534.

de Luise, M., Martin, T.J., Greenberg, P.B. and Michelangeli, V. (1972).
Metabolism of porcine, human and salmon calcitonin in the rat.
J. Endocr. 53: 475-482.

MacIntyre, I. and Parsons, J.A. (1966).
Blood-bone calcium equilibrium in the perfused cat tibia and the effect of thyroid calcitonin.
J. Physiol.(Lond.) 183: 31P.

MacIntyre, I., Arnett, T.R., Brown, D.J., Galan Galan, F., Girgis, S.I., Rogers, R.M., Spanos, E., Stevenson, J.C. and Bone, Q. (1979).
The interrelation of the calcium regulating hormones: some recent findings.
In: Molecular Endocrinology I, Proceedings of the International Congress, pp. 193-201. Eds. I. MacIntyre and M. Szelke. Amsterdam: Elsevier/North-Holland Biomedical Press.

Marchalonis, J.J. (1969).
An enzymatic method for the trace iodination of immunoglobulins and other proteins.
Biochem. J. 113: 299-305.

Margules, D.L., Flynn, J.J., Walker, J. and Cooper, C.W. (1979).
Elevation of calcitonin immunoreactivity in the pituitary and thyroid glands of genetically obese rats (fa/fa).
Brain Res. Bull. 4: 589-591.

Martin, T.J., Hunt, N.H., Ellison, M., Underwood, J.C.E. and Michelangeli, V.P. (1977).
Physiology and biochemical pharmacology of calcitonin action.
In: Molecular Endocrinology - Proceedings of Endocrinology '77, pp. 179-192. Eds. I. MacIntyre and M. Szelke. Amsterdam: Elsevier/North-Holland Biomedical Press.

Martin, T.J., Robinson, C.J. and MacIntyre, I. (1966).
The mode of action of thyrocalcitonin.
Lancet i: 900-902.

Martin, T.J. and Melick, R.A. (1969).
The acute effects of porcine calcitonin in man.
Aust. Ann. Med. 18: 258-263.

Marx, S.J., Woodward, C.J. and Aurbach, G.D. (1972).
Calcitonin receptors of kidney and bone.
Science 178: 999-1001.

Melvin, K.E.W. and Tashjian, A.H., Jr. (1968).
The syndrome of excessive thyrocalcitonin produced by medullary carcinoma of the thyroid.
Proc. Natl. Acad. Sci. U.S.A. 59: 1216-1222.

Meyer, J.S. and Abdel-Bari, W. (1968).
Granules and thyrocalcitonin-like activity in medullary carcinoma of the thyroid glands.
New Engl. J. Med. 278: 523-529.

Milhaud, G., Perault and, A.M. and Moukhtar, M.S. (1965).
Etude du mecanisme de l'action hypocalcemiante de la thyrocalcitonine.
C. R. Acad. Sci. (Paris) 261: 813-816.

Milhaud, G., Tubiana, M., Parmentier, C. and Coutris, G. (1968).
Epithelioma de la thyroide secretant de la thyrocalcitonine.
C. R. Acad. Sci. (Paris) 266: 608-610.

Milhaud, G., Galmette, C., Taboulet, J., Julienne, A. and Moukhtar, M.S. (1974).
Hypersecretion of calcitonin in neoplastic conditions.
Lancet i: 462-463.

Miller, H.H., Melvin, K.E.W., Gibson, J.M. and Tashjian, A.H., Jr. (1972).
Surgical approach to early familial medullary carcinoma of the thyroid gland.
Amer. J. Surg. 123: 438-443.

Morgan, C.R. and Lazarow, A. (1963).
Immunoassay of insulin: two antibody system.
Diabetes 12: 115-126.

Morikawa, T., Munekata, E., Sakakibara, S., Noda, T. and Otani, M. (1976).
Synthesis of eel-calcitonin and /$^{-}$Asu^{1,7}/-eel-calcitonin: contribution of the disulfide bond to the hormonal activity.
Experientia 32: 1104-1106.

Morrison, M., Bayse, G.S. and Webster, R.G. (1971).
Use of lactoperoxidase catalyzed iodination in immunochemical studies.
Immunochemistry 8: 289-297.

Moseley, J.M., Matthews, E.W., Breed, R.H., Galante, L., Tse, A. and MacIntyre, I. (1968).
Ultimobranchial origin of calcitonin.
Lancet i: 108-110.

Moukhtar, M.S., Jullienne, A., Tharaud, D., Taboulet, J. and Milhaud, G. (1973).
Extraction and concentration of human plasma calcitonin using coupled antibodies on a solid support.
C. R. Acad. Sci. (D) (Paris) 276: 3445-3448.

Moya, F., Nieto, A. and Candela, J.L.R. (1975).
Calcitonin biosynthesis: evidence for a precursor.
Eur. J. Biochem. 55: 407-413.

Mutt, V., Jorpes, J.E. and Magnusson, S. (1970).
Structure of porcine secretin: the amino acid sequence.
Eur. J. Biochem. 15: 513-519.

McCredie, D.A., Dixon, S.R., Martin, T.J., Melick, R.A., Rotenberg, E. and Shipman, R. (1971).
In: Proc. XIII Int. Congress Ped., Vienna, pp. 155.
Quoted by Martin et al. 1977.

McLean, F.C. and Urist, M.R. (1961).
Bone. An Introduction to the Physiology of Skeletal Tissue.
2nd Edit. The University of Chicago Press, Chicago, Illionois, U.S.A.

Nagant de Deuxchaisnes, C., Rombouts-Lindemans, C., Huaux, J.P., Malghem, J. and Maldague, B. (1977).
Roentgenologic evaluation of the efficacy of calcitonin in Paget's disease of bone.
In: Molecular Endocrinology - Proceedings of Endocrinology '77, pp. 213-233. Eds. I. MacIntyre and M. Szelke. Amsterdam: Elsevier/North-Holland Biomedical Press.

Nakhla, A.M. and Nandi Majumdar, A.P. (1978).
Calcitonin-mediated changes in plasma tryptophan and brain 5-hydroxytryptamine and acetylcholinesterase activity in rats.
Biochem. J. 170: 445-448.

Neher, R., Riniker, B., Zuber, H., Rittel, W. and Kahnt, F. (1968) (a).
Thyrocalcitonin II. Structur von α thyrocalcitonin.
Helv. Chim. Acta 51: 917-924.

Neher, R., Riniker, B., Maier, R., Byfield, P.G.H., Gudmundsson, T.V. and MacIntyre, I. (1968) (b).
Human calcitonin.
Nature (Lond.) 220: 984-986.

Neher, R., Riniker, B., Rittel, W. and Zuber, H. (1968) (c).
Menschliches calcitonin. III. Struktur von calcitonin M und D.
Helv. Chim. Acta 51: 1900-1905.

Niall, H.D., Keutmann, H.T., Copp, D.H. and Potts, J.T., Jr. (1969).
The amino acid sequence of salmon ultimobranchial calcitonin.
Proc. Natl. Acad. Sci. U.S.A. 64: 771-778.

Noda, T. and Narita, K. (1976).
Amino acid sequence of eel calcitonin.
J. Biochem. (Tokyo) 79: 353-359.

Nonidez, J.F. (1930).
The origin of the "parafollicular" cell, a second epithelial component of the thyroid gland of the dog.
Amer. J. Anat. 49: 479-505.

Odell, W.D., Abraham, G.A., Skowsky, W.R., Hescox, M.A. and Fisher, D.A. (1971).
Production of antisera for radioimmunoassays.
In: Principles of Protein-Binding Assays, pp. 57-76.
Eds. Odell and Daughaday. Philadelphia and Toronto:
J.B. Lippincott Company.

O'Dor, R.K., Parkes, C.O. and Copp, D.H. (1969).
The amino acid composition of salmon calcitonin.
Cand. J. Biol. 47: 873-892.

O'Hare, M.J. and Nice, E.C. (1979).
Hydrophobic high-performance liquid chromatography of hormonal polypeptides and proteins on alkylsilane-bonded silica.
J. Chromat. 171: 209-226.

Osborne, N.N., Neuhoff, V., Ewers, E. and Roberson, H.A. (1979).
Putative neurotransmitters in the cerebral ganglia of the tunicate Ciona intestinalis.
Comp. Biochem. Physiol. 630: 209-213.

Otani, M., Yamauchi, H., Meguro, T., Kitazawa, S., Watanabe, S. and Orimo, H. (1976).
Isolation and characterization of calcitonin from pericardium and esophagus of eel.
J. Biochem. (Tokyo) 79: 345-352.

Palmieri, G.M.A., Yalow, R.S. and Berson, S.A. (1971).
Adsorbent techniques for the separation of antibody-bound from free peptide hormones in radioimmunoassay.
Horm. Metab. Res. 3: 301-305.

Parthemore, J.G., Bronzert, D., Roberts, G. and Deftos, L.J. (1974).
A short calcium infusion in the diagnosis of medullary thyroid carcinoma.
J. Clin. Endocrinol. Metab. 39: 108-111.

Parthemore, J.G., Deftos, L.J. and Bronzert, D. (1975).
The regulation of calcitonin in normal human plasma as assessed by immunoprecipitation and immunoextraction.
J. Clin. Invest. 56: 835-841.

Pearse, A.G.E. (1966).
The cytochemistry of the thyroid C cells and their relationship to calcitonin.
Proc. Roy. Soc. B. 164: 478-487.

Pearse, A.G.E. and Carvalheira, A.F. (1967).
Cytochemical evidence for an ultimobranchial origin of rodent thyroid C cells.
Nature (Lond.) 214: 929-930.

Pearse, A.G.E. (1968).
Common cytochemical and ultrastructural characteristics of cells producing polypeptide hormones (the APUD series) and their relevance to thyroid and ultimobranchial C cells and calcitonin.
Proc. Roy. Soc. B (Lond.) 170: 71-80.

Pearse, A.G.E. and Polak, J.M. (1971).
Cytochemical evidence for the neural crest origin of mammalian ultimobranchial C-cells.
Histochemie 27: 96-102.

Pecile, A., Ferri, S., Braga , P.C. and Olgiati, V.R. (1975).
Effects of intracerebroventricular calcitonin in the conscious rabbit.
Experientia 31: 332-333.

Potts, J.T., Jr., Niall, H.D., Keutmann, H.T., Brewer, H.B. and Deftos, L.J. (1968).
The amino acid sequence of porcine thyrocalcitonin.
Proc. Natl. Acad. Sci. U.S.A. 59: 1321-1328.

Potts, J.T., Jr., Niall, H.D., Keutmann, H.T., Deftos, L.J. and Parsons, J.A. (1970).
Calcitonin: recent chemical and immunological studies.
In: Calcitonin 1969, Proceedings of the 2nd International Symposium, pp. 56-73. Ed. S. Taylor. London: Heinemann Medical Books.

Potts, J.T., Jr., Keutmann, H.T., Niall, H.D. and Tregear, G.W. (1971).
The chemistry of parathyroid hormone and the calcitonins.
Vitam. Horm. 29: 41-93.

Queener, S., Fleming, J. and Bell, N. (1974).
Solubilization of calcitonin-responsive renal cortical adenylate cyclase.
J. Clin. Invest. 53: 62a.

Rasmussen, H. (1961).
Parathyroid hormone: nature and mechanism of action.
Amer. J. Med. 30: 112-128.

Raulais, D., Hagaman, J., Ontjes, D.A., Lundblad, R.L. and Kingdon, H.S. (1976).
The complete amino-acid sequence of rat thyrocalcitonin.
Eur. J. Biochem. 64: 607-611.

Rebel, A., Malkani, K., Basle, M. and Bregeon, C.L. (1976).
Osteoclast ultrastructure in Paget's disease.
Calcif. Tiss. Res. 20: 187-199.

Redshaw, M.R. and Lynch, S.S. (1974).
An improved method for the preparation of iodinated antigens for radioimmunoassay.
J. Endocr. 60: 527-528.

Reynolds, J.J., Dingle, J.T., Gudmundsson, T.V. and MacIntyre, I. (1968).
Bone resorption in vitro and its inhibition by calcitonin.
In: Symposium on Thyrocalcitonin and the C-Cells, pp. 223-229.
Ed. S.F. Taylor. London: Heinemann Medical Books.

Riniker, B., Neher, R., Maier, R., Kahnt, F.W., Byfield, P.G.H., Gudmundsson, T.V., Galante, L. and MacIntyre, I. (1968).
Menschliches calcitonin. I. Isolierung und charakerisierung.
Helv. Chim. Acta 51: 1738-1742.

Rittel, W., Brugger, M., Kamber, B., Riniker, B. and Sieber, P. (1968).
Thyrocalcitonin. III. Die syntheses des α-thyrocalcitonins.
Helv. Chim. Acta 51: 924-928.

Rittel, W., Maier, R., Brugger, M., Kamber, B., Riniker, B. and Sieber, P. (1976).
Structure-activity relationship of human calcitonin. III. Biological activity of synthetic analogues with shortened or terminally modified peptide chains.
Experientia 32: 246-248.

Robinson, C.J., Martin, T.J. and MacIntyre, I. (1966).
Phosphaturic effect of thyrocalcitonin.
Lancet ii: 83-84.

Robinson, C.J., Martin, T.J., Matthews, E.W. and MacIntyre, I. (1967).
Mode of action of thyrocalcitonin.
J. Endocr. 39: 71-79.

Robinson, C.J., Matthews, E.W. and MacIntyre, I. (1969).
The effect of parathyroid hormone and thyrocalcitonin on the intestinal absorption of calcium and magnesium.
In: Les Tissues Calcifies, Vth Symposium Europeen, pp. 279-282.

Rojanasthit, S., Rosenberg, E. and Haddad, J.G. (1974).
Paget's bone disease: response to human calcitonin in patients resistant to salmon calcitonin.
Lancet ii: 1412-1415.

Roos, B.A., Okano, K. and Deftos, L.J. (1974).
Evidence for a procalcitonin.
Biochem. Biophys. Res. Commun. 60: 1134-1140.

Rosselin, G., Assan, R., Yalow, R.S. and Berson, S.A. (1966).
Separation of antibody-bound and unbound peptide hormones labelled with iodine-131 by talcum powder and precipitated silica.
Nature (Lond.) 212: 355-357.

Russell, R.G.G. and Fleisch, H. (1968).
The renal effects of thyrocalcitonin in the pig and dog.
In: Calcitonin, Proceedings of Symposium on Thyrocalcitonin and C-Cells, pp. 297-305. Ed. S. Taylor. London: Heinemann Medical Books.

Sachs, D.Y. and Winn, H.J. (1970).
The use of glutaraldehyde as a coupling agent for ribonuclease and bovine serum albumin.
Immunochemistry 7: 581-585.

Salacinski, P., Hope, J., McLean, C., Clement-Jones, V., Sykes, J., Price, J. and Lowry, P.J. (1979).
A new simple method which allows theoretical incorporation of radioiodine into proteins and peptides without damage.
J. Endocr. 81: 131P.

Samaan, N.A., Hill, C.S., Jr., Beciero, J.R. and Schultz, P.N. (1973).
Immunoreactive calcitonin in medullary carcinoma of the thyroid and in maternal and cold serum.
J. Lab. Clin. Med. 31: 671-681.

Schimke, R.N. and Hartmann, W.H. (1965).
Familial amyloid-producing medullary thyroid carcinoma and pheochromocytoma: a distinct genetic entity.
Ann. Int. Med. 63: 1027-1039.

Schimke, R.N., Hartmann, W.H., Prout, T.E. and Rimoin, D.L. (1965).
Syndrome of bilateral pheochromocytoma, and medullary thyroid carcinoma, and multiple neuromas.
New Engl. J. Med. 279: 1-7.

Schwyzer, R. (1968).
Relationship of structure to activity of polypeptide hormones.
In: Protein and Polypeptide Hormones, pp. 201-207.
Ed. M. Margoulies. Amsterdam: Excerpta Medica Foundation.

