A RADIOIMMUNOASSAY FOR HUMAN CALCITONIN

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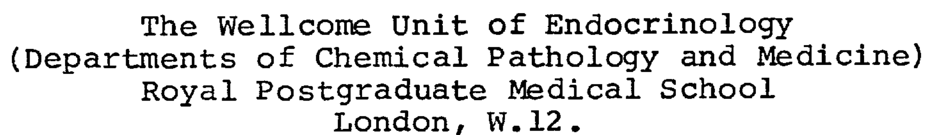
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ABSTRACT

This thesis describes studies which led to the development and refinement of a radioimmunoassay for human calcitonin, and investigations in which the assay was exploited to study levels in man, the structure of calcitonin itself and the pharmokinetics of the hormone.

Chapters I and II are short general reviews on calcitonin and radioimmunoassay respectively and jointly serve to introduce the corpus of original work described.

The assay which was developed employed antiserum raised in a rabbit to extracts of tumours from patients with medullary carcinoma of the thyroid. This antiserum proved to be specific for the human hormone and did not cross-react with several other peptide hormones or significantly with calcitonins from other species. The original assay detected 160 pg, but could measure no less than 10 ng/ml of hormone in plasma. This assay was used (1) to demonstrate that calcitonin was present in normal thyroid tissue, (2) to identify the antigenic determinants of the hormone, (3) to diagnose patients with medullary carcinoma of the thyroid, to study the results of surgery, the diurnal variation and factors that affect the secretion and release of the hormone in this disease, and (4) to investigate the presence of calcitonin in other tissues including adrenal glands and tumours from patients with carcinoid syndrome.

A thorough re-examination of the conditions of the assay was undertaken in order to improve sensitivity. By prolonging incubation time and by late addition of labelled hormone, the

limits of detection were improved 10-fold, enabling as little as 8-16 pg to be detected. Despite this improvement, not less than 500 pg per ml could be detected in plasma, and levels in normal subjects could not be measured. For this reason the improved assay was exploited to study the metabolic fate of human calcitonin in dogs. In these investigations the following conclusions were drawn: (1) calcitonin and/or its fragments are concentrated by liver and kidney, (2) of liver and kidney, kidney is principally responsible for the removal of hormone from plasma, accounting for a loss of 8% per minute, (3) the removal of hormone by the kidney is due to degradation and not excretion, (4) there are two half-times of disappearance for the hormone, one of 3 minutes and another in the order of 40 minutes, (5) nephrectomy partially abolishes the more rapid initial disappearance of the hormone, and (6) functional hepatectomy does not affect the disappearance of the hormone.

This thesis concludes with a tentative hypothesis. It is proposed that species variations in the amino acid composition of calcitonin may determine the organ by which the hormone is inactivated. If this is true, it should prove possible to prepare analogues to calcitonin with prolonged biological activity.

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PREFACE

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Chapter I

CALCITONIN

Summary:

The major developments in calcitonin research are reviewed in this chapter to serve as an introduction to the thesis. The topics covered include: the discovery of the hormone, the histology and embryology of the calcitonin-secreting cells, the chemistry of the various calcitonins, the bioassay of the hormone, mechanisms of its action, factors which affect its secretion and release, the metabolic fate of the hormone, studies in man, the therapeutic uses of calcitonin and its possible physiological significance.

DISCOVERY

For many years, plasma calcium was thought to be controlled by parathyroid hormone. This hormone is secreted by the parathyroid glands in response to hypocalcaemia. By acting on the bone to mobilise bone mineral, on the kidney to increase tubular reabsorption of calcium, and on the gut to increase intestinal reabsorption of this cation, it increases the circulating level of calcium (Rasmussen, 1961; Nordin and Peacock, 1970). As the level rises, the stimulus for the secretion of the hormone is reduced. This concept of a negative feedback system to control calcium homeostasis was formally proposed by McLean and Urist (1961). It is no longer in doubt that their postulate is correct. However, it is now appreciated that the original thesis was incomplete and that a second calcium-regulating hormone, whose action opposes that of parathyroid hormone, is secreted in response to hypercalcaemia.

This second hormone was discovered in 1961 by Copp and his colleagues (Copp, Cameron, Cheney, Davidson and Henze, 1962) who were testing the McLean hypothesis to see whether or not

hypercalcaemia inhibited the secretion of parathyroid hormone. They perfused the thyroparathyroid apparatus of dogs with hypercalcaemic blood and produced a fall in plasma calcium. This fall was greater than that which occurred when the parathyroid glands were removed. It was therefore not possible to explain the fall in plasma calcium produced by hypercalcaemic perfusion of the glands by simple inhibition of parathyroid hormone secretion. To account for the findings, Copp proposed that a second calcium-regulating hormone existed, that it was secreted by the parathyroid glands under the influence of hypercalcaemia, and that it produced hypocalcaemia. The name "calcitonin" was given to this hormone.

Independently, Hirsch, Gauthier and Munson (1963) concluded that a thyroid hypocalcaemic factor might exist. They observed that the fall in plasma calcium in animals following parathyroidectomy by electrocautery was greater than that following surgical removal of the parathyroid glands. Investigation of this difference led them to the conclusion that trauma to the thyroid, induced by electrocautery, was responsible for the release of a calcium-lowering substance from this tissue. Because it was then thought that calcitonin originated from the parathyroid, they gave the name "thyrocalcitonin" to the hypocalcaemic principle to distinguish it from the hormone that was thought to be secreted by the parathyroids.

The studies which resolved the question of the origin of the hormone were carried out by Foster and his colleagues (Foster, Baghdiantz, Kumar, Slack, Soliman and MacIntyre, 1964). Following perfusion of the external parathyroid gland of the goat with hypercalcaemic blood, no significant change in systemic

plasma calcium occurred. However, when the thyroid was perfused, a striking fall was seen. The presence of a calcium-lowering substance in the thyroid was confirmed by demonstrating that an extract of thyroid, when injected into goat, produced a similar fall in plasma calcium. From these studies, it was concluded that calcitonin was released by hypercalcaemic stimulation, that the active principle could be extracted from the gland and that calcitonin and thyrocalcitonin were one and the same substance.

HISTOLOGY AND EMBRYOLOGY

Calcitonin is now known to be secreted by cells which are distinct from those which produce thyroxine and triiodothyronine. Using histological and cytochemical techniques, Foster, MacIntyre and Pearse (1964) studied the thyroid glands of dogs which had been perfused with varying concentrations of calcium. They observed that in thyroid tissue stimulated with hypercalcaemic blood, striking changes occurred in the parafollicular cells. The affected cells were shown to contain relatively high levels of mitochondrial alpha-glycerophosphate dehydrogenase. Since similar high concentrations of this enzyme occur in other specialised cells known to secrete endocrine hormones, they proposed that these parafollicular cells were the most probable source of calcitonin. Immunofluorescent studies confirmed this to be true (Bussolati, Carvalheira and Pearse, 1968; Kalina, Foster, Clark and Pearse, 1970). In addition to having a distinctive enzymatic profile, the cells are morphologically different from follicular cells known to secrete the classical thyroid hormones. Their most distinctive feature is the presence of electron-dense granules within the cytoplasm. These granules

are presumed to be the secretory product and are discharged when stimulated by hypercalcaemic blood (Pearse, 1966).

Unlike other cells of the thyroid, the calcitonin-secreting cells have an avidity _in vivo_ for 5-hydroxytryptamine. Use of this property enabled Pearse and his colleagues (Cavalheira and Pearse, 1968) to demonstrate that these cells arose from the last ultimobranchial pouch. By injecting pregnant mice with this substance, they were able to show in the embryos that these cells migrate from their embryological anlage to the thyroid where they fuse and become incorporated within the thyroid matrix. This discovery suggested that the ultimobranchial gland, which exists as a separate organ in fish, amphibians, reptiles and birds, was, in fact, a calcitonin-secreting endocrine organ. This was confirmed by the demonstration that extracts of ultimobranchial tissue from chickens, (Copp, Cockcroft, Kueh and Melville, 1968), pigeons, lizards (Moseley, Matthews, Breed, Galante, Tse and MacIntyre, 1968), frogs (Robertson, 1970) and fish (Copp, Brooks, Low, Newsome, O'Dor, Parkes, Walker and Watts, 1970; Byfield, 1971) contained a potent hypocalcaemic factor. The hypocalcaemic factor has now been proved to be calcitonin since the hormone from salmon has been isolated (Keutmann, Parsons, Potts and Schleuter, 1970) and characterised (Niall, Keutmann, Copp and Potts, 1969).