Sela, M., Schechter, B., Schechter, I. and Borek, F. (1967).
Antibodies to sequential and conformational determinants.
Cold Spring Harbor Symposia on Quantitative Biology 32: 537-545.

Sieber, P., Brugger, M., Kamber, B., Riniker, B. and Rittel, W. (1968).
Menschliches calcitonin. IV. Die synthese von calcitonin M.
Helv. Chim. Acta 51: 2057-2061.

Sieber, P., Brugger, M., Kamber, B., Riniker, B., Rittel, W., Maier, R. and Staehelin, M. (1970).
Synthesis and biological activity of peptide sequences related to porcine α-thyrocalcitonin.
In: Calcitonin 1969, Proceedings of the 2nd International Symposium, pp. 28-33. Ed. S.F. Taylor. London: Heinemann Medical Books.

Silva, O.L., Becker, K.L., Primack, A., Doppman, J. and Snider, R.H. (1973).
Ectopic production of calcitonin.
Lancet ii: 317.

Silva, O.L., Becker, K.L., Primack, A., Doppman, J. and Snider, R.H. (1974) (a).
Ectopic secretion of calcitonin by oat-cell carcinoma.
New Engl. J. Med. 290: 1122-1124.

Silva, O.L., Becker, K.L., Snider, R.H. and Shaloub, R.J. (1974) (b).
Serum calcitonin in renal disease.
Clin. Res. 22: 720A.

Silva, O.L., Snider, R.H. and Becker, K.L. (1974) (c).
Radioimmunoassay of calcitonin in human plasma.
Clin. Chem. 20: 337-339.

Sipple, J.H. (1961).
The association of pheochromocytoma with carcinoma of the thyroid.
Amer. J. Med. 31: 163-166.

Singer, F.R., Aldred, J.F., Neer, R.M., Krane, S.M., Potts, J.T. and Bloch, K.J. (1972).
An evaluation of antibodies and clinical resistance to salmon calcitonin.
J. Clin. Invest. 51: 2331-2338.

Singer, F.R. and Habener, J.F. (1974).
Multiple immunoreactive forms of calcitonin in human plasma.
Biochem. Biophys. Res. Commun. 61: 710-716.

Singer, F.R., Melvin, K.E.W. and Mills, B.G. (1976).
Acute effects of calcitonin on osteoclasts in man.
In: Molecular Endocrinology, Proceedings of Endocrinology 1975, Clin. Endocr. 5, Suppl., pp. 333s-340s.
Eds. I. MacIntyre and A.G.E. Pearse. London: Blackwell Scientific Publications.

Sizemore, G.W., Go, V.L.W., Kaplan, E.L., Sanzenbacher, L.J., Holtermullen, K.H. and Arnaud, C.D. (1973).
Relations of calcitonin and gastrin in Zollinger-Ellison syndrome and medullary carcinoma of the thyroid.
New Engl. J. Med. 288: 641-644.

Sizemore, G.W., Heath III, H. and Larson, J.M. (1975).
Immunochemical heterogeneity of calcitonin in plasma of patients with medullary thyroid carcinoma.
J. Clin. Invest. 55: 1111-1118.

Staehelin, M. (1972).
The calcitonins: an example of unusual evolution.
J. Molec. Evol. 1: 258-262.

Steiner, D.F. and Oyer, P.E. (1967).
The biosynthesis of insulin and a possible precursor of insulin by a human islet cell adenoma.
Proc. Natl. Acad. Sci. U.S.A. 57: 473-481.

Stevenson, J.C., Hillyard, C.J., MacIntyre, I., Cooper, H. and Whitehead, M.I. (1979).
A physiological role for calcitonin: protection of the maternal skeleton.
Lancet ii: 769-770.

Talmage, R.V., Grubb, S.A., Norimatsu, H. and Vanderwiel, C.J. (1980).
Evidence for an important physiological role for calcitonin.
Proc. Natl. Acad. Sci. U.S.A. 77: 609-613.

Tashjian, A.H., Jr., Howland, B.G., Melvin, K.E.W. and Hill, C.S., Jr. (1970).
Immunoassay of human calcitonin clinical measurement, relation to serum calcium and studies in patients with medullary thyroid carcinoma.
New Engl. J. Med. 283: 890-895.

Tashjian, A.H., Wolfe, H.J. and Voelkel, E.F. (1974).
Human CT. Immunologic assay, cytological localization, and studies on medullary thyroid carcinoma.
Amer. J. Med. 56: 840-849.

Tauber, S.D. (1967).
The ultimobranchial origin of thyrocalcitonin.
Proc. Nat. Acad. Sci. U.S.A. 58: 1684-1687.

Utiger, R.D., Parker, M.L. and Daughaday, W.H. (1962).
Studies on human growth hormone. I. A radioimmunoassay for human growth hormone.
J. Clin. Invest. 41: 254-261.

Van der Donk, J.A., Lips, C.J.M., Hackeng, W.H.L., Van Dam, R.H. and Goudswaard, J. (1978).
The synthesis of calcitonin: some characteristics of its precursors in man and rat.
J. Mol. Med. 3: 95-104.

van Houten, M., Posner, B.I., Kopriwa, B.M. and Brawer, J.R. (1980).
Insulin binding sites localized to nerve terminals in rat median eminence and arcuate nucleus.
Science 207: 1081-1083.

Vaitukaitis, J., Robbins, J.B., Nieschlag, E. and Ross, G.I. (1971).
A method for producing specific antisera with small doses of immunogen.
J. Clin. Endocrinol. Metab. 33: 988-991.

Watzka, M. (1933).
Vergleichende untersuchungen uber den ultimobranchialen korper.
Zeitschrift f. mikr. anat. Forschung 34: 485-533.
Translated by Oonagh Prendiville.

Weber, E., Voigt, K.H., Mains, R.E. and Eipper, B.A. (1979).
Calcitonin is not contained within the common precursor to corticotropin and endorphin in the rat.
Biochem. Biophys. Res. Commun. 89: 360-367.

Wermer, P. (1963).
Endocrine adenomatasis and peptic ulcer in a large kindred: inherited multiple tumours and mosaic pleiotropism in man.
Amer. J. Med. 35: 205-212.

Wide, L. and Porath, J. (1966).
Radioimmunoassay of proteins with the use of Sephadex-coupled antibodies.
Biochem. Biophys. Acta 130: 257-260.

Wide, L. (1971).
Solid phase antigen-antibody system.
In: Radioimmunoassay Methods, pp. 405-412. Eds. K.E. Kirkham and W.M. Hunter. Edinburgh: Churchill Livingstone.

Williams, E.D. (1965).
A review of 17 cases of carcinoma of the thyroid and pheochromocytoma.
J. Clin. Pathol. 18: 288-292.

Williams, E.D. (1966).
Histogenesis of medullary thyroid carcinoma of the thyroid.
J. Clin. Path. 19: 114-118.

Wong, G.L. and Cohn, D.V. (1975).
Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces.
Proc. Natl. Acad. Sci. U.S.A. 72: 3167-3171.

Woodhouse, N.J.Y., Bordier, P., Fisher, M., Joplin, G.F., Reiner, M., Kalu, D.N., Foster, G.V. and MacIntyre, I. (1971).
Human calcitonin in the treatment of Paget's disease.
Lancet i: 1139-1143.

Yalow, R.S. and Berson, S.A. (1959).
Assay of plasma insulin in human subjects by immunological methods.
Nature (Lond.) 184: 1648-1649.

Yalow, R.S. and Berson, S.A. (1960).
Immunoassay of endogenous plasma insulin in man.
J. Clin. Invest. 39: 1157-1175.

Ziegler, R., Holz, G., Raue, F., Minne, H. and Delling, G. (1977).
Therapeutic studies with human calcitonin.
In: Human Calcitonin and Paget's Disease, pp. 167-177.
Ed. I. MacIntyre. Bern: Hans Huber Publishers.

APPENDIX

MATERIALS AND EQUIPMENT

MATERIALS AND EQUIPMENT

Synthetic human calcitonin, its fragments and analogues, bovine insulin, Synacthen (corticotrophin-(1-24)-tetracosapeptide) were kindly supplied by Dr. W. Rittel, CIBA-GEIGY A.G., Basle, Switzerland.

Synthetic salmon calcitonin for iodination was kindly supplied by Armour Pharmaceutical Company Ltd., Hampden Park, Eastbourne, East Sussex.

MRC and WHO standard preparations of human and salmon calcitonin were donated by the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, NW3 6RB.

Materials

Amberlite CG 400	:	BDH Chemicals Ltd., Poole, Dorset, England.
Co:Pell ODS	:	Whatman Ltd., Springfield Mill, Maidstone, Kent, U.K.
Charcoal (Norit SX2) replaced by activated charcoal (Sigma)	:	Sigma (London) Chemical Company Ltd., Fancy Road, Poole, Dorset, England.
Dextran T 70	:	Pharmacia (Great Britain) Ltd., Prince Regent Road, Hounslow, Middlesex, U.K.
Freunds complete adjuvent	:	Difco Laboratories, Detroit 1, Michigan, U.S.A.

Gel chromatography materials:

- Bio-gel P-30	:	Bio-Rad Laboratories Ltd., Caxon Way, Watford, Hertfordshire, England.

- Sephadex G-50 superfine	:	Pharmacia (Great Britain) Ltd., Prince Regent Road, Hounslow, Middlesex, U.K.
Glutaraldehyde	:	Sigma (London) Chemical Company Ltd., Fancy Road, Poole, Dorset, England.
^{125}I (Iodine-125)	:	The Radiochemical Centre, Amersham, P.O. Box 16, Buckinghamshire, HP7 OYB, England.
Porasil A (35-70 µm)	:	Water Associates, Northwich, Cheshire, England.
Octadecyltrichlorosilane	:	Aldrich Chemical Co. Ltd., The Old Brickyard, New Road, Gillingham, Dorset, England.
Ovalbumin	:	Sigma (London) Chemical Company Ltd., Fancy Road, Poole, Dorset, England.
Trasylol (Aprotinin-Proteinase Inhibitor)	:	Bayer U.K. Ltd., Pharmaceutical Division, Haywards Heath, West Sussex, U.K.
Trifluoroacetic acid Laboratory grade	:	BDH Chemicals Ltd., Poole, Dorset, U.K.

Equipment

Chance "Repette"	:	Jencons (Scientific) Ltd., Mark Road, Hemel Hempstead, Hertfordshire, England.
Cold tray	:	ChemLab Instruments, Ilford, Essex, U.K.
Diluter (LKB-Automatic 2075 diluter)	:	LKB Instruments Ltd., Addington Road, South Croydon, Surrey, England.
Fraction collector (Ultrorac)	:	LKB Instruments Ltd., Addington Road, South Croydon, Surrey, England.
Gamma Counters:		
- Auto-Gamma scintillation: spectrometer Packard 5160		Packard Instruments Ltd., Church Road, Caversham, Reading, Berkshire, U.K.

- NE 1600 Gamma counter provides simultaneous counting of 16 tubes	:	Nuclear Enterprises Ltd., Sighthill, Edinburgh, Scotland.
High pressure pump (Milton-Roy)	:	Dosapro Milton-Roy (U.K.) Ltd., Chertsey, Surrey, U.K.
"Hamilton" repeating syringes i) Capacity 5 ml delivers 100 µl ii) Capacity 2.5 ml delivers 50 µl	:	V.A. Howe, Peterborough Road, Fulham, London, S.W.6., England.
Luckham LP3 tubes and caps	:	Luckham Ltd., Labro Works, Victoria Gardens, Burgess Hill, Sussex, U.K.
Magnetic stirrer/ thermostat hot plate (Gallenkamp)	:	A. Gallenkamp & Co. Ltd., Christopher Street, London, E.C.2., U.K.
Partisil ODS analytical column for HPLC and accessories	:	Whatmann Ltd., Springfield Mill, Maidstone, Kent, U.K.
Pen recorder (LKB 2210, 1-channel recorder)	:	LKB Instruments Ltd., Addington Road, South Croydon, Surrey, England.
Spectrophotometer CE 212A ultraviolet variable wave length	:	Cecil Instruments Ltd., Milton Industrial Estate, Cambridge Road, Milton, Cambridge, U.K.
Racks to fit Luckham tubes (hold 13 tubes)	:	Kartell Plastic (U.K.) Ltd., Unit L, Broad Lane, Cottenham, Cambridge, U.K.

PUBLICATIONS

Secretion of immunoreactive calcitonin by human breast carcinomas

R C COOMBES, G C EASTY, S I DETRE, C J HILLYARD, U STEVENS, S I GIRGIS, L S GALANTE, L HEYWOOD, I MACINTYRE, A M NEVILLE

Summary

Twenty-three out of 28 patients with metastatic breast carcinoma and one out of 13 patients with localised disease had raised levels of plasma immunoreactive calcitonin. Monolayer cultures of breast carcinomas maintained for up to 10 weeks released immunoreactive calcitonin, and a primary breast carcinoma passaged in "nude" mice for over a year contained material immunologically and chromatographically resembling the monomeric form of human calcitonin.

These studies indicate that breast carcinomas can produce calcitonin and that plasma calcitonin measurements may be useful in staging patients with breast carcinomas.

Introduction

Extrathyroidal secretion of calcitonin has been suggested by finding increased concentrations of immunologically or biologically active hormone in a variety of endocrine tumours[1-3] and an oat-cell carcinoma of the bronchus.[4] Using extraction procedures we have detected immunoreactive calcitonin in non-endocrine tumours, including a squamous-cell carcinoma of the bronchus and seven out of eight consecutive breast carcinomas.[5] The latter association is of particular interest since patients with breast carcinoma may have high levels of circulating immunoreactive calcitonin.[6]

This observation, and the urgent need for a satisfactory marker for breast cancer,[7] prompted us to carry out studies in vitro and in vivo to establish whether calcitonin is actually produced by breast carcinomas, and not simply stored or adsorbed by them. We also wanted to determine whether calcitonin estimations could be useful in the staging of this disease.

Patients and methods

Fasting venous blood samples were obtained from 72 patients with breast disease. The samples were placed on ice and immediately centrifuged for 10-20 minutes, and the plasma was separated and stored at −20°C until immunoassay, which was within two weeks of collection.

Thirty-five consecutive patients were awaiting surgery at the Royal Marsden Hospital, 17 consecutive patients were attending the radiotherapy department of Hammersmith Hospital for postoperative radiotherapy, and the remaining 20 patients were inpatients on the radiotherapy wards. All except two were normocalcaemic and were not suffering from renal failure, liver failure, or any other form of malignant or endocrine disease known to be associated with hypercalcitoninaemia.

Each patient was placed in one of four groups depending on the histology of the tumour removed and the presence or absence of distant metastases as disclosed by full biochemical profile, bone and liver scans, bone marrow aspiration, and skeletal survey. Fourteen patients had benign disease, 13 had cancer localized to breast and lymph nodes, 17 were recovering from mastectomy, and 28 had metastatic disease. Those patients from whom samples were obtained three to four weeks after mastectomy had no evidence of metastatic disease as determined by these criteria. The control group of 69 patients has been described.[6]

PREPARATION OF MONOLAYER CULTURES OF BREAST CARCINOMAS

Twenty-one consecutive breast tumours were studied: 15 were histologically proved carcinomas and six were fibroadenomas (see table). Tumour tissue, removed at operation, was immediately placed in medium 199 (Bio-Cult Labs) containing 0·05% penicillin and

Unit of Human Cancer Biology, Ludwig Institute for Cancer Research and Royal Marsden Hospital, London
R C COOMBES, MRCP, MRC clinical research fellow
G C EASTY, PHD, senior grade scientist
S I DETRE, BSC, junior technical officer
U STEVENS, technical officer
A M NEVILLE, MD, MRCPATH, professor of experimental pathology

Endocrine Unit, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0HS
C J HILLYARD, BSC, scientific officer
S I GIRGIS, MB, BCH, honorary research assistant
L S GALANTE, PHD, honorary assistant lecturer
L HEYWOOD, junior technician
I MACINTYRE, PHD, FRCPATH, professor and director of unit

0·05% streptomycin and delivered to the laboratory within two hours. Contaminating fat and connective tissue were removed. The remaining tumour was cut into 0·5-mm slices which were then reduced to a fine "mince," transferred to a sterile centrifuge tube containing medium 199, shaken manually for five minutes, and then allowed to stand for five minutes. The supernatant containing single and small aggregates of cells was removed with a Pasteur pipette, transferred to a 25-ml Falcon flask, and incubated with 5-ml medium RPMI 1640 with 10% fetal calf serum at 37°C. When the cells had attached (within three days) they were rinsed with medium RPMI 1640 containing 10% lamb serum and refed with this medium, which was replaced every two or three days. The cell-exposed media were stored at −30°C for subsequent calcitonin immunoassay. Selected cultures (1, 2, and 3, see table) were incubated for 24 hours with 10-mM dibutyryl cyclic adenosine monophosphate (AMP) (Sigma) or calcium chloride (7·5 mmol/l (15 mEq/l)).

CULTURE OF BREAST TUMOURS IN IMMUNE-DEFICIENT MICE

Athymic nude mice (obtained from the MRC Laboratory Animal Centre, Carshalton, and Anglia Laboratory Animals) were delivered to our laboratory pathogen-free and were housed in a sterile environment.

A breast tumour (grade 2, infiltrating and intraduct carcinoma) was obtained at operation, and blocks 2 × 2 × 3 mm were implanted subcutaneously into four sites of four female nude mice. These xenografts grew to an average size of 1 cm.[3] One mouse was killed seven months after the original implant and a 2 × 2 × 3-mm sample from each graft site was implanted into another nude mouse. After five months further growth had occurred and the tumour was removed and extracted with 0·1-M HCl.[4] Control mouse salivary gland and thyroid tissue from 11 BALB-C mice were extracted in a similar manner. A monolayer culture of some of the mouse-grown breast tumour was prepared (see above) and the medium immunoassayed for calcitonin, after incubation with the cells for three days.

CHROMATOGRAPHY

Media from breast carcinoma cultures 9 and 11 (see table) were collected over a week, pooled, and lyophilised after removing material of less than 1000 molecular weight by filtration using AMICON UM2 filters. This material and an extract of mouse-grown breast tumour and control mouse tissue were chromatographed using a G50 Sephadex column (130 × 1 cm) equilibrated with 0·1-M formic acid and 10% isopropanol (pH 2·2). The column was calibrated with dextran 2000 and ^{125}I-labelled synthetic human calcitonin. Fractions were collected at room temperature, lyophilised, and dissolved in 0·05-M phosphate buffer before immunoassay.

IMMUNOASSAY

The immunoassay for plasma calcitonin using an antiserum directed against synthetic human calcitonin has been reported elsewhere.[6] The immunoassay for carcinoembryonic antigen (CEA) has also been described,[8] and the method for human chorionic gonadotrophin (HCG) immunoassay was that of Vaitukaitis *et al*,[10] but using Burroughs-Wellcome antiserum RD01. Insulin was measured using a kit (Gruppo Lepetit, Italy).

For tissue culture samples, similar methods were followed but unincubated tissue culture medium was used in the standard curve in the place of plasma. Control culture medium from fibroblasts, incubated under similar conditions to the breast carcinomas, was included in each immunoassay; all samples were assayed in duplicate and in at least two dilutions.

Results

PLASMA IMMUNOREACTIVE CALCITONIN IN PATIENTS

Immunoreactive calcitonin was undetectable (<0·1 μg/l) in plasma samples from our control population of 69 subjects[6] and in the plasma of 14 patients with benign breast disease. Only one of 13 patients with apparently localised disease and three out of 17 patients who had had "curative" mastectomy had raised plasma calcitonin levels. One of the latter subsequently developed distant metastases. In contrast, 23 out of 28 patients with metastatic disease had high levels of circulating immunoreactive calcitonin (fig 1).

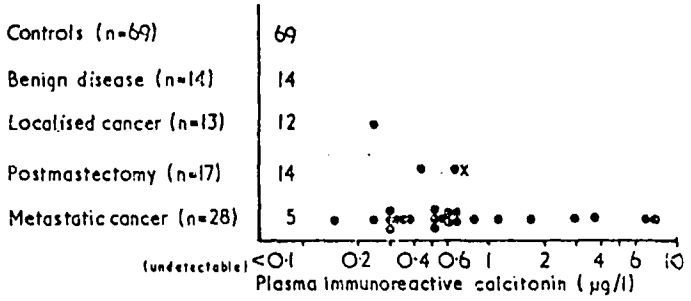


FIG 1—Plasma calcitonin in patients with breast disease. x = Patient who developed metastases three months after sample was taken.

BREAST TUMOUR MONOLAYER CULTURES

Within three to four days clusters of epithelial cells had settled on the plastic substrate of the Falcon flasks. Significant proliferation of the cultures of epithelial cells was rarely observed, but all cultures survived for at least two weeks and three survived for four to 10 weeks before overgrowth by fibroblasts occurred (see table).

Eight out of 15 cultures of breast carcinoma cells released a material which, in at least two dilutions, inhibited the binding of ^{125}I-labelled calcitonin to antibody in a similar manner to synthetic human calcitonin. In contrast, immunoreactive material was not detected in media from fibroadenoma or fibroblast cultures which were incubated under identical conditions.

Three cultures continued to release hormone for four, six, and ten weeks respectively. In these cultures the rate of production of immunoreactive calcitonin was not altered by incubation with 10-mM dibutyryl cyclic AMP or by increasing the calcium concentrations of the medium incubated with the cells. Two out of six breast carcinomas also produced a material which inhibited the binding of ^{125}I-labelled CEA to anti-CEA, and three out of 12 released immunoreactive HCG. Immunoreactive insulin was not detected in any of the three cultures tested (N1, 2, and 3).

Gel filtration of medium incubated with breast carcinoma cultures 9 and 11 showed that some immunoreactive material was eluted at a similar position to ^{125}I-labelled calcitonin, some eluted before, and some later, indicating the presence of multiple immunoreactive forms.

TABLE I—*Breast tumour monolayer cultures*

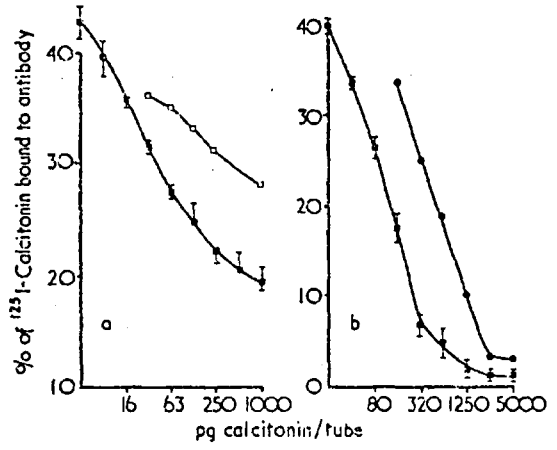
Culture No.	Tumour*	Time over which calcitonin was measured (weeks)	Maximum calcitonin concentration (μg l medium^{-1} 24 h^{-1})	Other products measured	
				CEA (μg l^{-1} 24 h^{-1})	HCG (U l^{-1} 24 h^{-1})
1	Carcinoma	10	1·2	ND	ND
2	Carcinoma	4	1·0	ND	1·6
3	Carcinoma	6	0·76	66	ND
4	Carcinoma†	2	ND		
5	Carcinoma	2	ND	ND	ND
6	Carcinoma	2	ND	ND	20
7	Carcinoma	2	1·25		ND
8	Carcinoma	2	ND		ND
9	Carcinoma†	1	0·92		8·6
10	Carcinoma	2	1·28		ND
11	Carcinoma	1	0·16		
12	Carcinoma‡	2	1·0		ND
13	Carcinoma†	2	ND		ND
14	Carcinoma	2	ND	100	
15	Carcinoma	2	ND		ND
16–21	Fibro-adenomas	2	ND		ND (2)

*Unless otherwise stated all carcinomas were infiltrating ductal carcinomas.
†Mucus-secreting carcinomas.
‡Medullary carcinoma.
ND = Not detectable.

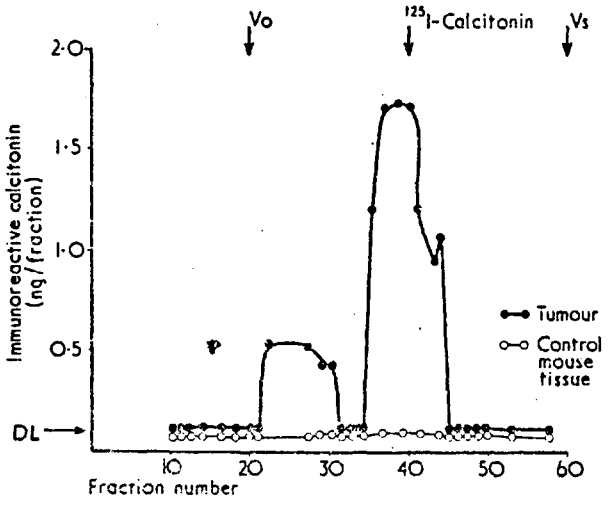
BREAST TUMOUR GROWN IN NUDE MICE

The breast xenograft studied had an identical histological appearance to the specimen removed from the patient at operation one year

earlier. The extract contained a material (20 ng/g wet tissue) which, in six dilutions, paralleled the standard curve of synthetic human calcitonin (fig 2b) in the immunoassay. When chromatographed, the immunoreactive material eluted as two peaks, one running before and one coincident with ^{125}I-labelled calcitonin (fig 3). An extract of control mouse tissue was similarly chromatographed, but no peak of immunoreactivity was found. The extract of mouse thyroid glands contained a material which, in several dilutions, inhibited the binding of ^{125}I-labelled calcitonin to antibody, but, in contrast to the xenograft extract, this inhibition did not parallel that produced by the synthetic standard (fig 2a). Subsequently monolayer culture of this tumour was shown to release a material which resembled synthetic human calcitonin immunologically (3-5 μg l tissue culture medium $^{-1}$day^{-1}).



IG 2—Comparison of displacement of ^{125}I-labelled calcitonin from antibody y synthetic human calcitonin (■—■) and by (a) mouse thyroid extract (□—□), and (b) mouse-grown human breast carcinoma extract (●—●).



G. 3—G50 elution pattern of extract of human breast tumour grown in ude mouse. DL = Detection limits of assay. Vo = Exclusion volume. s = Salt volume.

iscussion

Many plasma constituents circulate in abnormally high ncentrations in patients with advanced malignancies. Most ften these represent secondary effects of the tumour on normal ssues, but occasionally they represent release of substances by e neoplastic cell.[7]

Our results indicate that in the case of the association of reast carcinomas with calcitonin the tumours themselves roduce calcitonin. This is shown by the release of immuno- ctive calcitonin by eight out of 15 consecutive breast rcinoma monolayer cultures for up to 10 weeks and by the ility of a human breast carcinoma to release calcitonin after prolonged culture in immune-deficient mice. Calcitonin from this tumour resembled human calcitonin M and not mouse calcitonin, indicating that a "sponge" phenomenon[11] in which circulating calcitonin is adsorbed by the tumour could not account for our findings.

Other methods of showing hormone production by tumours, such as the measurement of an arteriovenous difference of hormone concentration across a tumour bed or incorporation of labelled amino-acids into peptide by tumour cells could be applied to calcitonin production by breast cancer. We believe, however, that the former method may lead to misleading results owing to fluctuation in levels, intermittent release of adsorbed products, and intra-assay variation. Difficulty in isolating and satisfactorily identifying a peptide after labelled amino-acid incorporation and the problems of ensuring adequate controls make the latter method even more difficult to achieve with any degree of reproducibility.

Using culture methods, we have shown for the first time that short-term monolayer culture systems of breast carcinoma can release peptides, and that immune-deficient mice can support not only the growth of human breast tumours but also the production of a peptide by this tumour. This is important for those investigating the functional pathology of tumours, since these culture systems could be of value in studying tumour products and further clarifying their relationship to tumour mass, suggested here by finding raised circulating levels in those patients with metastatic disease. Although the clinical data suggest that plasma calcitonin estimations may be useful in staging breast carcinoma patients, all except four of those with raised levels had metastatic disease which was evident clinically or on investigation. Furthermore, as is the case for several other possible markers for breast carcinoma, such as CEA[12], casein,[13] and HCG,[11 14] the test is not specific for breast carcinoma.

The data obtained from the monolayer cultures indicate, however, that an approach involving the use of several markers may well be valuable since these studies show that some tumours can secrete calcitonin, while others secrete HCG or CEA.

We thank Mr J-C Gazet, Dr T Powles and Dr H T Ford of the Royal Marsden Hospital, Sutton, and the consultants of the radiotherapy department, Hammersmith Hospital, for permission to study patients under their care.

These investigations were supported by programme and project grants from the Medical Research Council to IM and AMN. They were also supported by the Ludwig Institute for Cancer Research and the Wellcome Trust. RCC is in receipt of a Medical Research Council clinical research fellowship. We gratefully acknowledge the technical help of Miss T Billingsley.

Requests for reprints should be sent to RCC.

References

1 Milhaud, G, *et al*, *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences*, 1970, 29, 8775.
2 Milhaud, G, *et al*, in *Calcium, Parathyroid Hormone and the Calcitonins*, ed R V Talmage and P L Munson, p 56. Amsterdam, Excerpta Medica, 1972.
3 Voelkel, E F, *et al*, *Journal of Clinical Endocrinology and Metabolism*, 1973, 37, 297.
4 Silva, O L, *et al*, *New England Journal of Medicine*, 1974, 290, 1122.
5 Hillyard, C J, *et al*, *Clinical Endocrinology*, in press.
6 Coombes, R C, *et al*, *Lancet*, 1974, 1, 1080.
7 Neville, A M, and Cooper, E H, *Annals of Clinical Biochemistry*, in press.
8 Roos, B A, Okano, K, and Deftos, L J, *Biochemical and Biophysical Research Communications*, 1974, 60, 1134.
9 Laurence, D J R, *et al*, *British Medical Journal*, 1972, 3, 605.
10 Vaitukaitis, J L, Braunstein, G D, and Ross, G T, *American Journal of Obstetrics and Gynecology*, 1972, 113, 751.
11 Unger, R H, Lochner, J V, and Eisentraut, A M, *Journal of Clinical Endocrinology*, 1964, 24, 823.
12 Chu, T M, and Nemoto, T, *Journal of the National Cancer Institute*, 173, 51, 1119.
13 Hendrick, J C, and Franchimont, P, *European Journal of Cancer*, 1974, 10, 725.
14 McArthur, J W, *Progress in Obstetrics and Gynaecology*, vol IV, p 146. Heinemann, London, 1963.
15 Braunstein, G D, *et al*, *Annals of Internal Medicine*, 1973, 78, 39.

Printed in Great Britain by Qualitex Printing Limited, Cardiff

Clinical Endocrinology (1976) 5, Suppl., 387s-396s.

THE ECTOPIC SECRETION OF CALCITONIN BY LUNG AND BREAST CARCINOMAS

R. C. COOMBES, M. L. ELLISON, G. C. EASTY, C. J. HILLYARD,* R. JAMES, L. GALANTE,* S. GIRGIS,* L. HEYWOOD,* I. MACINTYRE* AND A. M. NEVILLE

*Unit of Human Tumour Biology, London Branch, Ludwig Institute of Cancer Research and Royal Marsden Hospital, London, and *Royal Postgraduate Medical School, London*

SUMMARY

Many patients with advanced non-thyroid malignancies have elevated plasma immunoreactive calcitonin concentrations. Breast and bronchial carcinomas contain immunoreactive calcitonin and an epidermoid bronchial carcinoma has been shown to produce immunoreactive calcitonin *in vitro*.

We have established monolayer cultures of breast carcinomas and eight out of fifteen consecutive carcinomas released immunoreactive calcitonin; some released HCG (human chorionic gonadotrophin) or CEA (carcinoembryonic antigen).