More recently, Pearse and Polak (1971) and Le Douarin and Le Lièvre (1971) have independently suggested that cells in the ultimobranchial body may derive from the neural crest. If this is true, the implications are important, since the calcitonin-secreting cells may therefore be found elsewhere in the body than in the thyroid. There is already evidence that this is so.

In man, calcitonin-like activity is extractable from both parathyroid and thymus tissue (Galante, Gudmundsson, Matthews, Tse, Williams, Woodhouse and MacIntyre, 1968), and in rabbit and dog calcitonin-secreting cells have been demonstrated in the parathyroids (Welsch and Pearse, 1969).

CHEMISTRY

Calcitonins from several different species have now been isolated, including pig (Kahnt, Riniker, MacIntyre and Neher, 1968), man (Riniker, Neher, Maier, Kahnt, Byfield, Gudmundsson, Galante and MacIntyre, 1968), salmon (Keutmann, Parsons, Potts and Schleuter, 1970), cow (Brewer and Ronan, 1969) and sheep (Potts, Niall, Keutmann, Deftos and Parsons, 1970). Of these, porcine (Neher, Riniker, Zuber, Rittel and Kahnt, 1968; Potts, Niall, Keutmann, Brewer and Deftos, 1968; Bell, Barg, Colucci, Davies, Dziobkowski, Englert, Heyder, Paul and Snedeker, 1968), salmon (Niall, Keutmann, Copp and Potts, 1969), human (Neher, Riniker, Maier, Byfield, Gudmundsson and MacIntyre, 1968; Riniker, Neher, Maier, Kahnt, Byfield, Gudmundsson, Galante and MacIntyre, 1968), cow and sheep (Potts, Niall, Keutmann, Deftos and Parsons, 1970) have had their structure determined, and porcine calcitonin (Rittel, Brugger, Kamber, Riniker and Sieber, 1968; St. Guttman, Pless, Sandrin, Jaquenoud, Bossert and Willems, 1968), salmon calcitonin (St. Guttman, Pless, Huguenin, Sandrin, Bossert and Zehnder, 1968) and human calcitonin (Sieber, Brugger, Kamber, Riniker and Rittel, 1968) have been synthesised.

The hormone from each of these species is a single chain polypeptide consisting of thirty-two amino acids. It is characterised by a 1-7 disulphide bridge and a terminal proline

amide. As shown in Figure 1, considerable variation in the amino acid sequence may exist between species. Compared to porcine calcitonin, the human hormone differs in 18 of its 32 amino acid residues. Partial degradation of any of the hormones results in almost complete loss of biological activity.

The human hormone was originally isolated from tumour tissue from patients with medullary carcinoma of the thyroid. Since the original demonstration of its presence in this neoplasm (Meyer and Abdel Bari, 1968; Melvin and Tashjian, 1968; Tashjian and Melvin, 1968; Milhaud, Tubiana, Parmentier and Coutris, 1968; Dube, Bell and Aliapoulios, 1969), it has been shown that tumour tissue may contain as much as 5000 times more calcium-lowering activity than normal thyroid (Cunliffe, Black, Hall, Johnston, Hudgson, Shuster, Gudmundsson, Joplin, Williams, Woodhouse, Galante and MacIntyre, 1968). During isolation of the active principle from neoplastic tissue, it became apparent that there were three active components. The two major components have been identified as the monomer and antiparallel dimer forms of the hormone. The third is a lipophilic faction which has yet to be studied (Byfield, 1970). The significance of the occurrence of both monomer and dimer in man is not yet known. It has, however, been found that the potency of the human dimer as assayed in rat is much lower than that of the monomer and may be completely inactive (Galante, Gudmundsson, Horton, Woodhouse, MacIntyre, Burrows and Robinson, 1970). Whether the dimer occurs in other species, or whether human dimer is active in species other than rat, has yet to be demonstrated.

BIOASSAY

The isolation of the hormone was made possible by the

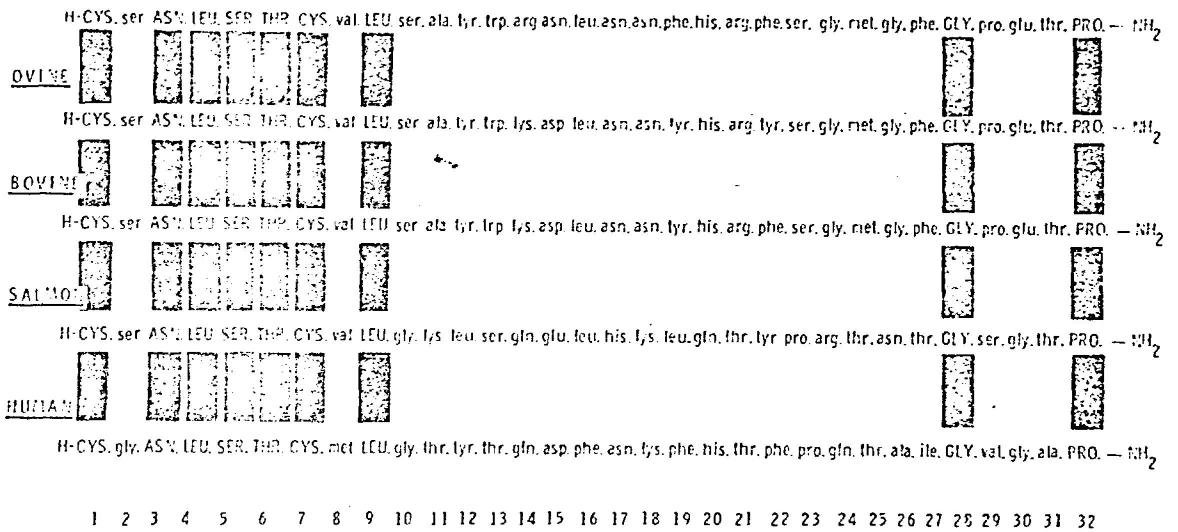

Figure 1. The amino-acid sequences of calcitonin isolated from five different species. Of the nine conserved residues, shown joined by vertical bars, seven are found at the C-terminal and two at the amino-terminal end of the molecule.

development of biological assays which measured the hypocalcaemic response of the active principle in rats. The assays used have all been modifications of the original assays described by Kumar, Slack, Edwards, Soliman, Baghdiantz, Foster and MacIntyre (1965) and Hirsch, Voelkel and Munson (1964). The assay used in our laboratory employs 50 gm female Wistar rats, starved overnight, and injected with assay preparations intravenously. Falls in plasma calcium observed at 30 minutes with unknown preparations are compared to the falls observed with the MRC Research Standard Preparation for porcine calcitonin at two dose levels. This assay has a detection limit of 10 nanograms/ml (1 MRC mU/ml) and an index of precision of less than 0.2.

PHYSIOLOGY

Calcitonin induces both hypocalcaemia and hypophosphataemia. These effects are brought about by the action of the hormone on bone and kidney. In addition, the hormone may also affect gut. The action on each of these target tissues is discussed below.

Bone. Calcitonin acts on bone by inhibiting the resorption of calcium. Calcium ions in the blood are in constant interchange with bone. When resorption is inhibited, these ions continue to enter bone from blood but are prevented from leaving and thus hypocalcaemia results. This conclusion is supported by bone culture studies (Friedman and Raisz, 1965; Aliapoulios, Goldhaber and Munson, 1966; Reynolds, Dingle, Gudmundsson and MacIntyre, 1968), kinetic data (Milhaud, Pérault and Moukhtar, 1965; Johnston and Deiss, 1966), perfusion experiments (MacIntyre, Parsons and Robinson, 1967), urine excretion studies of

bone metabolites (Robinson, Martin, Matthews and MacIntyre, 1967) and demonstration that the number of osteoclasts is reduced both in vitro (Gaillard, 1967) and in vivo (Foster, Doyle, Bordier and Matrajt, 1966). Despite earlier conflicting evidence, it now appears certain that the hormone does not directly promote bone formation (Goldhaber, Stern, Glimcher and Chao, 1968; Kalu and Foster, 1971).

Kidney. Calcitonin decreases the urinary excretion of both calcium and magnesium and promotes phosphaturia and natriuresis. The effect on calcium excretion is due primarily to the action of the hormone on the bone. By inhibiting bone resorption, calcitonin lowers the plasma calcium level and therefore the filtered calcium load. The decrease in magnesium excretion may be explained by postulating a common pathway for tubular reabsorption shared by calcium and magnesium (Hanna, Harrison, MacIntyre and Fraser, 1960). If this is accepted, the decrease in magnesium excretion results from the reduction in the filtered load of calcium, thereby permitting increased tubular reabsorption of magnesium.