In addition, a primary human breast carcinoma has been shown to release and contain calcitonin after being passaged in 'nude' mice over 1 year.

Chromatography of extracts and culture media of a bronchial carcinoma demonstrated that, in contrast with the other tumours, it secreted a form or forms of calcitonin having size, charge and immunological differences when compared to calcitonin M.

Preliminary evaluation of plasma immunoreactive calcitonin estimations in patients with breast carcinoma showed that twenty-three out of twenty-eight patients with metastatic disease had elevated plasma calcitonin concentrations, whereas only one out of thirteen with localized disease had high levels.

The finding of elevated levels of circulating immunoreactive calcitonin in twenty-one out of forty-six unselected patients with a variety of advanced non-thyroid malignancies (Coombes *et al.*, 1974) is of considerable interest. Several reports (Milhaud *et al.*, 1970, 1972; Silva *et al.*, 1974a; Rosen & Weintraub, 1974) have shown that endocrine-type tumours contain increased amounts of immunologically or biologically active calcitonin, and this indicates that the elevated levels of calcitonin in the plasma of patients with these tumours may reflect ectopic production of calcitonin.

However, most of the patients in the series referred to above had non-endocrine-type

Correspondence: Dr R. C. Coombes, Unit of Human Tumour Biology, London Branch, Ludwig Institute of Cancer Research and Royal Marsden Hospital, London SW3 6JB.

tumours, and eight patients with abnormally high levels of circulating calcitonin had breast carcinomas. Furthermore, some extracts of breast carcinomas contain immunoreactive calcitonin and have hypocalcaemic activity in the rat bioassay system, whilst extracts of benign breast tumours do not (Hillyard *et al.*, 1976). In this same publication it was reported that an epidermoid bronchial carcinoma contained immunoreactive calcitonin; more recently a proliferative monolayer culture of a poorly differentiated epidermoid bronchial carcinoma has been shown to produce immunoreactive calcitonin (Ellison *et al.*, 1975).

Both these reports indicated that immunological differences existed between tumour and synthetic human calcitonin. These observations and the finding that circulating calcitonin in patients with medullary thyroid carcinomas is heterogeneous (Singer & Habener, 1974; Deftos *et al.*, 1975; Sizemore *et al.*, 1975) prompted us to carry out gel filtration studies of material released by a variety of tumours *in vitro*.

In addition, using different culture systems, we have sought to determine whether ectopic production of calcitonin by breast carcinomas can occur. We have also measured plasma immunoreactive calcitonin in a variety of patients with breast disease in order to determine whether any relationship exists between the level of immunoreactive calcitonin and the stage of disease.

MATERIALS AND METHODS

1. *Culture systems*

(a) *Monolayer cultures.* One medullary thyroid carcinoma, two bronchial carcinomas (one a poorly differentiated epidermoid and the other an oat cell carcinoma) and twenty-one unselected breast tumours were studied. Of the breast tumours, fifteen were carcinomas and six fibroadenomas. Routine histology was carried out in all cases. Tumour tissue, removed at operation, was immediately placed in medium 199 with antibiotics and delivered to the laboratory within 2 h.

The method for initiating the medullary carcinoma culture was similar to that for the bronchial carcinomas and has been previously described (Ellison *et al.*, 1975). The method for establishing the breast tumour monolayer cultures was as follows: contaminating fat and connective tissue was removed and the tumour tissue cut into 0·5 mm slices, which were then reduced to a fine 'mince', placed in medium 199, transferred to a sterile centrifuge tube, shaken manually for 5 min and then allowed to stand. The supernatant containing single and small aggregates of cells was removed with a pasteur pipette, transferred to a 25 ml Falcon Fask and incubated with 5 ml medium RPMI 1640 with either 10% fetal calf serum or 10% lamb serum.

The bronchogenic carcinoma cultures, in contrast to those of the medullary thyroid carcinoma and the breast tumours, were proliferative and medium was obtained for study at subculture passages 22–24 (the oat cell carcinoma) and 64–70 (poorly differentiated carcinoma). Medium from the breast tumours was collected at 2–3 day intervals for up to 10 weeks. The medium from the medullary thyroid carcinoma was collected within 2 weeks of establishing the monolayer culture.

Some flasks of medullary carcinoma cells were incubated with leucine-free medium 199 (Bio-Cult) and subsequently with 100 μCi ^{3}H-leucine (Radiochemical Centre, Amersham) with leucine-free medium for 6 h. Cells and medium were then separated and immediately frozen at -40°C.

When extracts of cells were required, they were extracted with 0·1 N HCl according to the method of Roos *et al.* (1974).

(b) *Culture of breast carcinoma in 'nude' mice.* 'Nude' mice (obtained from Laboratory Animal Centre, MRC, Carshalton; Anglia Laboratory Animals) were delivered to our laboratory pathogen-free and were housed in a sterile environment.

A breast tumour (grade II, infiltrating and intraduct carcinoma) was obtained at operation, and 2 × 2 × 3 mm blocks were implanted subcutaneously into four sites of four female nude mice. These xenografts grew to an average size of 1 cm^3. One mouse was killed 7 months after the original implant and a 2 × 2 × 3 mm sample from each graft site was implanted into another 'nude' mouse. After 5 months, further growth had occurred and the tumour was removed and extracted with 0·1 HCl (see above). Control mouse thyroid tissue from eleven BALB-C mice were extracted in a similar manner. A monolayer culture of some of the mouse-grown tumour was prepared (see above) and the medium assayed for calcitonin following incubation with the cells for three days.

2. *Chromatography*

Medium from both lung carcinoma cell lines was processed in batches of 50–500 ml using Amicon UM2 filters to remove material of less than 1000 MW and P30 filters to remove material of more than 30 000 MW, since no immunoreactive material could be detected in either of these two fractions.

Prior to sephadex chromatography of labelled material, free ^{3}H-leucine was removed by desalting on a 2 × 20 cm Bio-Gel P2 column.

A G-50 (superfine) sephadex column (130 × 1 cm) equilibrated with 0·1 M formic acid and 10% isopropanol (pH 2·2) was used and 3·4 ml fractions were collected at room temperature. The column was calibrated with Dextran 2000, ^{125}I-labelled synthetic human calcitonin and ^{125}I-labelled growth hormone. In some experiments, material was chromatographed after prior equilibration at 2 h at room temperature with either 8 M urea or 10 mM mercaptoethanol.

Ion-exchange chromatography was carried out using a 10 cm × 0·5 cm carboxymethylcellulose (CM52, Whatman) column with a linear gradient from 0·01 M ammonium acetate, pH 4·5, to 0·35 M ammonium acetate, pH 6·5. 3·3 ml fractions were collected.

Recovery of chromatographed material in both methods ranged from 60 to 70%. Fractions were frozen and lyophilized before immunoassay, or, in the case of ^{3}H-labelled samples, aliquots were prepared for liquid scintillation counting.

3. *Patients*

Fasting blood samples were obtained from seventy-two patients with breast disease. These were immediately placed on ice, centrifuged for 10–20 min, separated and the plasma stored at −20°C. They were immunoassayed within 2 weeks of collection.

Thirty-five consecutive patients were awaiting surgery at the Royal Marsden Hospital over a 6 week period; seventeen consecutive patients were attending the Radiotherapy Department of Hammersmith Hospital for post-operative radiotherapy and the remaining twenty patients were in-patients on the radiotherapy wards. They were normocalcaemic (with two exceptions) and were not suffering from renal failure, liver failure, or any other form of malignant or endocrine disease known to be associated with hypercalcitoninaemia.

Each patient was placed in one of four groups (Fig. 4) depending on the histology of the

tumour removed and the presence or otherwise of distant metastases as disclosed by full biochemical profile, bone and liver scans, bone marrow aspiration and skeletal survey. Those in group 3, i.e. the patients from whom samples were obtained 3–4 weeks following mastectomy, had no evidence of metastatic disease. The control group of sixty-nine patients has already been described (Coombes *et al.*, 1974).

4. *Radioimmunoassay*

The immunoassay for plasma calcitonin employing an antiserum directed against synthetic human calcitonin, has been reported elsewhere (Coombes *et al.*, 1974). For tissue culture samples, a similar method was followed but unincubated tissue culture medium was used in the standard curve in place of plasma.

Control culture medium from fibroblasts, incubated under similar conditions to the breast carcinomas, was included in each immunoassay.

Lyophilized chromatographic fractions were dissolved in 0·5 ml 0·05 M phosphate buffer and tissue extracts in 0·1 M formic acid and 0·05 M phosphate buffer (v/v 1 : 2·5) before immunoassay. All samples were assayed in duplicate in at least two dilutions.

The immunoassay for carcinoembryonic antigen (CEA) has been described (Laurence *et al.*, 1972) and the method for human chorionic gonadotrophin was that of Vaitukaitis *et al.* (1972), but using Burroughs–Wellcome antiserum RDO1.

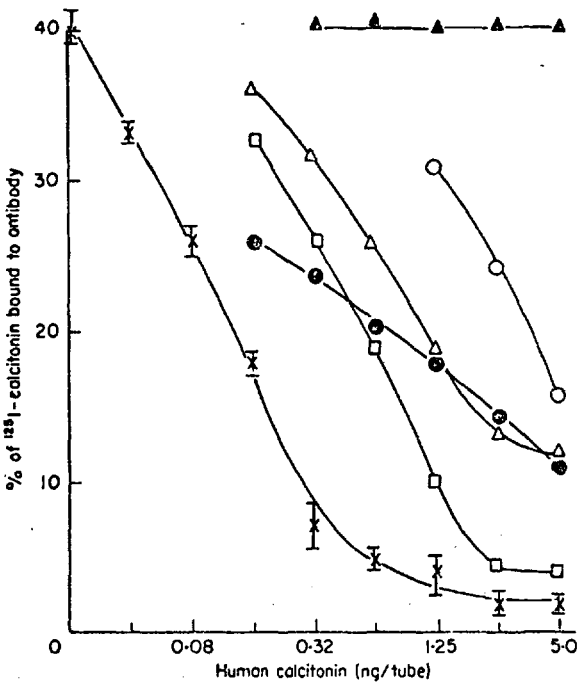


FIG. 1. Comparison of displacement of ^{125}I-labelled calcitonin from antibody by calcitonin and by culture medium or extracts of cultured cells of various malignancies. ▲, Fibroblasts; ○, medullary thyroid carcinoma (culture medium); ●, poorly differentiated bronchial carcinoma (medium or cell extract); △, oat cell bronchial carcinoma (culture medium); □, mouse-grown breast carcinoma; ×, standard.

RESULTS

(a) *Immunoassay of cultured materials*

The medullary carcinoma of the thyroid monolayer cultures released immunoreactive calcitonin into the culture medium in amounts ranging from 200 to 300 ng/ml of culture medium per 24 h. The oat cell bronchogenic carcinoma also released immunoreactive calcitonin (0·2–0·8 ng/ml of culture medium per 24 h) and both this material and the material released by the medullary thyroid carcinomas produced inhibition of binding of ^{125}I-labelled calcitonin in a similar manner to synthetic human calcitonin (Fig. 1). The proliferative epidermoid bronchogenic carcinoma cultures released material which, in contrast to that from the other cultures, was immunologically distinct from synthetic human calcitonin. Extracts of the cells behaved in the immunoassay in a similar way (Fig. 1). Eight out of fifteen monolayer cultures of breast carcinoma cells released a material which inhibited the binding of ^{125}I-labelled calcitonin to antibody (Table 1). We could not detect immunoreactive material in medium from breast fibroadenoma or fibroblast cultures, which were incubated under identical conditions. One monolayer culture of breast carcinoma cells released immunoreactive calcitonin for 10 weeks. Two out of six cultures also released immunoreactive CEA and three out of twelve released immunoreactive HCG.

TABLE 1. Products measured in medium from breast monolayer cultures

Tumour type	Time in culture (weeks)	Number releasing:		
		Calcitonin	CEA	HCG
Carcinoma	1–10	8/15	2/6	3/12
Fibroadenoma	1–2	0/6	—	0/2

The breast carcinoma xenograft cultured in 'nude' mice had an identical histological appearance to that of the specimen removed from the patient 1 year previously. The extract contained immunoreactive calcitonin (20 ng/g wet weight) (Fig. 1) and the monolayer prepared from this tumour released immunoreactive calcitonin (3–5 ng/ml medium/24 h). In contrast, the extract prepared from mouse thyroid glands was immunologically distinct from synthetic human calcitonin, although inhibiting the binding of ^{125}I-labelled calcitonin to the antiserum.

(b) *Chromatography*

G-50 Sephadex chromatography (Fig. 2) of the various materials discussed above clearly demonstrates that differences can exist between tumour-derived calcitonin and calcitonin M. Although a small amount of immunoreactive calcitonin elutes earlier, the predominant form of calcitonin secreted by the medullary carcinoma co-elutes with calcitonin M (Fig. 2a). The elution pattern of incorporated ^{3}H-leucine and immunoreactivity of extracts of this tumour confirms this finding (Fig. 3a).

In contrast, the main immunoreactive peak of the material secreted by the poorly

differentiated bronchogenic carcinoma occurs nearer the void volume of the G-50 column, which indicates the presence of larger-molecular-weight form or forms of the hormone. This elution pattern did not change following prior equilibration with 8 M urea or 10 mM mercaptoethanol (Fig. 2b). Ion-exchange chromatography also indicated that this material was distinct from the major peak of calcitonin secreted by the medullary thyroid carcinoma.

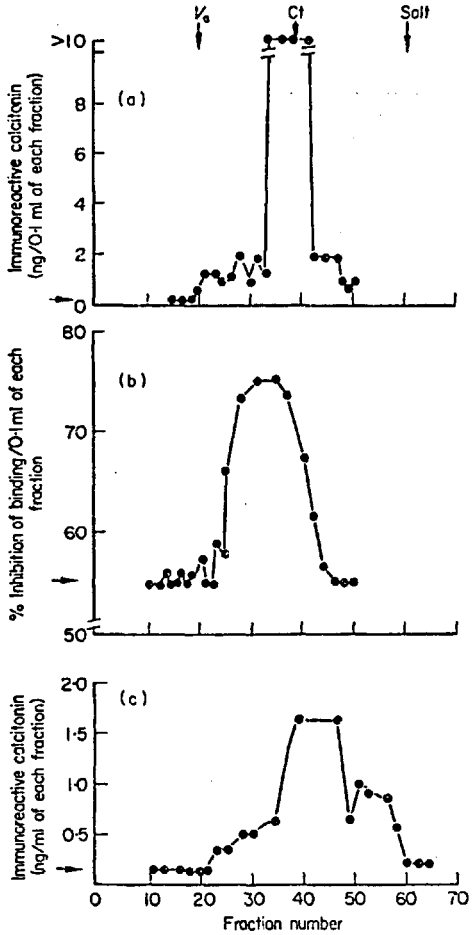


FIG. 2. Sephadex G-50 elution patterns of culture media from monolayer cultures. (a) Medullary carcinoma of the thyroid; (b) poorly differentiated epidermoid carcinoma of the bronchus; (c) oat cell carcinoma of the bronchus. →, Detection limits of assay. Ct, Elution position of ^{125}I-labelled calcitonin.

Most of the immunoreactive material secreted by the oat-cell bronchogenic carcinoma co-eluted with ^{125}I-labelled calcitonin but there was a peak occurring later than this, indicating the presence of smaller-molecular-weight forms (Fig. 2c). The elution pattern of the mouse-grown breast carcinoma extract indicated that the majority of the immunoreactive material co-eluted with ^{125}I-labelled calcitonin (Fig. 3b).