Calcitonin probably increases the renal excretion of phosphate by a direct action on the renal tubules. The phosphaturic effect of the hormone is more apparent in the absence of the parathyroid glands (Pors Nielsen, Buchanan Lee, Matthews, Moseley and Williams, 1970). This difference is explained as follows: in parathyroidectomised rats the serum phosphate is low and as a consequence the filtered load is low. A decrease in tubular reabsorption by calcitonin therefore causes a net increase in phosphate excretion (Williams, Matthews, Moseley and MacIntyre, in press).

In addition to inducing phosphaturia, calcitonin promotes excretion of sodium (Ardaillou, Vuagnat, Milhaud and Richet, 1967). The extent of the natriuresis depends on which calcitonin is being administered. Salmon and dogfish calcitonins produce a much greater effect than do either the human or the porcine hormones (MacIntyre, in press). The significance of this action is not apparent, but from an evolutionary point of view it may be that the original function of the hormone was to regulate ions other than calcium.

Gut. Calcitonin does not appear to affect the absorption of calcium from the gastrointestinal tract. Support for this conclusion is of two kinds. First, the hormone effectively lowers plasma calcium when the gastrointestinal tract has been removed (Aliapoulios, Savery and Munson, 1965). Second, calcitonin does not promote the absorption of isotopic calcium from isolated segments of gut in the parathyroidectomised animal (Robinson, Matthews and MacIntyre, 1968).

MECHANISM OF ACTION

The mode of action of calcitonin is not known. Several interesting theses have been proposed. Of these, two can be rejected: calcitonin acts neither by inhibiting parathyroid hormone (Hirsch, Gauthier and Munson, 1963; Aliapoulios, Savery and Munson, 1965; Gudmundsson, MacIntyre and Soliman, 1966; Tashjian, 1965), nor by increasing the synthesis of messenger ribonucleic acid (Tashjian, 1965; Gudmundsson, MacIntyre and Soliman, 1966) as many other hormones do. Four other possibilities exist, which are separately discussed.

First, calcitonin may regulate the influx or efflux of calcium at the cellular level. The effect of the hormone on a "calcium pump" remains, however, uncertain. Borle (1969) has presented suggestive evidence in kidney cells that calcitonin may be responsible for the ingress of calcium. Conversely, Parkinson and Radde (1970) believe that the hormone has the opposite effect. The latter investigators have found that calcitonin enhances the activity of calcium-activated ATPase of red cell ghosts, thereby increasing the rate of calcium efflux. This observation is in accord with current popular speculations (MacIntyre, 1970; Tenenhouse, Rasmussen and Nagata, 1970). Since the presence of intracellular calcium may be the signal to osteoclasts to initiate bone resorption (Rasmussen, Feinblatt, Nagata and Pechet, 1970), calcitonin, by effecting the release of calcium from these cells, may subsequently lead to the inhibition of bone resorption. This hypothesis is attractive in that it suggests how both calcitonin and parathyroid hormone may affect bone (Rasmussen and Pechet, 1970). It cannot, however, be completely accepted since there is as yet insufficient experimental support.

Second, calcitonin may act directly on bone cells to regulate their resorbing activity. This could be brought about in at least two ways. Calcitonin, by regulating local pH, may decrease the solubility of bone mineral adjacent to osteoclasts. Evidence that this is so has been presented by Nisbit and Nordin (1968). They observed that citric acid levels in bone culture, which are raised by parathyroid hormone, are depressed by calcitonin. In addition to changes in pH, bone resorption may be mediated by changes in cellular lysosomal activity. It is now well accepted that acid phosphatase activity contained

within lysosomes, is diminished by calcitonin (Doty, Schofield and Robinson, 1968). More recently, it has been observed that parathyroid hormone increases the number of acid phosphatase-rich lysosomes adjacent to the brush borders of parathyroid-stimulated osteoclast resorbing bone (Goshi, Scott and Foster, in press). These lysosomes appear to pass via the brush borders directly to the adjacent bone. Calcitonin has three effects on this process. It reduces the number of lysosomal granules adjacent to the brush border, causes the disappearance of the brush border and induces the cells to retract from adjacent bone surfaces.

Third, calcitonin may affect the production, distribution and differentiation of bone cells. MacManus and Whitfield (1970) find that calcitonin, in contrast to parathyroid hormone, inhibits mitotic activity. Whether or not this is a general effect on all cell types is not known. Evidence suggests that osteoclasts and osteoblasts may be affected in different ways. Both in vitro (Gaillard, 1967) and in vivo (Foster, Doyle, Bordier, Matrajt and Tun-Chot, 1967), calcitonin decreases the number of osteoclasts and increases the number of osteoblasts. These effects may be relatively rapid. In both rats and rabbits, administration of the hormone produces a significant decrease in the number of osteoclasts within 30 minutes (Mills, Haroutinian, Bordier and Tun-Chot, in press). This acute effect can be explained in one of three ways: (1) cells may be acutely destroyed by the hormone, (2) production of new cells may be inhibited, and (3) the osteoclast may be transformed into another cell type. It is unlikely that the decrease in the number of osteoclasts can be accounted for by the inhibition of the production of new cells. Since the life of the osteoclast

(Toto and Magon, 1966) is in the order of 48-72 hours, cessation of production of new cells within 30 minutes could only account for a 2-4% decrease. As the decrease in the number of osteoclasts is associated with an increase in multinucleated cells, it is more likely that the osteoclasts are transformed into another cell type. How this could occur is not yet known.

Fourth, the action of calcitonin may be mediated via cyclic AMP. Initially, it was thought that calcitonin activated the enzyme phosphodiesterase to destroy cyclic AMP (Wells and Lloyd, 1968), which mediates parathyroid-induced bone resorption. This hypothesis is untenable since calcitonin is now known to decrease adenyl-cyclase or cyclic nucleotide phosphodiesterase from skeletal tissue (Chase, Fedak and Aurbach, 1969). It now appears more likely that the hormone activates adenyl-cyclase. In both bone and kidney tissues, this enzyme is activated and enhances the production of cyclic-3'5'-adenosine monophosphate (Marcus, Heersche and Aurbach, 1971).

Each of the foregoing possible modes of action requires further confirmation. None, however, need to be mutually exclusive.

RELEASE AND SECRETION

The secretion of calcitonin is regulated by the level of calcium in the blood. Studies that initially proved the existence of the hormone (Copp, Cameron, Cheney, Davidson and Henze, 1962; Kumar, Foster and MacIntyre, 1963) and demonstrated that its source was the thyroid gland (Foster, Baghdiantz, Kumar, Slack, Soliman and MacIntyre, 1964) support this

conclusion. As assayed in the rat, calcitonin-like activity is present in the venous effluent of pig thyroid glands perfused with hypercalcaemic blood (Care, Cooper, Duncan and Orimo, 1968). No activity is measurable following perfusion with either normocalcaemic or hypocalcaemic blood. These findings have been confirmed by studies which have demonstrated that the level of the hormone, measured by radioimmunoassay, may be increased a thousand-fold in the thyroid effluent blood of a rabbit when this animal is infused with calcium (Potts, 1970). Furthermore, levels measured both *in vitro* (Talmage and Klein, 1968) and *in vivo* (Potts, 1970) were reduced under hypocalcaemic conditions compared to normocalcaemic conditions.

In addition to calcium, several other substances are thought to promote the release or secretion of calcitonin from the thyroid. These include streptomycin (Galante, Horton, Wiggins, Gudmundsson and MacIntyre, 1970), magnesium (Care, Cooper, Duncan and Orimo, 1968; MacIntyre, 1970), caerulin (Care and Bruce, 1971), gastrin (Cooper, Deftos and Potts, 1971) and glucagon (Melvin, Voelkel and Tashjian, 1970; Care, Bates and Gitelman, 1969). It is uncertain how these substances bring about their effect.

It has been suggested that other endocrine hormones may possibly affect the secretion of calcitonin. Both the parathyroids (Gittes and Irving, 1965) and the pituitary (Zileli, Kanra, Urunay, Guner and Caglar, 1968) have been proposed to elaborate releasing factors. At the present time, however, evidence that such factors exist from these organs has not been confirmed.

METABOLIC FATE

Relatively little is known about the distribution and metabolic fate of calcitonin. Recent evidence suggests that the porcine hormone is degraded by liver and to a lesser extent by kidney (de Luise, Martin and Melick, 1970). Since the studies which led to this conclusion were carried out with radioiodinated porcine hormone, the conclusions must be interpreted with caution.