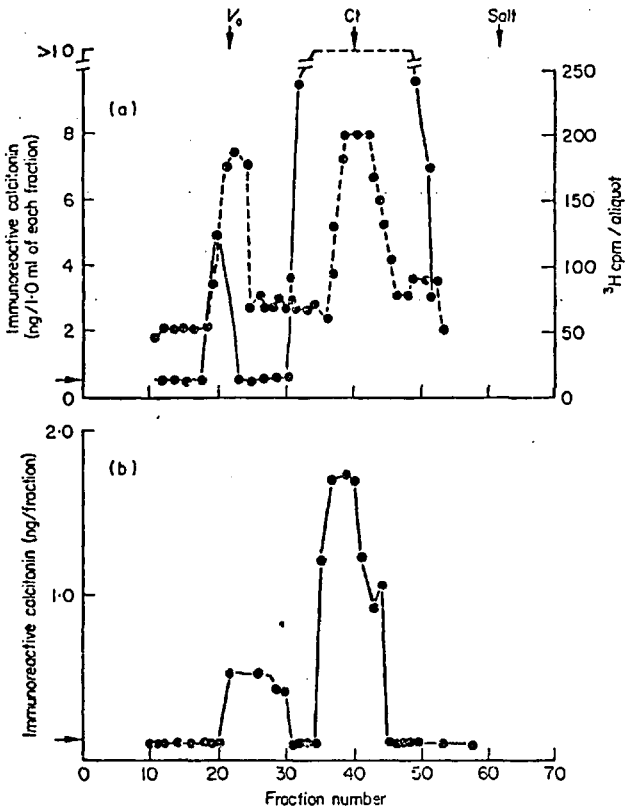


FIG. 3. Sephadex G-50 elution patterns of extracts of tumour cells. (a) Medullary carcinoma of the thyroid; ——, immunoreactive calcitonin; – – –, ^{3}H cpm/aliquot. (b) Mouse-grown human breast carcinoma. Ct, Elution position of ^{125}I-labelled calcitonin. →, Detection limits of assay.

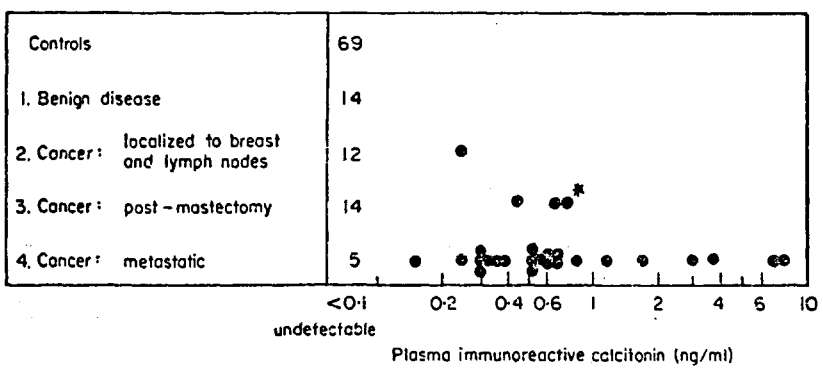


FIG. 4. Plasma calcitonin in patients with breast disease. * Developed metastases 3 months later. (From *British Medical Journal*, by permission of the Editor.)

(c) *Clinical studies* (Fig. 4)

Immunoreactive calcitonin was undetectable (<0·1 ng/ml) in plasma samples obtained from our control population of sixty-nine subjects (Coombes *et al.*, 1974) and in the plasma of fourteen patients with benign breast disease. Only one out of thirteen patients with apparently localized disease and three out of seventeen patients following mastectomy had elevated levels of calcitonin. One of the latter group has subsequently developed clinically obvious metastases. In contrast to these groups, twenty-three out of twenty-eight patients with metastatic disease had high levels of circulating immunoreactive calcitonin.

DISCUSSION

Hypercalcitoninaemia has been reported in patients with several conditions, including endocrine-type tumours (Milhaud *et al.*, 1974; Silva *et al.*, 1974a), hypercalcaemia (Tashjian *et al.*, 1970), hypocalcaemia (Deftos *et al.*, 1973), renal failure (Silva *et al.*, 1974b), acute pancreatitis (Marshall *et al.*, 1975) and Zollinger–Ellison syndrome (Sizemore *et al.*, 1973). Many other conditions will probably be added to this list.

Thus, our original finding of hypercalcitoninaemia in patients with a variety of advanced common malignancies (Coombes *et al.*, 1974) could have been due to factors other than secretion of the hormone by the tumour. In addition to this, patients with disseminated malignancy tend to have elevated levels of many normal and abnormal plasma constituents (Neville & Cooper, 1976) and thus artefacts of radioimmunoassay may be considered to occur with greater frequency in this group of patients. For these reasons, in addition to clinical studies, a multidisciplinary approach involving different aspects of tissue culture and chemical techniques is essential to evaluate tumour 'products'. In this way we may hope to determine whether the 'product' is actually secreted by the tumour and not by the normal source as a reaction to the tumour's presence. Chemical studies are important to establish whether any differences occur between 'native' and tumour-derived materials since, if this is the case, specific methods of measuring the tumour product could be developed.

In the case of calcitonin, we feel we have demonstrated production of the hormone by breast and lung carcinomas since both tumour types have been shown to be capable of releasing the hormone after prolonged maintenance in culture systems. This effectively rules out the release of stored or adsorbed material by breast or lung carcinoma tissues.

Preliminary data presented here indicates that although in some cases the size and immunological characteristics of the predominant form of tumour calcitonin are similar to calcitonin M, this is not always the case. The material secreted by the poorly differentiated epidermoid bronchial carcinoma clearly has size, charge and immunological differences from calcitonin M. This material may be heterogeneous and may well be similar to the earlier eluting immunoreactive forms of the hormone secreted by the medullary thyroid carcinoma.

Thus, we have not yet established that this tumour-product is distinct from 'native' hormone. Ectopic 'big ACTH' appears identical to 'big ACTH' found in normal pituitaries (Yalow & Berson, 1971) and 'big gastrin' isolated from pancreatic tumours has its counterpart in the normal subject (Yalow & Berson, 1970), and so it seems reasonable to suppose that 'big calcitonin' may well be similar in this respect.

The reason for our findings concerning the association of calcitonin with breast car-

cinoma is not clear. Medullary carcinoma of the thyroid and oat cell lung carcinomas are composed of cells with 'APUD' characteristics and some cells with these properties are associated with calcitonin production. Clear cells with argyrophilic properties have been described in normal breast and in breast tumours (Feyrter, 1972); but breast carcinoma tissue with a high content of immunoreactive calcitonin, and monolayer cultures releasing immunoreactive calcitonin were examined specifically for the histochemical and electron microscopic characteristics of 'APUD' cells by Dr J. Polak and Miss S. Van Noorden (Pearse & Polak, 1974): these properties could not be demonstrated. Although these findings do not exclude calcitonin production by 'APUD' cells in such tumours, alternative explanations of calcitonin production must be considered. These include the expression of genes usually repressed or the occurrence of tumours arising in primitive and undifferentiated cells (Cohnheim, 1889).

The suggestion has been made that measurements of plasma immunoreactive calcitonin in malignancy could assist clinical management (Coombes *et al.*, 1974; Silva *et al.*, 1974a). The preliminary data presented here concerning the relationship of plasma calcitonin concentrations to the stage of breast carcinoma indicate that this may well be the case. The patients with metastatic disease tended to have elevated levels whereas those with 'localized' disease generally had undetectable levels of plasma calcitonin. Of particular interest is the patient who had an elevated plasma immunoreactive calcitonin concentration following mastectomy and subsequently developed clinically obvious metastases.

The tissue culture preparations release not only immunoreactive calcitonin but also CEA and HCG, and since some tumours release different materials, a multiparametric approach to monitoring breast cancer, involving measurement of calcitonin, CEA and HCG may be more valuable than measurement of a single substance.

Finally, the relationship of calcitonin production to the development of abnormalities in calcium metabolism which may complicate cancer, is not clear. However, serum calcium and calcium absorption and excretion in a group of patients with malignancy having undetectable levels of calcitonin did not differ from those having elevated levels, but the mean ^{47}Ca space (a measurement of bone turnover rate) of those with raised circulating calcitonin was considerably higher than the other patients (Coombes *et al.*, to be published). This association could simply mean that patients with higher plasma calcitonin levels are likely to have a greater tumour mass (and hence skeletal deposits) and therefore concomitant elevation of the ^{47}Ca space.

ACKNOWLEDGMENTS

We thank Miss T. Billingsley and Mrs D. Lamb for their invaluable assistance with the culture studies. R.C.C. is in receipt of a Medical Research Council Clinical Research Fellowship. This work was supported by Programme and Project grants to I.M. from the M.R.C. and to A.M.N. from the M.R.C. and the Ludwig Institute for Cancer Research.

REFERENCES

COHNHEIM, J. (1889) Lectures in general pathology (transl. by A. B. McKee). *The New Sydenham Society, London*, **7**, 789.

COOMBES, R.C., HILLYARD, C.J., GREENBERG, P.B. & MACINTYRE, I. (1974) Plasma immunoreactive calcitonin in patients with non-thyroid tumours. *Lancet*, **i**, 1080–1083.

DEFTOS, L.J., POWELL, D., PARTHEMORE, J.G. & POTTS, J.T., JR (1973) Secretion of calcitonin in hypocalcaemic states in man. *Journal of Clinical Investigation*, **52**, 3109–3114.

DEFTOS, L.J., ROOS, B.A., BRONZERT, D. & PARTHEMORE, J.G. (1975) Immunochemical heterogeneity of calcitonin in plasma. *Journal of Clinical Endocrinology and Metabolism*, **40**, 409–412.

ELLISON, M., WOODHOUSE, D., HILLYARD, C., DOWSETT, M., COOMBES, R.C., GILBY, E.D., GREENBERG, P.B. & NEVILLE, A.M. (1975) Immunoreactive calcitonin production by human lung carcinoma cells in culture. *British Journal of Cancer*, **32**, 373–379.

FEYRTER, F. (1972) The clear cell system: the peripheral endocrine (paracrine) glands. *Endocrinology 1971: Proceedings of Third International Symposium* (ed. by Selwyn Taylor), pp. 134–144. Heinemann, London.

HILLYARD, C.J., COOMBES, R.C., GREENBERG, P.B., GALANTE, L.S. & MACINTYRE, I. (1976) Calcitonin in breast and lung cancer. *Clinical Endocrinology*, **5**, 1–8.

LAURENCE, D.J.R., STEVENS, U., BETTELHEIM, R., DARCY, D., LEESE, C., TURBERVILLE, C., ALEXANDER, P., JOHNS, E.W. & NEVILLE, A.M. (1972) Role of plasma carcinoembryonic antigen in diagnosis of gastrointestinal, mammary and bronchial carcinoma. *British Medical Journal*, **iii**, 605–609.

MARSHALL, J.P., WINNACKER, J.L. & ANAST, C.S. (1975) Hypercalcitoninemia in acute pancreatitis. (Abstract). *Clinical Research*, **23**, 325A.

MILHAUD, G., CALMETTE, C., RAYMOND, J.P., BIGNON, J. & MOUKHTAR, M.S. (1970) Carcinoid secretant de la thyrocalcitonin. *Compte Rendu Hebdomadaire des Séances de l'Académie des Sciences*, D, **270**, 2195.

MILHAUD, G., CALMETTE, C., JULIENNE, A., THARAUD, D., BLOCH-MICHEL, H., CAVAILLON, J.P., COLIN, R. & MOUKHTAR, M.S. (1972) A new chapter in human pathology: calcitonin disorders and therapeutic uses. *Calcium, Parathyroid Hormone and the Calcitonin* (ed. by R. V. Talmage and P. L. Munson), p. 56. Excerpta Medica, Amsterdam.

MILHAUD, G., CALMETTE, C., TABOULET, J., JULIENNE, A. & MOUKHTAR, M.S. (1974) Hypersecretion of calcitonin in neoplastic conditions. *Lancet*, **i**, 462–463.

NEVILLE, A.M. & COOPER, E.H. (1976) Biochemical monitoring of cancer. *Annals of Clinical Biochemistry*, (to be published).

PEARSE, A.G.E. & POLAK, J.M. (1974) Endocrine tumours of neural crest origin. Neurolophomas, apudomas and the APUD concept. *Medical Biology*, **53**, 3–18.

ROOS, B.A., OKANO, K. & DEFTOS, L.J. (1974) Evidence for a pro-calcitonin. *Biochemical and Biophysical Research Communications*, **60**, 1134–1140.

ROSEN, S.W. & WEINTRAUB, B.D. (1974) Ectopic production of the isolated alpha subunit of the glycoprotein hormones. *New England Journal of Medicine*, **290**, 1441–1447.

SILVA, O.L., BECKER, K.L., PRIMACK, A., DOPPMAN, J. & SNIDER, R.H. (1974a) Ectopic secretion of calcitonin by oat-cell carcinoma. *New England Journal of Medicine*, **290**, 1122–1124.

SILVA, O.L., BECKER, K.L., SNIDER, R.H. & SHALHOUB, R.J. (1974b) Serum calcitonin in renal disease. (Abstract). *Clinical Research*, **22**, 720A.

SINGER, F.R. & HABENER, J.F. (1974) Multiple immunoreactive forms of calcitonin in human plasma. *Biochemical and Biophysical Research Communications*, **61**, 710–716.

SIZEMORE, G.W., GO, V.L.W., KAPLAN, E.L., SANZENBACHER, L.J., HOLTERMULLER, K.H. & ARNAUD, C.D. (1973) Relations of calcitonin and gastrin in the Zollinger–Ellison syndrome and medullary carcinoma of the thyroid. *New England Journal of Medicine*, **288**, 641–644.

SIZEMORE, G.W., HEATH, H. & LARSON, J.M. (1975) Immunochemical heterogeneity of calcitonin in plasma of patients with medullary thyroid carcinoma. *Journal of Clinical Investigation*, **55**, 1111–1118.

TASHJIAN, A.H., JR, HOWLAND, B.G., MELVIN, K.E.W. & STRATTON HILL, C. (1970) Immunoassay of human calcitonin. *New England Journal of Medicine*, **283**, 890–895.

VAITUKAITIS, J.L., BRAUNSTEIN, G.D. & ROSS, G.T. (1972) A radioimmunoassay which specifically measures human chorionic gonadotropin in the presence of human luteinizing hormone. *American Journal of Obstetrics and Gynecology*, **113**, 751–758.

YALOW, R.S. & BERSON, S.A. (1970) Size and charge distinctions between endogenous human plasma gastrin in peripheral blood and hectadecapeptide gastrins. *Gastroenterology*, **58**, 609–615.

YALOW, R.S. & BERSON, S.A. (1971) Size heterogeneity of immunoreactive human ACTH in plasma and in extracts of pituitary glands and ACTH-producing thymoma. *Biochemical and Biophysical Research Communications*, **44**, 439–445.

ISOLATION OF CALCITONIN FROM RAT THYROID MEDULLARY CARCINOMA

P. G. H. BYFIELD, E. W. MATTHEWS, J. N. M. HEERSCHE*, G. A. BOORMAN**, S. I. GIRGIS and I. MACINTYRE

*Endocrine Unit, Royal Postgraduate Medical School, Ducane Road, London W12 OHS, UK, *Laboratory for Cell Biology and Histology, University of Leiden, Leiden, The Netherlands, and **Institute for Experimental Gerontology TNO, Rijswijk (ZH), The Netherlands*

Received 9 February 1976

1. Introduction

The calcitonins show great differences in amino acid sequence between species. In the eight sequences so far known (human, pig, cow, sheep, eel and salmons I, II and III) only nine of the 32 residues are conserved; those at positions 1,3,4,5,6,7,9,28 and 32. Surprisingly, however, the full sequence is required for biological activity [1]. This paper describes the isolation of calcitonin from the rat as the preliminary step to determining its sequence in order to further understand the relationship between structure and function in this hormone. Studies on the structure of rat calcitonin are described in the accompanying paper [2].

The concentration of calcitonin in normal mammalian thyroid and fish or avian ultimobranchial glands is so low that large quantities of tissue must be collected. Medullary carcinoma of the thyroid in mammals is however, a tumour of the calcitonin producing C-cells and the cancers are extremely rich in the hormone [3]. Our previous isolation of human calcitonin [4,5] was only made practical by the use of such tumours in place of normal tissue. The discovery of medullary carcinomata of the thyroid in rats which contained high concentrations of calcitonin [6] prompted us to attempt the isolation of the hormone from this species for comparison with other known varieties.

2. Experimental

2.1. *Isolation of rat calcitonin*

The production of second and third generation carcinoma explants in rats has been described elsewhere [6]. First, second and third generation tumour tissue was pooled for use in this study. Finely-divided freeze dried tumours (8.38 g) were twice extracted with butanol–acetic acid–water, (150 : 15 : 42, by vol.) using 15 ml per g tissue overnight at room temperature and finally for 4 h using 10 ml solvent per g tissue. The extracts were then combined and dried by rotary evaporation. The sticky, brown solid was partitioned between 0.1 M formic acid and diethyl ether (approx. 25 ml of each phase per g original dry tissue). The ether layer was discarded and the aqueous layer taken and dried by rotary evaporation to give a brown solid (1.61 g) termed the crude extract.

For further purification the crude extract was dissolved in 0.1 M formic acid (containing 10%, v/v, propan-2-ol and 8 M urea) and chromatographed on a Bio Gel P-6 column (95 × 2.5 cm) using 0.1 M formic acid–propan-2-ol (9:1, v/v) as eluent. Pure rat calcitonin was then isolated by column partition chromatography as described by Yamashiro [7] using the solvent pair butanol–pyridine–acetic acid–water (1000 : 96 : 16 : 1000, by vol) and Bio Gel P-10 (100–200 mesh) as the support. Samples were dissolved in 400 μl of the upper phase of the solvent mixture and applied to a column (0.9 × 41.5 cm) previously equilibrated with both phases and then eluted with the upper phase collecting 500 μl fractions.

2.2. *Analytical methods*

Progress of the isolation was followed by either bioassay [8] or a radioimmunoassay for human calcitonin [9]. Quantitative estimations of protein were made by the Folin-Lowry method [12].

North-Holland Publishing Company – Amsterdam

Thin-layer chromatography was performed on cellulose plates (Merck, Darmstadt, GFR) using the solvent butanol–pyridine–acetic acid–water, (42 : 24 : 4 : 30 by vol). Peptide spots were located with ninhydrin, by a modified Reindel-Hoppe method [10] or with fluorescamine [11].

Amino acid analyses were performed on a JEOL 6AH instrument (JEOL Ltd., Tokyo, Japan) using a 10 mm path-length flow-cell to increase sensitivity. Samples were hydrolysed with 6 M HCl in the presence of 10 μg phenol under nitrogen in sealed tubes at 110°C for 20 h.

Dansylation, identification of dansyl amino acids and dansyl-Edman degradation were carried out as described by Hartley [13].

Enzyme digests were carried out using an estimated 5 μg hormone and 0.1 μg enzyme; for thermolysin 20 μl 0.2 M pyridine acetate pH 7.1, 16 h, 37°C, for trypsin 20 μl 0.5 M ammonium bicarbonate, 60 min, 37°C.

3. Results and discussion

Gel filtration of the crude extract of rat thyroid medullary carcinoma (fig.1) demonstrated two peaks of biological activity. They corresponded in molecular size to the monomeric and dimeric calcitonins found in extracts of human thyroid medullary carcinoma [4] and were therefore assumed to be monomeric and dimeric rat calcitonins. The dimer could be located only when samples were first allowed to stand for several hours in the assay buffer before bioassay in rats. This conversion of an inactive dimer to active

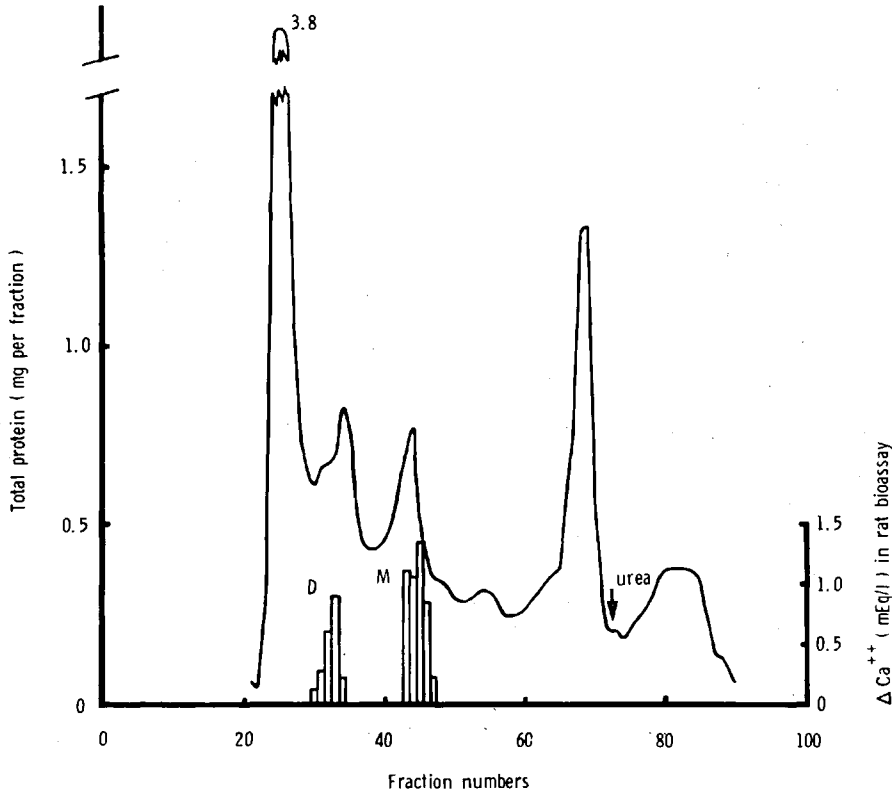


Fig.1. Gel filtration of crude extract of rat thyroid medullary carcinoma. The sample (158 mg containing 43 mg protein) was added to 3.0 g urea, 0.6 ml propan-2-ol and 1 μl mercaptoethanol, made up to 6.0 ml total volume with 0.1 M formic acid and chromatographed on a Bio Gel P-6 (200–400 mesh) (95 × 2.5 cm) column using 0.1 M formic acid–propan-2-ol, (9:1, v/v) as eluent. Fractions of 6.0 ml were collected. The continuous line shows the protein content per fraction and the histogram shows the two biologically active regions; D = dimeric, M = monomeric rat calcitonins.

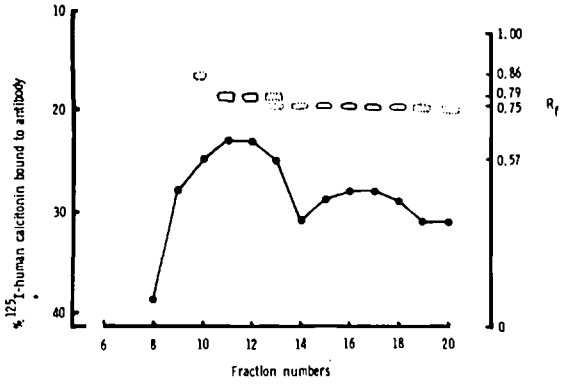


Fig.2. Partition chromatography of monomeric rat calcitonin purified by gel filtration. Two peaks of calcitonin were detected by the radioimmunoassay for human calcitonin (solid line). Superimposed on this graph is a t.l.c. analysis of a sample from each fraction. Selected R_f values are shown in the right-hand axis; on the same plate human calcitonin ran at R_f 0.75 and bovine insulin at R_f 0.57. The two peaks of immunological activity coincide with pure peptides.

Table 1
Amino acid composition of rat calcitonin

Amino acid	Residues per mole peptide (average of 3 values)	Integer value (human)
Lys	0.9	1 (1)
His	1.1	1 (1)
Arg	0	0 (0)
Asx	2.6	3 (3)
Thr	4.0	4 (5)
Ser	2.0	2 (1)
Glx	2.4	2 (2)
Pro	2.4	2 (2)
Gly	3.9	4 (4)
Ala	1.6	2 (2)
½ $(Cys)_2$	n.d.	2[a] (2)
Val	1.1	1 (1)
Met	0.8	1 (1)
Ile	1.0	1 (1)
Leu	2.9	3 (2)
Tyr	0.8	1 (1)
Phe	2.0	2 (3)

[a] N-terminal half-cystine shown by dansylation and the second residue assumed by analogy with all other calcitonins.

n.d. = not determined.

monomer is also observed with human calcitonin dimer [14] and dogfish calcitonin dimer [15] and is presumed to represent reduction by the free thiol group of bovine serum albumin present in the assay buffer. Calcitonin dimers are formed by rearrangement of the intrachenic disulphide bonds to interchain links [20].

After reduction with 4 M mercaptoethanol of all dimeric hormone to the monomeric form and its subsequent rechromatography, all monomeric rat calcitonin was pooled and fractionated by partition chromatography (fig.2). The fractions from the column were analysed by radioimmunoassay and t.l.c. but protein estimations were not made in order to conserve material. It is evident that two immunoreactive peptides were eluted from this column both of which appeared homogenous on t.l.c. In further examination both peptides gave the same amino acid composition after acid hydrolysis and the same tryptic and thermolytic peptide patterns on one-dimensional t.l.c. It is likely, therefore, that they both have the same sequence but the modification responsible for the difference in polarity remains unknown.

The amino acid composition of rat calcitonin is given in table 1. Except for cystine, which was not determined, the residue numbers are very similar to those for human calcitonin. The provisional values reported by Burford et al. [16] are also similar to our present values. However, the inability of these workers to assign whole residue numbers for some amino acids must reflect a degree of impurity in their preparation.

Following oxidation with performic acid, dansylation revealed cysteic acid as the amino terminal residue. This is characteristic of all other known calcitonins.

The biological potency of rat calcitonin was not determined rigourously but estimated to be of the order of a few hundred MRC units per mg in common with other mammalian species; Burford et al. reported 400 MRC U/mg [16]. The biological activity was destroyed on mild oxidation with hydrogen peroxide as is the case with human calcitonin where the methionine residue at position 8 is oxidised to its sulphoxide. Pig and salmon calcitonin are not affected in this way.

Several publications have reported the cross-reaction of rat calcitonin in radioimmunoassays for human calcitonin [16,17,18]. We confirm this and

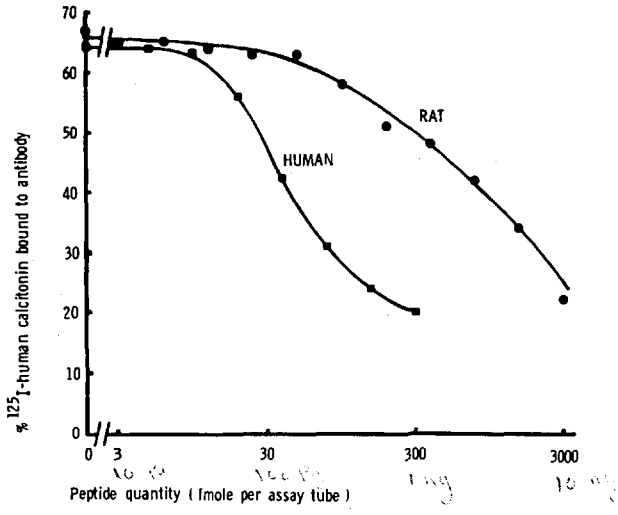


Fig.3. Comparison on a molar basis of the reaction of rat (•) and human (■) calcitonins with an anti-human calcitonin antiserum (336/6) at a final dilution of 1:24 000) using ^{125}I-human calcitonin as tracer. Human calcitonin was the MRC synthetic standard 70/50 and rat calcitonin was quantitated by amino acid analysis.

have been able to use the human calcitonin radioimmunoassay to follow the progress of isolation of the rat hormone. A direct comparison on a molar basis for the reaction of pure rat and human calcitonins with antiserum 336/6 at a final dilution of 1 : 24 000 is shown in fig.3. Rat calcitonin appears to reach the same maximum displacement of ^{125}I-human calcitonin from antibody but only when present in approx. 10-fold higher molar concentration. This degree of cross-reaction clearly demonstrates a large degree of sequence homology between rat and human calcitonins, at least in the region of the binding site which is at the C-terminus for this particular antiserum [19].

This work shows the importance of medullary carcinoma of the thyroid as a general source tissue in the isolation of calcitonins. The concern that peptides from tumours may not have the natural sequence is to some extent supported by the fact that rat calcitonin and human calcitonin (both isolated from tumours) are similar to each other but dissimilar to all other known calcitonins. However the provisional amino acid composition reported by Burford et al. [16] for rat calcitonin from normal thyroid tissue is broadly in agreement with our results for tumour-derived rat calcitonin. It seems likely, therefore, that 'normal' and 'tumour' rat calcitonin are identical.

Acknowledgements

This work was supported in part by the Medical Research Council. We thank Mrs. C. J. Hillyard for preliminary radioimmunoassays.

References

[1] Sieber, P., Brugger, M., Kamber, B., Riniker, B., Rittel, W., Maier, R. and Staehelin, M. (1970) Calcitonin 1969: Proc. 2nd. Int. Symp. (Taylor, S. and Foster, G. V., eds.) p. 28–33. Heinemann, London.
[2] Byfield, P. G. H., McLoughlin, J. L., Matthews, E. W. and MacIntyre, I. (1976) FEBS Lett., following paper.
[3] Cunliffe, W. J., Hall, R., Hudgson, P., Gudmundsson, T. V., Williams, E. D., Black, M. M., Johnston, I. D. A., Shuster, S., Joplin, G. F., Woodhouse, N. J. Y., Galante, L. and MacIntyre, I. (1968) Lancet ii 63–66.
[4] Riniker, B., Neher, R., Maier, R., Kahnt, F. W., Byfield, P. G. H., Gudmundsson, T. V., Galante, L. and MacIntyre, I. (1968) Helv. chim. Acta 51, 1738–1742.
[5] Byfield, P. G. H., Turner, K., Galante, L., Gudmundsson, T. V. and MacIntyre, I. (1968) Biochem. J. 111, 13p–14p.
[6] Boorman, G. A., Heersche, J. N. M. and Hollander, C. F. (1974) J. Nat. Cancer. Inst. 53, 1011–1014.
[7] Yamashiro, D. (1964) Nat. 201, 76–77.
[8] Sturtridge, W. C. and Kumar, M. A. (1968) J. Endocr. 42, 501–503.
[9] Clark, M. B., Boyd, G. W., Byfield, P. G. H. and Foster, G. V. (1969) Lancet, i. 74–77.
[10] Von Arx, E. and Neher, R. (1963) J. Chromatogr. 12, 329–341.
[11] Furlan, M. and Beck, E. A. (1974) J. Chromatogr. 101, 244–246.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Hartley, B. S. (1970) Biochem. J. 119, 805–822.
[14] MacIntyre, I. (1970) in: Calcitonin 1969: Proc. 2nd. Int. Symp. (Eds. Taylor, S. and Foster, G. V., eds.) p. 1–13, Heinemann, London.
[15] Byfield, P. G. H. and Matthews, E. W., unpublished.
[16] Burford, H. J., Ontjes, D. A., Cooper, C. W., Parlow, A. F. and Hirsch, P. F. (1975) Endocrinology 96, 340–348.
[17] Moukhtar, M. S., Tharaud, D., Jullienne, A., Raulais, D., Calmettes, C. and Milhaud, G. (1974) Experentia 30, 553–555.
[18] Triggs, S. M., Hesch, R. D., Woodhead, J. S. and Williams, E. D. (1975) J. Endocr. 66, 37–43.
[19] Byfield, P. G. H. and Hillyard, C. J., unpublished.
[20] Neher, R., Riniker, B., Rittel, W. and Zuber, H. (1968) Helv. chim. Acta 51, 1900–1905.