Varying half-lives of the hormone have been reported in man and animals (Melvin and Tashjian, 1968; Tashjian and Melvin, 1968; Neer, Parsons, Krane, Deftos, Shields and Potts, 1970; West, O'Riordan and Care, 1969; Cunliffe, Black, Hall, Johnston, Hudgson, Shuster, Gudmundsson, Joplin, Williams, Woodhouse, Galante and MacIntyre, 1969). Most of the evidence suggests that the half-life of the hormone is less than 15 minutes, and that less than 1% of the circulating hormone is excreted in the urine (Melvin, Voelkel and Tashjian, 1970).

This subject is reviewed in detail in Chapter VII. For this reason, further discussion will be delayed.

STUDIES IN MAN

Relatively little is known about levels of calcitonin in man. Radioimmunoassays have been insufficiently sensitive to measure normal levels in man and biological assays have not been entirely satisfactory. Besides requiring large volumes of blood to allow for the extraction of hypocalcaemic activity, the results formerly obtained are now open to question and are thought

to be too high (MacIntyre, in press).

What is known can be briefly summarised. The only disease in which high levels are found with certainty is medullary carcinoma of the thyroid (Chapter V). In addition to this condition, Milhaud and his colleagues have reported that the hormone is elevated in carcinoid, benign C cell adenomas, and trabecular thyroid cancer (Milhaud, Calmettes, Raymond, Bignon and Moukhtar, 1970). (Whether or not the last two conditions are, in fact, genuine clinical entities remains to be proven).

While reservations must be made as to the levels reported by bioassay, several interesting observations have been made by Gudmundsson and his colleagues (Gudmundsson, Galante, Horton, Matthews, Woodhouse, MacIntyre and Nagant de Deuxchaisnes, 1970). These investigators have carried out studies in both normal subjects and in patients with various diseases. They have found in healthy volunteers that the circulating level of calcitonin in the blood is raised postprandially and following calcium gluconate infusions. These findings have subsequently been supported by radioimmunoassay (Tashjian, Howland, Melvin and Hill, 1970). In patients with hyperparathyroidism, levels are either elevated or in the upper range of normal, relative to those found in normal subjects. This finding is not surprising since the hypercalcaemia associated with this condition is likely to stimulate the hypersecretion of calcitonin. In three patients in whom the thyroid was totally removed surgically, and in whom there was no evidence of residual tissue, low normal levels were found. This is evidence that sources other than the thyroid may be secreting the hormone in man. In three patients with osteopetrosis, low normal or low values have been obtained.

The results are interpreted as evidence that this condition is not due to hypersecretion of the hormone as previously speculated. Likewise, patients with pseudohypoparathyroidism have been studied. The levels reported are variable, ranging from below to above normal, presumptive evidence that probably this condition is not due to hypercalcitoninism. High values have, however, been detected by immunoassay following calcium infusion in patients with this disease (Deftos, Bury, Mayer, Habener, Singer, Powell and Potts, in press). This finding supports the contention that the thyroid accumulates the hormone in this condition (Aliapoulios, Voelkel and Munson, 1966; Sturtridge and Kumar, 1968; Tashjian, Frantz and Lee, 1966).

THERAPEUTIC USES

Potentially, calcitonin may prove of clinical use in the treatment of Paget's disease, osteoporosis and hypercalcaemia. Insufficient studies have been carried out in each of these conditions to warrant definitive conclusions. However, some of the preliminary findings suggest that the hormone may have therapeutic uses.

Of the four conditions, Paget's disease appears the most amenable to treatment. Many studies are now in progress but a comparative assessment of the results is at present difficult, since different groups are using different preparations of hormone from different species. One or two general conclusions, however, can be drawn. Of the different calcitonins assayed in man on a weight basis, the salmon hormone appears to be the most potent when changes in plasma calcium are measured. Both salmon and porcine calcitonin have the potential disadvantage of

inducing the formation of antibodies. The fact that some patients treated with these hormones become refractive to treatment is preliminary evidence that this may be true (Dube, Goldsmith, Arnaud and Arnaud, 1971). For this reason, human hormone may prove to be the hormone of choice for use in man. The most encouraging results so far reported have been those obtained in our laboratory. Several Pagetic patients, with bone pain and with various degrees of skeletal involvement, have obtained both clinical and biochemical remission of their disease when treated for 3-18 months (Woodhouse, Reiner, Bordier, Kalu, Fisher, Foster, Joplin and MacIntyre, 1971).

Whether or not calcitonin will be of use in the treatment of osteoporosis remains in doubt. Since the rate of bone turnover in man is relatively slow and since calcitonin does not augment the rate of bone formation, it is unlikely that the hormone will ever produce dramatic results. However, long-term treatment may prove to be of value in preventing the progress of the disease.

Calcitonin effectively lowers plasma calcium in patients with hypercalcaemia. Initial studies with porcine hormone have demonstrated that it is effective in lowering plasma calcium levels in patients with hyperparathyroidism, hypercalcaemia secondary to malignancy, idiopathic hypercalcaemia and in children with vitamin D intoxication (Foster, Joplin, MacIntyre, Melvin and Slack, 1966; Milhaud and Job, 1966). Preliminary reports suggest that both porcine and salmon calcitonin are relatively ineffective in maintaining normal calcium levels in patients with hypercalcaemia (Dube, Goldsmith, Arnaud and Arnaud, 1971; Potts, personal communication; Hamilton, personal

communication). Since this failure may be due to the development of antibodies, the therapeutic value of calcitonin for the treatment of hypercalcaemia remains to be shown.

PHYSIOLOGICAL SIGNIFICANCE

The physiological role of calcitonin is still unknown. Since the preponderance of research effort has been carried out in mammalian species, attention has been focussed on the role of calcitonin in the regulation of calcium and skeletal metabolism. In mammals, calcitonin may be of physiological importance for one of three reasons. Implicit in each of these is its interrelationship with parathyroid hormone. First, calcitonin may be important in maintaining constant levels of calcium in biological fluids by preventing oscillations in plasma calcium induced by variations in secretion of parathyroid hormone. Second, calcitonin, together with parathyroid hormone, may effectively regulate the normal processes of bone growth and bone maintenance. Since both adult bone and growing bone undergo continuous bone resorption and accretion during remodelling, both hormones together may regulate these parameters. Third, both hormones, acting in concert, may augment positive calcium balance. Since calcitonin prevents the deleterious effects of parathyroid hormone on bone resorption, it may permit parathyroid hormone to effect increased calcium absorption both from the gut and renal tubule.

From an evolutionary point of view, calcitonin appears to be an older hormone than parathyroid hormone, having made its appearance in fish before the parathyroid glands. Since calcitonin is secreted by the ultimobranchial body in cartillagenous

fish (Copp, Brooks, Low, Newsome, O'Dor, Parkes, Walker and Watts, 1970), it is reasonable to suppose that its primeval function might have been other than the regulation of calcium and skeletal metabolism. The recent findings that both salmon and dogfish calcitonin have natriuretic and diuretic actions (Williams, Matthews, Moseley and MacIntyre, in press) and that ultimobranchial glands of eels affect osmolarity (Orimo, Fujita, Ohata, Okamo and Yoshikawa, in press) suggests that in phylogenetically primitive forms of life, this hormone may have been concerned with the regulation of salt and water. However, this thesis, while attractive, requires further substantiation.

Chapter II

RADIOIMMUNOASSAY

Summary:

In this chapter, the following topics are reviewed: the general principles of radioimmunoassay, methods for raising antisera, techniques for radioiodinating hormone, methods of separating hormone bound to antibody from free hormone, dilution and standard curves, mathematical concepts of antigen-antibody binding, correlation of results obtained by bioassay and radioimmunoassay and recent developments in methods of automating radioimmunoassays.

In 1956, Berson, Yalow, Bauman, Rothschild and Newerly demonstrated that radioiodinated insulin disappeared more slowly from plasma in patients receiving long-term treatment with insulin than in normal subjects. The delay in the disappearance of labelled insulin was shown to be due to the binding of the hormone to an acquired globulin, which proved to be an antibody to the insulin molecule. This observation was the first demonstration of antibodies to a relatively low molecular weight peptide hormone.

Subsequently, Berson and Yalow (1959) raised antibodies to bovine insulin in guinea pigs, and, with these, developed an assay to measure plasma levels of circulating insulin in man. In the past twelve years, their techniques have been applied to many other peptide hormones, and more recently to some non-peptide molecules as well.

GENERAL PRINCIPLES

To develop a radioimmunoassay, antibodies specific for the hormone to be measured, radioisotopically labelled hormone and pure hormone are required. The principle underlying the method

is as follows. Labelled hormone will compete with unlabelled hormone for binding sites on the antibody to the hormone. If varying amounts of unlabelled hormone are added to constant amounts of antiserum and labelled hormone, the percentage of labelled hormone binding to antibody will be inversely proportional to the amount of unlabelled hormone present in the system.

The principal advantages of radioimmunoassays are their sensitivity, specificity and convenience. Most assays can detect less than 100 pg of hormone, which is many times more sensitive than most biological assays. Chemical differences in storage and circulating forms of a hormone may be detected; hormones which vary even slightly in their amino-acid sequence may show different binding capacities relative to standard hormone (Berson and Yalow, 1968). In contrast to most bioassays, the results produced with radioimmunoassay are more precise and reproducible. In addition, the method permits the simultaneous estimation of many samples.

METHODS OF IMMUNISATION

Since the sensitivity and the specificity of any given radioimmunoassay depend on the antiserum used, the production of an antibody, with high affinity for the hormone to be measured, is fundamental to the development of a practical assay.

Antibodies are raised by giving repeated injections of the hormone, usually emulsified with an adjuvant (Freund, 1951), to animals of a species different from that of the hormone used as antigen. For this purpose, rabbits and guinea pigs are commonly used. However, high titre antibodies have also been produced in

a variety of other species (Tashjian, Bell and Levine, 1970), including birds (Reiss and Canterbury, 1968).

High affinity antibodies to large proteins are readily raised. In contrast, there is often difficulty in producing satisfactory antisera to smaller peptides for use in radioimmunoassays. Several techniques have been developed to make small peptides more immunogenic. One method is to covalently link the peptide to a larger and more immunogenic molecule such as bovine serum albumin or poly-L-lysine. This method has been successfully exploited to raise high titre antibodies to angiotensin (Goodfriend, Levine and Fasman, 1964), calcitonin (Tashjian, Bell and Levine, 1970) and arginine vasopressin (Beardwell and Wright, 1968; Edwards, Chard, Kitau and Forsling, 1970), and other small peptides. Since the conjugated hormone may have a different conformation, the antibodies raised by this method may differ from those formed against the non-conjugated molecule, and may not react with the native peptide. For this reason other methods have been developed.

One approach has been to adsorb the antigen onto a large inert particle (Boyd and Peart, 1968). This technique has two potential advantages. It may protect small peptides from enzymatic degradation _in vivo_ and may allow the antigen to be released more slowly into the reticulo-endothelial system.

The routes of administration of the immunogen are various, and have been ranked in decreasing order of their effectiveness as follows: lymph nodes and spleen, intradermal, intramuscular, intraperitoneal, subcutaneous and intravenous (Hurn and Landon, 1971). The size of the dose given is usually limited by one of

three considerations: the availability of the immunogen, the physiological effect of the hormone and, to a lesser extent, the size of the animal being immunised.

IODINATION OF ANTIGEN

In order to demonstrate the presence of antibodies in plasma, their ability to bind radioisotopically labelled hormone must be shown.

Peptide hormones are usually labelled with 125-iodine or 131-iodine because of the ease with which the reaction may be accomplished and the efficiency of counting the gamma radiation of these isotopes. The labelling is carried out by substituting iodine into the tyrosine residues of the molecule using the method of Hunter and Greenwood (1962). To produce the iodine to be incorporated, from the sodium iodide form in which the isotope is supplied, an oxidising agent, chloramine T, is used. Iodination is carried out at room temperature at neutral pH and the reaction stopped within 10-20 seconds by the addition of a reducing agent, sodium metabisulphite. Unreacted iodine and peptide fragments are removed by gel filtration, ion exchange or adsorption. Before it is used in the assay, the purified labelled hormone is tested for its ability to bind to antibody.

SEPARATION TECHNIQUES

Following incubation of labelled and unlabelled hormone with antiserum, a dynamic equilibrium is reached in which some labelled hormone is bound to antibody and some is free. It is necessary to separate this non-precipitating antigen-antibody

complex from the free labelled hormone in order to determine the amounts of each present.

Methods of separation fall into four main categories: differential migration, precipitation, adsorption and solid phase systems.

Differential migration of bound and free labelled hormone can be accomplished by gel filtration, chromatography, electrophoresis or chromatoelectrophoresis.

Chromatoelectrophoresis was the first system to be used (Berson and Yalow, 1956). It rapidly separates antibody-bound from free labelled hormone and thereby reduces possible dissociation of the complex. The method is as follows. An aliquot of incubation mixture is applied to a strip of Whatman 3MC chromatography paper, moistened with buffer, and the strip laid over a plastic support with both ends dipping into vessels containing buffer. A high voltage is applied which causes an electrophoretic separation of the antigen and antibody-antigen complex. The rate of separation is increased by allowing heat to generate in the centre of the strip, causing the buffer to evaporate and thus increasing hydrodynamic flow. Free hormone remains at the site of application, while the bound hormone migrates with the gamma globulins. This method has the advantage that it separates not only antibody-bound but also damaged hormone from free labelled hormone. Its disadvantages are that it is time-consuming and inefficient. Since the size of the aliquot applied to the paper strip is limited and relatively large amounts of labelled hormone are required to give efficient counting along the strip, as a consequence, the sensitivity of

the assay is decreased, because of the high concentration of tracer hormone needed.

Several precipitation methods have been described. These include salt precipitation (Farr, 1958), solvent fractionation (Thomas, Nash and Ferrin, 1969) and precipitation of gamma globulins by a second antibody (Morgan and Lazarow, 1962). The volumes used in these procedures are not limited, as in chromato-electrophoresis, and the concentration of labelled hormone may be decreased, theoretically providing assays with greater sensitivity.

Free labelled hormone has been separated from the antibody complex by adsorption of the former onto charcoal (Herbert, Lau, Gottlieb and Bleicher, 1965), silica or talc (Rosselin, Assam, Yalow and Berson, 1966). Of these, charcoal is the most commonly used. In this method, charcoal is coated with a dextran intermediate in size between the peptide hormone being assayed and the gamma globulin antibody. This permits small peptides to pass through the dextran coat and adsorb to the charcoal while excluding hormone bound to antibody.

Solid phase methods are of two kinds. In one, the antibody is coated to tubes (Catt and Tregear, 1967; Catt, Tregear, Burgher and Skermer, 1970). In the other, the antibody is incorporated into discs made of an insoluble polymer (Catt, Niall and Tregear, 1967). In both instances, the antigen-antibody reaction takes place on a solid surface and free hormone remains in the liquid phase.

Each separation technique has its own relative advantages

and disadvantages. It is necessary to select the one which is most applicable to the hormone being assayed.

DILUTION AND STANDARD CURVES

The binding of a labelled hormone to antibody is studied over a range of antiserum concentrations in the presence of a constant amount of labelled hormone. From these results, the least amount of antiserum needed to bind 50% of the labelled hormone in a convenient period of time is chosen to give maximum sensitivity in the assay (Berson, Yalow, Glick and Roth, 1964). With this amount of antiserum and the same amount of labelled hormone, changes in binding over a range of concentrations of standard hormone can be studied to provide a standard curve.

The detection limit of the assay is affected by many factors including the specific activity of the labelled hormone, pH, buffer salt, molarity of the buffer system, duration of incubation, temperature, amount of labelled hormone, volume of incubation, etc. Each condition must be separately studied in order that optimal conditions can be used to achieve maximum sensitivity. These conditions are more fully discussed in Chapter VI.

MATHEMATICAL CONCEPTS

The two most important considerations in assay performance are the working range of the standard curve and the detection limit of that curve. The order of magnitude of the working range can be described by its midpoint. The detection limit is defined as "The smallest concentration which is greater than and statistically different from the apparent concentrations found in

assay blanks" (Borth, 1970). The detection limit depends, therefore, on the blank and on the number of replicates and the variation of their results. Assuming equality of blank and sample variances S^2, obtained from 20-30 observations, and using a t-test at $p=0.05$, the detection limit for single samples is approximately equal to the apparent blank concentration + 2.5S (Borth, 1970).

Several mathematical models have been proposed to relate the working range and detection limit of an assay to the equilibrium constant of the antibody being used (Berson and Yalow, 1959; Ekins, Newman and O'Riordan, 1968; Potts, Sherwood, O'Riordan and Aurbach, 1967; Chard, 1971). Although there is not general agreement on any one model to describe the assay system, there is general agreement that the smallest amounts of hormone may be measured by antibodies with a high equilibrium constant, and that the titre of the antibody is not of primary importance.

VALIDITY OF RESULTS

Immunoassays quantitatively estimate the amount of hormone present which binds to the antibody used. Binding sites, or antigenic determinants on a peptide antigen are thought to have a diameter of less than 15 Ångstrom units and to consist of 2-8 amino acids (Goodman, 1969).