Molecular Endocrinology, editors MacIntyre and Szelke

AN IMMUNOLOGICAL COMPARISON OF NORMAL CIRCULATING CALCITONIN WITH CALCITONIN FROM MEDULLARY CARCINOMA

S. I. Girgis , C. J. Hillyard, I. MacIntyre and M. Szelke
Endocrine Unit,
Royal Postgraduate Medical School,
Ducane Road,
London W12 0HS
ENGLAND

SUMMARY

One form of circulating calcitonin from normal subjects has been compared with calcitonin M (CT-M) which was originally isolated from medullary thyroid carcinoma. A radioimmunoassay system employing six different antisera was used. These antisera could detect amino acid substitutions at 12 distinct sites in the calcitonin molecule. Normal and tumour rat calcitonin were also studied by this means.

Immunological identity was found between one form of normal circulating human calcitonin and CT-M; normal and tumour rat calcitonins were also immunochemically identical.

These results strongly suggest that the circulating hormone in the normal human is calcitonin M.

INTRODUCTION

The amino acid sequence of calcitonin from the normal human is not yet known, and it is at present impractical to determine because of the small amounts of material available[1,2].

This paper describes an immunological approach to the characterization of circulating calcitonin in normal subjects.

MATERIALS AND METHODS

Radioimmunoassay for CT-M : An overnight equilibrium assay system was used as previously described[3], with the following six antisera: As 827/4 and As 336/6 directed against the whole molecule, in a final dilution of 1/48,000 and 1/12,000 respectively; As 12/2 and As 10/2 directed against fragment (11-32) of CT-M[4], both used in a final dilution of 1/6000 (details to be published); As 5/1 and As 7/2 directed against fragment (17-32) of CT-M[4] in a final dilution

of 1/24000 each.

Calcitonin analogues : 15 analogues of calcitonin M were kindly donated by Dr. Rittel, Ciba-Geigy, Basle. These are: Val^{8}-CT; Lys^{11}-CT, Leu^{12}-CT; Leu^{16}-CT; Leu^{19}-CT; Tyr^{22}-CT; Arg^{24}-CT; Asn^{26}-CT; Thr^{27}-CT; CT-OH; Ala^{29},Val^{31}-CT; Ser^{29},Thr^{31}-CT; Lys^{11},Arg^{24}-CT; Asn^{26},Thr^{27}-CT and Leu^{12},Thr^{27}-CT.

The binding of these analogues was compared with that of CT-M on a molar basis.

Calcitonin from normal subjects : Calcitonin was extracted from plasma pooled from 43 normal volunteers[1] (430ml). The extract was run on a Bio-Gel P-30 column (100cm x 1cm) in 0.1M formic acid containing 10% isopropanol. Fractions co-eluting with (^{125}I)-CT-M were combined, lyophilized and dissolved in 2ml buffer before duplicate assay.

Calcitonin from normal rat thyroid : Thyroids from 20 two-months old normal male rats were dissected and immediately frozen in liquid nitrogen. Calcitonin was extracted as previously described[5]. After purification on Bio-Gel P-30, the fractions co-eluting with (^{125}I)-CT-M were pooled, lyophilized and dissolved in 6ml buffer before assay. Calcitonin from rat medullary thyroid carcinomas, previously purified in this laboratory[5], was studied together with the extract from normal rat thyroids.

RESULTS

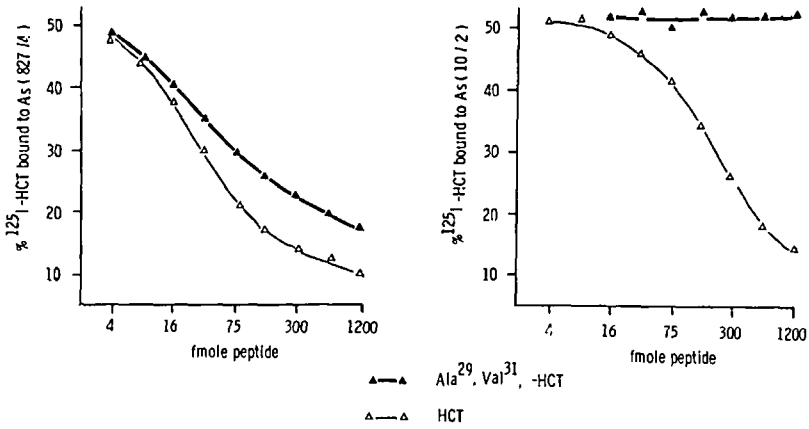
Calcitonin analogues : Replacement of amino acids at positions 8, 11, 12, 16, 19, 22, 24, 26, 27, 29, 31 and 32 in the CT-M molecule could be identified by one or more of the six antisera.

Single or double amino acid substitutions at the antigenic determinants of the hormone produce either a loss or inhibition of binding by the antiserum (Figure 1) or a change in the slope of the displacement curves (Figure 2).

Calcitonin from normal subjects : Five different immunoreactive forms of calcitonin were found after gel filteration on Bio-Gel P-30. The form co-eluting with synthetic CT-M produced displacement curves with all six antisera at several dilutions which were parallel to those obtained with CT-M.

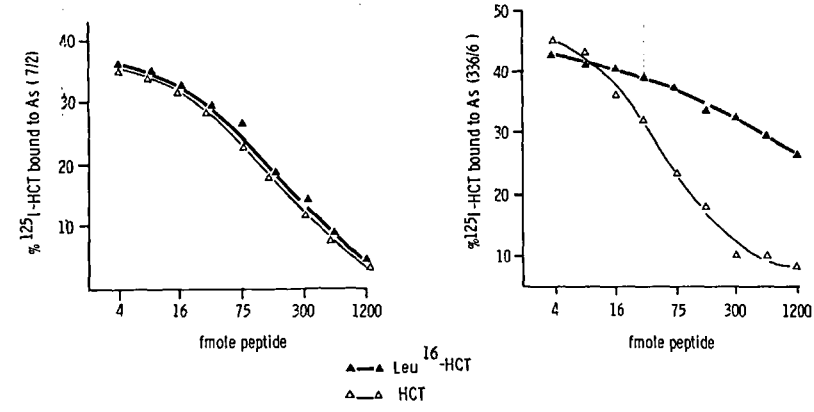
Calcitonin from normal rat thyroids : Similarly, displacement curves of normal and tumour rat calcitonins obtained with six

Figure 1



Legend to Figure 1 : Displacement of labelled calcitonin M by Ala^{29},Val^{31}-CT (▲—▲) and CT-M (△—△) from antiserum 827/4 (left) and antiserum (10/2) right.

Figure 2



Legend to Figure 2 : Displacement of labelled CT-M by Leu^{16}-CT (▲—▲) and CT-M (△—△) from antiserum 7/2 (left)and antiserum 336/6 (right).

different antisera were found to be parallel at several dilutions.

DISCUSSION

Our finding of immunological identity between normal and tumour rat calcitonins confirm earlier reports on the chemical identity of the two by the Chapel Hill group[6] and by our own laboratory[7].

The results of chemical and immunological studies in the rat, together with the finding of immunological identity between one form of normal circulating human calcitonin and CT-M, provide strong indication that CT-M circulates in normal man.

ACKNOWLEDGEMENT

We would like to thank Mrs Kay Colston for dissecting rat thyroids. S.I. Girgis is in receipt of an Egyptian Government Scholarship.

REFERENCES

1. Hillyard, Carmel J., Cooke, T.J.C. Coombes, R.C., Evans, Imogen M.A. and MacIntyre, I. (1977). Clinical Endocrinology, 6, 291-298.

2. Deftos, L.J., Lee, V. and Roos, B.A. (1976). Abstract 385, Vth International Congress of Endocrinology.

3. Clark, M.B., Boyd, G.W., Byfield, P.G.H. and Foster, G.V. (1969). Lancet, i, 74-77.

4. Girgis, S.I. and Hillyard, C.J. (1976). Abstract 382, Vth International Congress of Endocrinology, Hamburg.

5. Byfield, P.G.H., Matthews, E.W., Heersche, J.N.M., Boorman, G.A., Girgis, S.I. and MacIntyre, I. (1976). FEBS Letters, 65, 238-241.

6. Raulais, D., Hagaman, J., Ontjes, D.A., Lundblad, R.L. and Kingdon, H.S. (1976). Eur.J.Biochem., 64, 607-611.

7. Byfield, P.G.H., McLoughlin, J.L., Matthews, E.W. and MacIntyre, I. (1976). FEBS Letters, 65, 242-245.

[REPRINTED FROM THE PROCEEDINGS OF THE SOCIETY FOR ENDOCRINOLOGY, 24–25 MAY 1979]
JOURNAL OF ENDOCRINOLOGY, [1979], 83, 55P–56P
 PRINTED IN GREAT BRITAIN

Nature of normal human calcitonin in the circulation. By S. I. Girgis, C. McMartin* and I. MacIntyre. *Endocrine Unit, Royal Postgraduate Medical School, Ducane Road, London, W12 0HS and *Research Centre, Ciba–Geigy Pharmaceuticals Division, Wimblehurst Road, Horsham, West Sussex, RH12 4AB*

Calcitonin M (CT-M) was isolated from human medullary thyroid carcinoma (Neher, Riniker, Mair, Byfield, Gudmundsson & MacIntyre, 1968). However, the structure of the normal hormone is not yet known and it is at present impractical to determine it because of the extremely low level in plasma and thyroid tissue. In order to characterize the normal hormone in the circulation, we have used two indirect approaches: immunological study and high-pressure liquid chromatographic (HPLC) identification.

Pooled plasma (1 litre) from 20 normal volunteers was lyophilized and extracted with butanol : acetic acid : water mixture (75 : 7·5 : 21, by vol.) (Byfield, Matthews, Heersche, Boorman, Girgis & MacIntyre, 1976). The extract was chromatographed on Bio-Gel P-30 column and the fractions assayed for calcitonin immunoreactivity using a specific and sensitive immunoassay for CT-M (detection limit 4 pg/tube). Synthetic CT-M was added to blank plasma, extracted and chromatographed in the same way as a control.

Five immunoreactive peaks of calcitonin were seen; the peak co-eluting with CT-M was studied with six region specific antisera (Girgis, Hillyard, MacIntyre & Szelke, 1977). It was then applied to an octadecasilyl silica high pressure column and eluted with a linear solvent gradient from 20% methanol : 1% trifluoroacetic acid (TFA) to 80% methanol : 1% TFA. The separation of peptide was monitored by immunoassay. Previous studies have shown that analogues of CT-M differing by one or two amino acids only can be distinguished by HPLC alone (Dr W. Rittel, Ciba–Geigy, Basle, personal communication).

One of the peaks of normal plasma immunoreactive calcitonin was identical, both immunologically and on HPLC, to the CT-M control. This is strong evidence of the structural identity of one form of normal calcitonin and CT-M in the circulation.

REFERENCES

Byfield, P. G. H., Matthews, E. W., Heersche, J. N. M., Boorman, G. A, Girgis, S. I. & MacIntyre, I. (1976). *FEBS Letters* **65**, 238.

Girgis, S. I., Hillyard, C. J., MacIntyre, I. & Szelke, M. (1977). In *Molecular endocrinology: proceedings of endocrinology, '77*, p. 175. Eds I. MacIntyre & M. Szelke. Amsterdam: Elsevier/North Holland Biomedical Press.

Neher, R., Riniker, B., Mair, R., Byfield, P. G. H., Gudmundsson, T. V. & MacIntyre, I. (1968). *Nature* **220**, 946.

Molecular Endocrinology, editors MacIntyre and Szelke
© 1979 Elsevier/North-Holland Biomedical Press

THE INTERRELATION OF THE CALCIUM REGULATING HORMONES: SOME RECENT FINDINGS

IAIN MACINTYRE, TIMOTHY R. ARNETT, DOUGLAS J. BROWN, FERNANDO GALAN GALAN, SAMIA I. GIRGIS, RAYMOND M. ROGERS, EVANGELOS SPANOS, JOHN C. STEVENSON AND QUENTIN BONE*
Endocrine Unit, Royal Postgraduate Medical School, Du Cane Road, London W12 OHS, U.K. and * Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth PL1 2PB, U.K.

INTRODUCTION

The progress in this field since the discovery of calcitonin has been so great that a coherent scheme of calcium regulation rarely incorporates recent advances. In this paper I shall try to summarize the present views of the regulation of vitamin D metabolism and of the physiological place of calcitonin. There is insufficient space to treat parathyroid hormone but this subject has been well reviewed and is quite well understood. But there have been so many advances in the fields of vitamin D and calcitonin that no discussion of the interrelation of the calcium regulating hormones is feasible without a prior summary of the present state of knowledge.

VITAMIN D

It is now well established that the major circulating form of vitamin D_3 is 25-hydroxycholecalciferol. This metabolite has real biological activity but of rather low order. The most active metabolite, $1,25(OH)_2D_3$, is formed in the kidney and then secreted into the blood and carried to its various target tissues: the gut, the bone and perhaps the parathyroid gland and other tissues. But the kidney also forms a second metabolite, $24,25(OH)_2D_3$, which has a much lower biological activity, probably similar to that of $25\text{-}OHD_3$ itself. It is still not clear whether $1,25(OH)_2D_3$ is capable of exerting all the major biological effects of vitamin D or whether it requires the co-operative action of one or more of the other metabolites. At present it seems a reasonable hypothesis that $25\text{-}OHD_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ all exert a biological effect and that the effects of these metabolites do not differ qualitatively. But since the function of vitamin D is to maintain or enhance calcium and phosphorus absorption from the gut this can be achieved most effectively by varying the secretion of $1,25(OH)_2D_3$, by far the most active metabolite. The

key question is how the kidney regulates its production of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. There are two sets of regulators or control factors.

1) The first group increases and controls the production of $1,25(OH)_2D_3$ during the periods of major physiological calcium stress: growth, pregnancy, and lactation. These regulators are: growth hormone,[1] prolactin,[2,3,4] placental lactogen, the circulating calcium level and parathyroid hormone.[4] Although many of these effects have been described only recently they are quite widely accepted.

2) But there is a second and extremely important type of regulation: this is to adjust the output of $1,25(OH)_2D_3$ to make up for alterations in supply of the substrate ($25\text{-}OHD_3$) and thus to maintain a constant level of vitamin D activity.

We used to think that this was done by a direct action of $1,25(OH)_2D_3$ itself regulating the synthesis of the renal enzymes ($25\text{-}OHD_3$ 1α-hydroxylase and $25\text{-}OHD_3$ 24-hydroxylase) which produce the two renal metabolites.[5,6] But very recently we have found clear evidence in our laboratory that this view was incomplete in a very important respect. $25\text{-}OHD_3$ and $24,25(OH)_2D_3$ induce 24-hydroxylase activity in kidney cells culture much more rapidly than $1,25(OH)_2D_3$ itself (Figures 1 and 2).

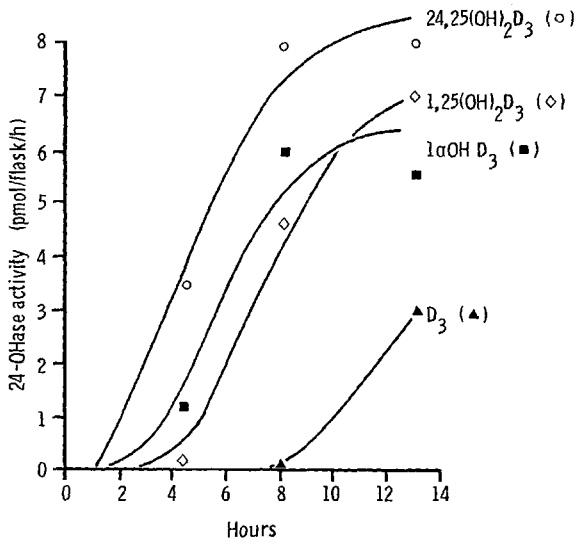


Fig. 1. The induction of 24-hydroxylase activity in chick kidney cell cultures.

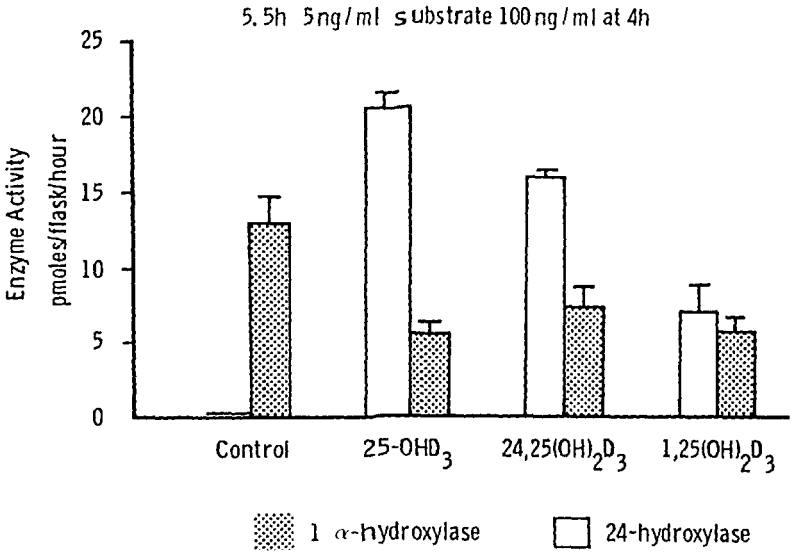


Fig. 2. The effect of D metabolites on enzyme activities after exposure of chick kidney cell cultures for 5.5 hours.

We believe that this effect of 25-OHD_3 is of major regulatory importance and much more important in practice than that of either 24,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$ since 25-OHD_3 exceeds the circulating concentrations of these metabolites by a factor of 10 and 1,000, respectively.

Thus, in summary, the body increases calcium and phosphorus absorption during growth, pregnancy and lactation by increasing the total vitamin D activity. And this is done most effectively by increasing 1,25$(OH)_2D_3$ production since this is the most active metabolite (Figure 3). The controlling factors in these situations are the pituitary and placental hormones (growth hormone, prolactin and placental lactogen) aided by the actions of parathyroid hormone and the circulating calcium level.

But in order to maintain a constant level of vitamin D activity during basal situations there is in addition a newly discovered major regulation of the renal enzymes by a direct action of 25-OHD_3 on the kidney. An excess of 25-OHD_3 levels will increase the activity of the 24-hydroxylase enzyme and diminish that of the 1α-hydroxylase enzyme, while a diminution of the circulating level of 25-OHD_3 will have the opposite effect. The effect will be to maintain the total vitamin D activity at a relatively constant level despite wide

fluctuations in the circulating level of 25-OHD_3 itself. This mechanism is an important one and is part of the reason why we do not suffer from vitamin D poisoning after sunbathing nor severe deficiency from an English winter.

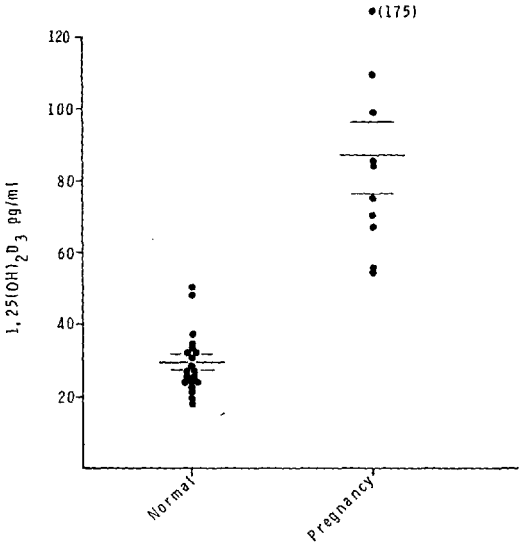


Fig. 3. The plasma $1,25(OH)_2D_3$ levels in pregnancy (33-34 weeks).

CALCITONIN

Chemistry. Calcitonin is produced by the parafollicular or C-cells in mammalian thyroid, and by cells in the ultimobranchial body in sub-mammalian vertebrates. Both types of cells are of neural crest origin. All the calcitonins so far isolated consist of a single chain polypeptide, 32 amino acids in length with a 1-7 disulphide bridge at the amino terminus and prolinamide at the carboxyl terminus. Major changes in the sequence are permissible with retention of biological activity and there are only 9 invariant residues. The sequences so far determined fall into three groups:[7] the primate-rodent group (e.g. man and the rat); the teleost group (e.g. eel and salmon); and the artiodactyl group (e.g. ox and sheep). The many differences in sequence have two consequences. These are that it is necessary to use antisera prepared against hormones from the same group if one wishes sufficient cross reaction for radioimmunoassay; and when the calcitonins are used therapeutically only the human hormone is free from the risk of production of antibodies when used in man.

Physiology. The function of calcitonin in man is far from clear. It certainly contributes to plasma calcium homoeostasis, especially in children, but this action may not be of great importance in adults. This is because calcitonin exerts its effect on plasma calcium via a direct action on osteoclasts and osteocytes to inhibit bone resorption. Although this is still seen in the adult it must contribute less to an effect on plasma calcium because bone turnover is so much slower than in the child. However, there is some suggestive evidence that the action of calcitonin on bone may be of considerable importance in preservation of the skeleton, apart from any question of regulation of plasma calcium. This is still an hypothesis but some support is lent to the idea by the finding that plasma calcitonin levels are greatly elevated during pregnancy and lactation as well as during growth. In these situations the plasma level of $1,25(OH)_2D_3$ is greatly increased and it is possible that, but for the enhanced concentration of calcitonin, this highly active metabolite of vitamin D would act unopposed on bone to enhance osteoclastic activity. And there is a large sex difference between men and women[8] (Figure 4).

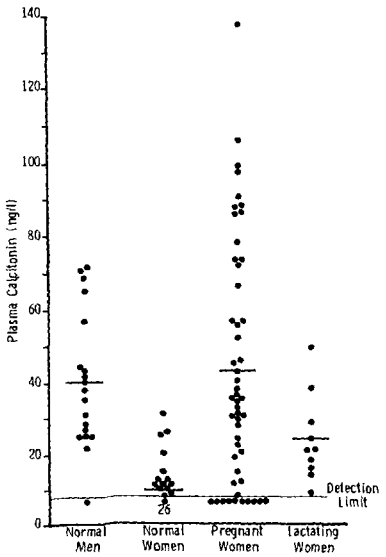


Fig. 4. Plasma calcitonin levels in normal men and women and during pregnancy and lactation. 26 of the normal women had undetectable levels (less than 8 ng/l).

The concentration of plasma calcitonin in women is 4 - 10 times less than in men; this may explain the greater liability of women to bone loss, whether after the menopause or during intestinal malabsorption (Doyle, personal communication). This major sex difference in plasma calcitonin was missed for some years for several reasons. The most obvious was that radioimmunoassays were of barely adequate sensitivity. But equally important factors were the failure to recognize the marked diurnal variation in plasma calcitonin[9] (Figure 5) and the fact that the contraceptive pill had a major effect in increasing plasma calcitonin levels.[8]

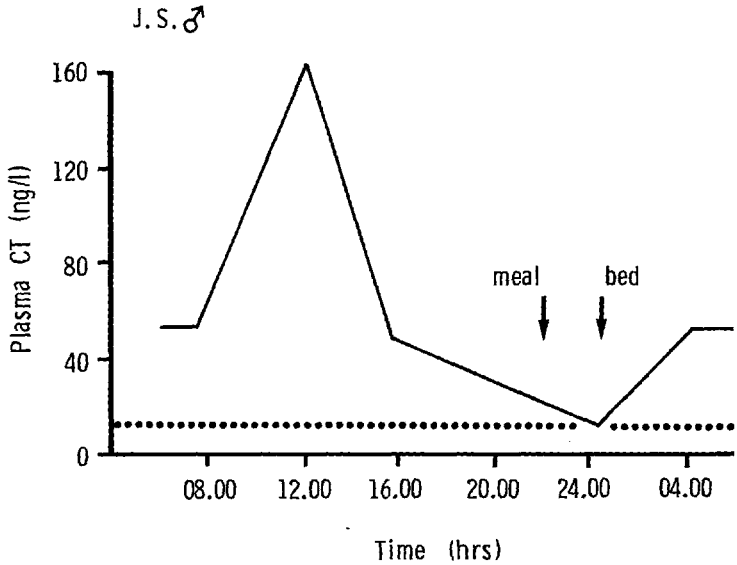


Fig. 5. Plasma calcitonin levels in one subject during 24 hours. No meal was taken until the evening.

When studies are made with proper control of these variables the sex difference becomes unmistakable. Much further detailed investigation is necessary but it now seems quite reasonable to believe that the altered physiology of calcitonin in women may play some role in the aetiology of post-menopausal osteoporosis.

Recent Findings. It is usually believed that calcitonin is not found in the agnatha, but we have recently observed that human calcitonin-like immunoassayable activity is present in the hagfish brain. The immuno-activity was confirmed by high pressure liquid chromatography which showed that the material behaved in a generally similar, but not identical, fashion to human calcitonin. These findings were at first surprising considering the major

differences in sequence between human and teleost calcitonin. We may presume that the primitive agnatha may have contained a similar molecule and that this molecule has been largely preserved in several mammalian groups including man. The many changes in sequence present in teleost calcitonin must then have taken place during the development of the chondrostei, holostei and teleostei. This interpretation is greatly strengthened by further recent findings. Calcitonin-like immunoassayable activity is present in several chordates[10] (Amphioxus, Ascidiella and Ciona) sometimes in very high concentrations (Figure 6).

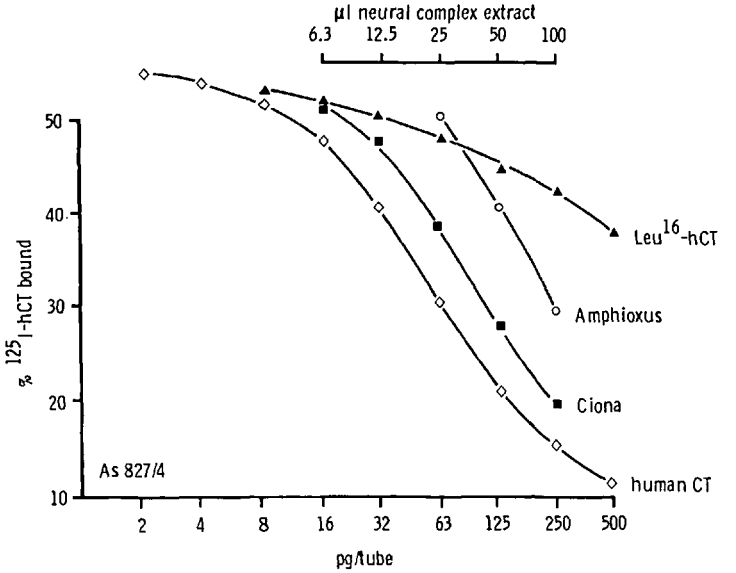


Fig. 6. Human calcitonin-like activity in chordates as detected in an assay for human calcitonin. The immunoassay detects the substitution of one amino acid (leu^{16}) in human calcitonin as shown.

In the case of the tunicates this activity is present in the excised neural complex and confirmed in Ciona by fluorescent localization,[11] but in the case of Amphioxus localization studies have still to be completed. However, these studies indicate that a molecule with very similar immunological characteristics to human calcitonin existed in the primitive nervous system of chordates. Further high pressure liquid chromatographic and gel filtration studies indicate that this molecule is indeed very similar to human calcitonin. These observations are in good agreement with other studies which show that mammalian hormones may be among those molecules which are highly conserved from our remote ancestors in whom they must have fulfilled completely different functions. It would be fashionable to suggest that calcitonin functioned as a

neurotransmitter in chordate brain but there is no direct evidence of this. Further, the large variations in concentration found in different samples raise the possibility that calcitonin may have had quite another role. However that may be, it is interesting to observe that calcitonin in mammals is still secreted by cells which are derived from the nervous system during embryological development. Whether these findings have any relevance to the presence of calcitonin-like immunoreactivity in the pituitary[12] is unknown. We find a small concentration of activity in rat pituitary (0.5% of that in the thyroid) but cannot confirm[13] the intense immunofluorescent staining reported in the pars intermedia.[14] In any case, the small amount of pituitary calcitonin cannot have much significance in the known function of calcitonin in regulating bone resorption. It is possible, however, that pituitary calcitonin is an evolutionary relic of an ancient brain peptide which fulfilled some unknown function in chordates.

SUMMARY

1) Vitamin D is a major factor in regulating calcium and phosphorus absorption. The enhanced absorption during growth, pregnancy and lactation is due to increased production of the most active metabolite, $1,25(OH)_2D_3$, controlled by growth hormone, prolactin and placental lactogen in association with parathyroid hormone and the circulating level of plasma calcium. But in the interval states between episodes of physiological calcium and phosphorus stress a constant level of activity of all the vitamin D metabolites taken together is achieved by a direct action of 25-hydroxy D_3 on the kidney to induce or suppress the appropriate enzyme activity.

2) Human calcitonin is a highly conserved molecule. It exists in an identical or similar form in several chordate species as well as in cyclostomes. It is present in the tunicate nervous system, and may be provisionally referred to as neurocalcitonin until its chemical identity has been clarified. Its function in the neural complex of these primitive species is completely unknown. The function of calcitonin in man is still only partly understood. It plays a role in plasma calcium homoeostasis, and it is possible that it may also have an important function in protecting the skeleton during periods of calcium stress. The relative deficiency of calcitonin in women may be a factor in the aetiology of post-menopausal osteoporosis.

ACKNOWLEDGEMENTS

These studies were supported in part by grants from the Medical Research Council, Wellcome Trust, Arthritis and Rheumatism Council and Nuffield Foundation. It is a pleasure to acknowledge the collaboration of Professor Lelio Orci and Dr. Godfrey Howard.

REFERENCES

1. Spanos, E., Barrett, D., MacIntyre, I., Pike, J.W., Safilian, E. and Haussler, M.R. (1978) Nature 273, 246-247.
2. Spanos, E., Pike, J.W., Haussler, M.R., Colston, K.W., Evans, I.M.A., Goldner, A.M., McCain, T.A. and MacIntyre, I. (1976) Life Sciences 19, 1751-1756.
3. MacIntyre, I., Colston, K.W., Robinson, C.J. and Spanos, E. (1977) Molecular Endocrinology: Proceedings of Endocrinology '77, Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 73-85.
4. MacIntyre, I., Brown, D.J. and Spanos, E. Proceedings of IV Workshop on Vitamin D (in press).
5. Larkins, R.G., MacAuley, S.J. and MacIntyre, I. (1974) Nature 252, 412-414.
6. Spanos, E., Barrett, D.I., Chong, K.T. and MacIntyre, I. (1978) Biochem. J. 174, 231-236.
7. MacIntyre, I. (1978) J. Endocrinol. Invest. 1, 277-284.
8. Hillyard, C.J., Stevenson, J.C. and MacIntyre, I. (1978) Lancet i, 961-962.
9. Hillyard, C.J., Cooke, T.J.C., Coombes, R.C., Evans, I.M.A. and MacIntyre, I. (1977) Clin. Endocr. 6, 291-298.
10. Galan Galan, F., Rogers, R.M., Girgis, S.I., Arnett, T.R., Orci, L., Ravazzola, M. and MacIntyre, I. (in preparation).
11. Fritsch, H.A.R., Van Noorden, S. and Pearse, A.G.E. (in press).
12. Deftos, L.J., Burton, D., Catherwood, B.D., Bone, H.G., Parthemore, J.G., Guillemin, R., Watkins, W.B. and Moore, R.Y. (1978) J. Clin. Endocr. & Metab. 47(2), 457-460.
13. Galan Galan, F., Rogers, R.M., Girgis, S.I., Arnett, T.R., Orci, L., Ravazzola, M. and MacIntyre, I. (in preparation).
14. Deftos, L.J., Burton, D., Bone, H.G., Catherwood, B.D., Parthemore, J.G., Moore, R.Y., Minick, S. and Guillemin, R. (1978) Life Sciences 23, 743-748.