Peptide hormones may circulate in blood as intact biologically active hormone and as biologically inactive fragments. If the antigenic determinant for the antibody is included in one of these fragments, it will be recognised by the antibody and

measured by the assay. This has been found true for at least one immunoassay for follicle stimulating hormone (Butt, 1969). The presence of fragments in a plasma can be demonstrated by assaying the plasma sample with several different antisera which react with different parts of the antigen molecule. Should the same concentration of hormone not be found with each antiserum, it is probable that fragments are present in the plasma and that spurious values for the amount of intact biologically active hormone are being obtained. Using an antiserum which reacts to portions of the molecule which are biologically inert, estimation of the rates of disappearance of hormone from plasma become grossly inaccurate since the fragments are not distinguished from intact hormone (Berson and Yalow, 1968; Midgley and Jaffe, 1968; Yen, Llerna, Little and Pearson, 1968).

A second cause of spurious results in immunoassay may arise from cross reactions due to common or similar sequences in several peptides in the unknown sample. Antibodies, raised against one hormone, with an antigenic determinant on the common sequence, will react with the other hormones. This has proved to be the case with the glycoprotein hormones; luteinising hormone (LH), follicle stimulating hormone (FSH), human chorionic gonadotrophic (HCG) and thyroid stimulating hormone, thyrotrophin (TSH).

This cross reaction has been turned to some advantage in the assay of LH where antisera raised to the more readily available HCG have been used in the immunoassay of LH (Wide, Roos and Gemzell, 1961; Midgley, 1966), but has created difficulties in the assay of FSH for which highly specific antisera have proved difficult to obtain (Faiman and Ryan, 1967; Franchimont, 1969).

Assays for thyrotrophin (TSH) have also had problems of non-specific antisera (Odell, Reichart and Bates, 1969) which cross-react with HCG and LH. This lack of specificity has been overcome in the immunoassay by using the antiserum to TSH in the presence of constant amounts of HCG (Raud and Odell, 1969; Hall, Amos, Garry and Buxton, 1970). This added HCG completely absorbs all sites binding with it, allowing an assay which is specific for TSH alone.

Correlation of results obtained by radioimmunoassay to those obtained by a bioassay is the only convincing proof that biologically active hormone is being measured by the immunological method. Bioassays, capable of measuring in the range of immunoassays, however, exist only for a limited number of polypeptide hormones, including the gonadotrophins and adrenocorticotrophin. By exploiting both assay techniques, the immunologic-biologic relationship of a hormone may be studied and information on the relationship of structure to activity of polypeptide hormones may be obtained (Schwyzer, 1969; Landon, Girard and Greenwood, 1969).

RELATED ASSAY METHODS

Three methods will be briefly reviewed: (1) assays which employ labelled antibody, (2) assays for non-protein hormones and (3) receptor site assays.

Labelled antibody methods.

Several variations of the original radioimmunoassay method, in which antibody instead of antigen is labelled, have been developed. These systems have the following advantages. First,

antibodies, being larger molecules, are more readily labelled to a high specific activity and are more stable. Labelling of small hormone molecules, which possibly decreases their binding affinity for antibodies, is thus avoided. Second, antibodies with the greatest affinity for the antigen can be selected. Third, all the hormone being assayed reacts with the labelled antibody, thereby potentially increasing the precision of the assay.

Three techniques have been described. The first of these was developed by Miles and Hales (1968). (Figure 1). In this method, insulin was chemically bound to cellulose to produce an immunoadsorbent and insulin antibodies, in antisera, were allowed to bind to this complex. These antibodies were then labelled, while bound to cellulose, with 125-iodine and eluted from the immunoadsorbent with acid. The assay technique itself consists of incubating standard or unknown hormone with the labelled antibody; subsequently labelled antibody bound to hormone is separated from free labelled antibody by adding hormone attached to immunoadsorbent. The immunoadsorbent binds the free antibody and can be removed by centrifugation.

Unfortunately, the increases in sensitivity and precision have not proved to be as great as had been anticipated. Furthermore, the amounts of both antibody for labelling and antigen required for the immunoadsorbent are wasteful.

The second labelled antibody technique is the "two site assay" (Addison and Hales, 1971). (Figure 2). In this method one antibody to a hormone is linked to a solid matrix such as cellulose filter paper, and allowed to bind to the unknown

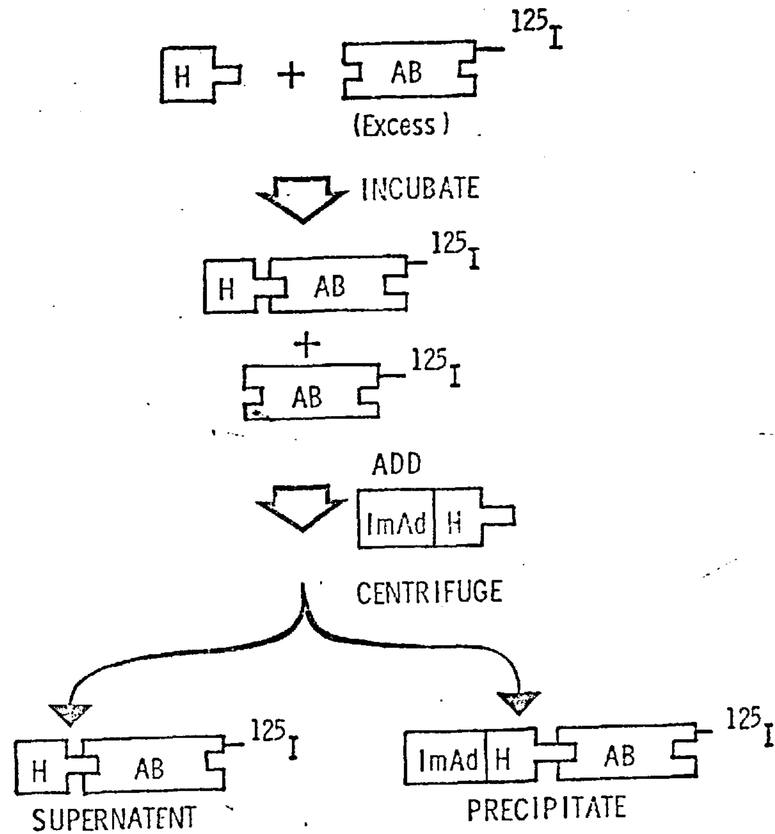

Figure 1. Principles underlying a radioimmunometric assay. Labelled antibody is incubated with plasma and binds to the hormone present in the solution. Excess free labelled hormone is removed by complexing with antigen attached to an insoluble immunoadsorbent. The insoluble complex formed is then removed by centrifugation and the labelled antibody attached to the hormone from plasma is counted.

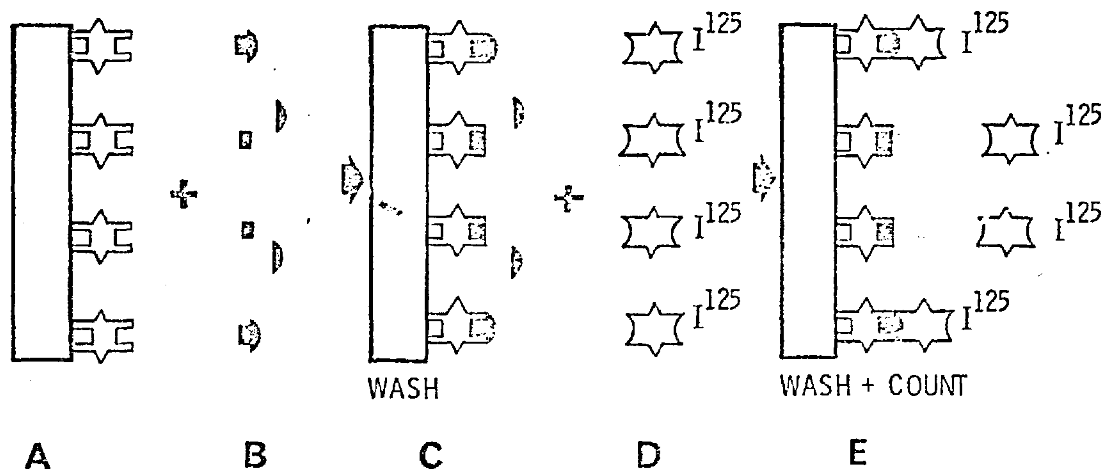

Figure 2. The two-site assay of Addison and Hales (1971). Antibodies linked to a solid matrix (A) are used to extract the hormone (symbol) from plasma (B). Cross-reacting hormones (symbol) are also extracted (C) but these do not bind the second labelled antibody (D) which is specific for the hormone being measured.

antigen in plasma. After washing, a ^{125}I-labelled antibody with a specificity to a different site on the antigen is reacted with this extracted antigen while it is still attached to the first antibody. After another wash, the labelled antibody, bound to the total insoluble complex, is counted, giving a measure of the antigen present. With this technique, two antigenic determinants on the hormone are recognised simultaneously, giving an increase in specificity. The method therefore offers a potential solution to the problem of cross-reaction between two similar antigens.

The third method (Wide, 1969 and 1971) uses cross-linked dextran (Sephadex) as an immunoadsorbent. Either the antigen or the antibody may be coupled to the immunoadsorbent, and by varying the technique, assays may be contrived to measure antigens, antibodies with different specificities or other biological compounds such as vitamin B12.

Assays for non-protein hormones.

Radioimmunoassays for measuring estrogens (Abraham, 1969; Midgley, Niswender and Ram, 1969; Mikhal, Wu, Ferin and Vande Wiele, 1970) and digoxin (Smith, Butler and Haber, 1969; Evered, Chapman and Hayter, 1970) have recently been developed. These substances are not immunogenic themselves, but are when chemically bound to large immunogenic proteins as haptenic groups. The antibodies produced do not cross-react with the protein conjugate, but, at least in the case of the estrogens, may cross-react with chemically similar molecules to the original haptenic group (Jeffcoate, 1971). Because of this non-specificity, these anti-estrogen antibodies do not allow the direct measurement of

the hormone in plasma; the substance to be assayed must first be extracted and purified. Despite this drawback, the method offers a practical alternative to the long and complicated chemical methods currently used for the measurement of steroids.

Receptor site assays.

A new approach to the assay of polypeptide hormones in plasma has been made with the development of an assay for ACTH using specific receptors from adrenal extracts instead of the antibody in the classical immunoassay (Lefkowitz, Roth and Pastan, 1970). Use of target tissue receptors in measurement of a polypeptide hormone has the principal advantage over current methods in that the assay is specific for the biologically active molecule.

The assay method for peptide hormones using tissue receptors is similar to the competitive protein binding assays currently used for measuring estradiol (Korenman, Perrin and McCallum, 1969) and aldosterone and cortisol (Murphy, 1967) in plasma. This latter technique is not applicable to peptide hormones as no specific binding proteins have been isolated for these molecules.

AUTOMATED TECHNIQUES

Until recently, radioimmunoassays have been principally used in research. Unquestionably, in the future, they will find more wide-spread application in clinical diagnosis.

Commercial kits of labelled hormone and antisera are now

available for both insulin and growth hormone (Radiochemical Centre, Amersham). No doubt similar preparations for the assay of other hormones will soon be marketed.

There is a pressing need in diagnostic laboratories for automated assays, and this need will increase as more immunoassays come into routine clinical use. Already some progress has been made in this direction. An automated method for assaying human chorionic gonadotrophin and luteinising hormone, using Technicon Autoanalyser equipment has been developed (Harris, Orr and Bagshawe, 1967). While the incubation time of the assay is less and some sensitivity is lost, subnormal levels can still be measured. Likewise, an assay for growth hormone has been automated (Knight, Caldwell and Audhya, 1971). For this purpose, an Analmatic preparation unit has been employed which has the advantage over the Autoanalyser equipment in that it has a discontinuous flow system and allows longer incubation times and no contamination between samples. In this system, reagents are automatically pipetted into tubes which are then incubated in a water bath at a controlled temperature. Bound and free hormone are separated by addition of anti-gamma-globulin. The resulting precipitate is automatically centrifuged and a fixed amount of supernatant aspirated and transferred to an automatic counter, which in turn presents the data in a suitable form for a computer to calculate and print out the results.

With these automatic techniques, the measurement of a hormone in plasma by radioimmunoassay may become a routine diagnostic laboratory procedure.

Chapter III

RADIOIMMUNOASSAY FOR HUMAN CALCITONIN

Summary:

The methods used for raising antibodies to human calcitonin, labelling the hormone with radioisotopic iodine, separating hormone bound to antibody from free hormone, and establishing the conditions for a useful radioimmunoassay requiring only an overnight incubation are described. This assay detected hormone over a range of 100 pg to 10 ng.

A homologous radioimmunoassay for human calcitonin only became feasible following the discovery that tumour tissue from patients with medullary carcinoma of the thyroid was rich in hormone (Meyer and Abdel Bari, 1968; Melvin and Tashjian, 1968; Milhaud, Tubiana, Parmentier and Coutris, 1968; Cunliffe, Black, Hall, Johnston, Hudgson, Shuster, Gudmundsson, Joplin, Williams, Woodhouse, Galante and MacIntyre, 1968). Crude extracts of this tumour, rich in calcitonin, could then be prepared for immunising animals, and the pure hormone isolated in sufficient quantities for both radioiodination and preparation of standards.

Preliminary attempts to assay human calcitonin using anti-serum raised to porcine calcitonin proved unsuccessful. This was not surprising when it became apparent that the sequences of the two hormones varied in eighteen of their thirty-two amino acids (Riniker, Neher, Maier, Kahnt, Byfield, Gudmundsson, Galante and MacIntyre, 1968).

In this chapter the methods used in the development of the first radioimmunoassay which could detect calcitonin in man are outlined (Clark, Boyd, Byfield and Foster, 1969).

PREPARATION OF ANTISERA

Material for immunisation was prepared in the following way. Ten per cent pure human calcitonin, containing 12 MRC units/milligram, was extracted with butanol-acetic acid-water (75:7.5:21) from tissue obtained at operation from two patients with medullary carcinoma of the thyroid (Neher, Riniker, Maier, Byfield, Gudmundsson and MacIntyre, 1968).

Ten milligrams of extract were dissolved in 1 ml of distilled water. To this 50 mg of finely divided carbon (Statex E.12., Columbian International (Great Britain) Limited) were added (Boyd and Peart, 1968). A suspension was prepared by ultrasonication for one minute and allowed to stand for five minutes. One hundred microlitres of a 10% Belloid solution (Geigy U.K., Limited) was added to enhance dispersion and the mixture again treated by ultrasonication for one minute. The resulting suspension was used on the same day for immunisation. Before injection, the fresh suspension was emulsified with 2 ml of complete Freund's adjuvant (Difco Bacto, Ltd.).

The immunisation procedure was as follows. The initial injections were administered directly into the spleen and popliteal and inguinal lymph nodes. A 1,500 gm New Zealand white rabbit was anaesthetised with veterinary Nembutal (0.5 ml/kg body weight) given intravenously and a respiratory stimulant (Daptazole) was kept available in the event of respiratory distress. Both groins of the animal were incised and the inguinal lymph nodes exposed. Approximately 0.5 ml of the antigen suspension was injected into these. Likewise, the popliteal nodes were exposed and injected with a similar dose. The spleen was

reached through a sub-costal incision and the remainder of the emulsion injected using a 25-gauge needle for this purpose. Post-operative recovery was uneventful.

The immunising technique used had one main advantage. It allowed small amounts of material to be used to greatest effect by direct injection into tissues of the reticulo-endothelial system. Had the antigen been given intramuscularly, much of it would have been locally destroyed. At the time these investigations were undertaken, even crude preparations of the hormone were in short supply.

Booster injections of freshly prepared suspensions of immunogen were given into the thigh muscles at irregular intervals over a period of eight months, and the animal bled periodically from a marginal ear vein. Plasma obtained from the final bleeding was used in the studies described in this chapter and is henceforth referred to as "Antibody I" in this thesis.

Subsequently the animal was further immunised for an additional nine months with the monomer form of synthetic human calcitonin similarly absorbed onto carbon, and antiserum collected at intervals. The antiserum obtained during the seventeenth month is referred to as "Antiserum II" and the results obtained with it are discussed in Chapters IV and VI.

DEMONSTRATION OF ANTIBODIES

Antibodies were confirmed by both neutralisation of biological activity and by demonstrating that isotopically labelled

hormone could be bound by the antiserum.

Neutralisation of biological activity.

This was proved by demonstrating that the hypocalcaemic effect of the hormone was abolished when incubated in antiserum.

In this investigation a modification of the original bio-assay of Kumar et al was used (Kumar, Slack, Edwards, Soliman, Baghdiantz, Foster and MacIntyre, 1965). Fifty gram female white Wistar rats, starved overnight, were injected intravenously, bled 30 minutes later and their plasma calciums measured by flame spectophotometry (MacIntyre, 1961). Four groups of five rats each were studied. Animals in group 1 received 0.4 ml of solvent buffer alone (0.15 M acetate, pH 3.0). Those in group 2 were given 1.8 MRC milliunits of human calcitonin in assay buffer. Animals in groups 3 and 4 were each given 1.8 MRC milli-units of human calcitonin which had been preincubated for 60 minutes at room temperature with 0.15 ml of antiserum and control rabbit serum respectively.

The hypocalcaemic effect of the hormone was abolished by calcitonin antiserum but not by control serum (Table 1). These findings are evidence that the serum from the rabbit destroyed the biological activity of the hormone.

Binding of labelled calcitonin to antibodies.

Because antibodies to small peptides cannot be precipitated when bound to antigen, other methods of demonstrating their presence and measuring their binding affinities were required.

GROUP	CALCITONIN 1·8mU/Rat	ANTISERUM 0·15ml/Rat	CONTROL PLASMA 0·15ml/Rat	Ca mEq/Litre
1	-	-	-	5·1 ± 0·1
2	+	-	-	4·4 ± 0·0
3	+	+	-	5·2 ± 0·1
4	+	-	+	4·4 ± 0·0

5 RATS IN EACH GROUP

Table 1. Effect of incubation of human calcitonin with antiserum. Five rats were studied in each group. Rats in group 1 were given 0.4 ml of the solvent buffer (0.15 M sodium acetate) intravenously and those in groups 2, 3 and 4, 1.8 MRC milliunits of calcitonin. Calcitonin given to each animal in group 3 was pre-incubated for 60 minutes with 0.15 ml antiserum at room temperature before injection. This resulted in complete loss of the hypocalcaemic effect of the hormone. In contrast, animals in group 4 received calcitonin which had been pre-incubated with normal rabbit plasma as a control. Full biological activity was retained.

In this study the presence of specific antibodies was confirmed by demonstrating that the antiserum bound radioisotopically labelled hormone.

^{125}I-labelled calcitonin was added to doubling dilutions of the antiserum in neutral phosphate buffer. The binding of labelled hormone to the antibody was confirmed by separating labelled antigen-antibody from any free labelled hormone by chromatoelectrophoresis and dextran-coated charcoal. (These techniques are described in detail later in this chapter). Between 85 and 90% of 200 pg of labelled calcitonin was bound to antibody in incubation tubes containing antiserum at a final dilution of 1:200 in a total volume of 300 µl. At a final dilution of 1:3000, 55-60% of the labelled hormone was bound. For each doubling dilution of antiserum, the percentage of labelled hormone bound to antibody decreased 18% (Figure 1). No binding of the labelled calcitonin was observed with normal rabbit serum under identical conditions.

When incubations of labelled hormone and antiserum were acidified to pH 2.0 prior to separation of bound and free hormone, all binding was reversed. This finding is evidence that antibody was present, since antigen-antibody complexes, formed by hydrogen ion bonding, are dissociated at acid pH.

The results of neutralisation of biological activity of the hormone and binding of the labelled antigen by the antiserum are strong evidence that an antibody to human calcitonin had been raised, using as immunogen an extract from a tumour from a patient with medullary carcinoma of the thyroid.

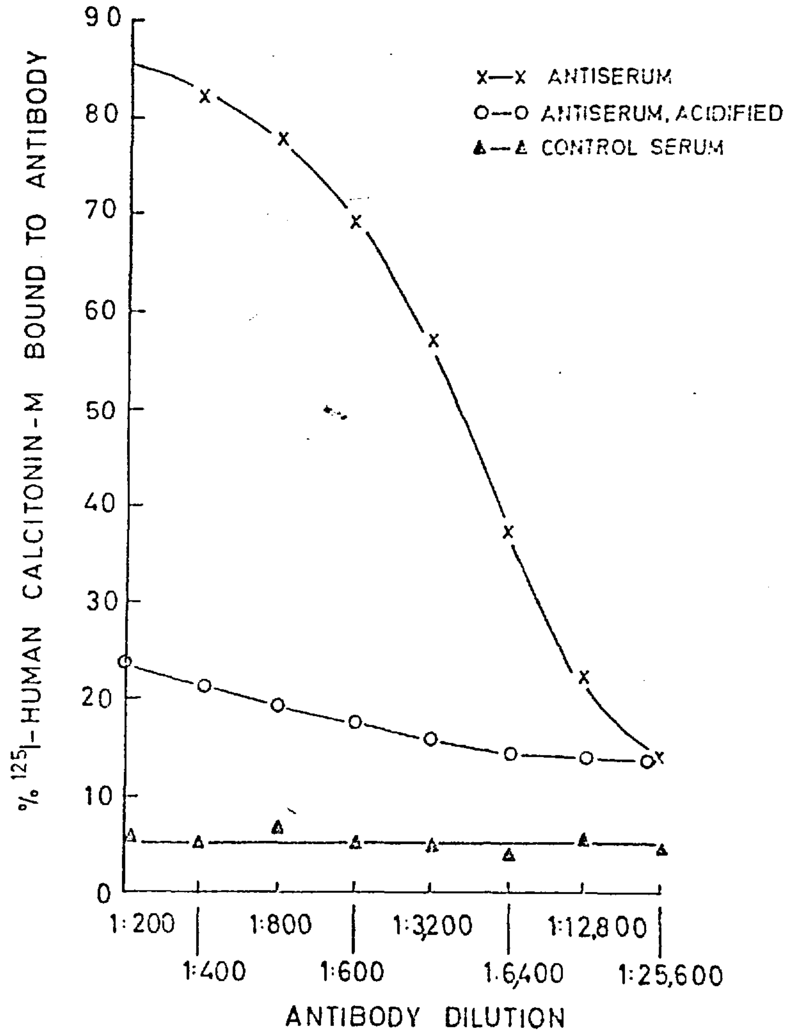

Figure 1. Antibody dilution curves for human calcitonin. Binding of 200 pg of labelled hormone was inversely related to the dilution of antiserum. On the steepest part of the curve, an 18% decrease occurred for each doubling dilution of antiserum. Binding was reversed by acidification before separation of bound and free hormone. Control serum bound no labelled hormone.

STANDARD SOLUTION OF CALCITONIN

Pure hormone is required both for standard and for preparation of radioiodinated hormone. For these purposes peptide free human calcitonin in the monomer form, isolated from a medullary carcinoma tumour by Dr. P.G.H. Byfield, was used.

One hundred microgrammes were weighed on a micro-balance. After the reading was checked by an impartial observer, the material was dissolved in 100 μl of 0.1 M formic acid. The latter was used since the hormone is most stable under acid conditions (Byfield, 1970).

The concentrated solution was divided into four aliquots, which were stored at -20°C and used for preparing radioiodinated hormone and as an internal standard for the assay. No loss of biological activity was found over a three month period of storage.

IODINATION OF HORMONE

To iodinate calcitonin, a modification of the method of Hunter and Greenwood (1962) is used. In this method Chloramine T is used to liberate iodine molecules from the isotopic iodine which is in the $Na^{+} I^{-}$ form. These labelled iodine molecules bind to the tyrosine residue of the hormone. The method allows peptide hormones to be labelled to a high specific activity.

The time of the reaction is critical since the peptide itself can become oxidised to a form which does not bind to antibody. To ensure optimum reaction conditions, a modification of

the method of Catt (1968) was used. For this, a micro-pipette is made by cementing a 25 µl capillary tube into the bottom of a polyethylene micro test tube (Beckman Spinco). The open end of the test tube is fitted onto a 2 ml disposable syringe. In this way reagents, separated from one another by air bubbles, can be drawn into the capillary tube and expelled simultaneously.

The procedure adopted is as follows. One millicurie of 125-iodine (as sodium iodide) in 10 µl 0.1 N sodium hydroxide is added to a 2.0 ml conical tube containing 20 µl of 0.5 M phosphate buffer pH 7.5. Into the prepared capillary syringe, chloramine T (40 µg in 25 µl 0.05 M phosphate buffer), air (20 µl), phosphate buffer (10 µl of 0.05 M, pH 7.5) as a wash, air (20 µl), and human calcitonin (4 µg in 4 µl 0.1 M formic acid) are drawn in that order. These are expelled into the buffered isotope solution. The reactants are mixed for 10 seconds and transferred, using the same capillary pipette, to a second 2.0 ml tube containing sodium metabisulphite (80 µg in 25 µl 0.05 M phosphate buffer pH 7.5). This reducing agent stops further oxidation.

To remove excess unreacted iodine, the reaction mixture is applied to a 1 x 6 cm column containing Amberlite CG 400 (100-200 mesh) in the acetate form,using the capillary pipette. Elution with 0.05 M acetate buffer pH 5.0 removes the labelled peptide and free iodine is retained by the ion exchange resin.

To assess the amount of iodine incorporated into the hormone, the damage which occurred during the reaction and the amount of free iodide remaining after purification, aliquots of the labelled preparation are chromatoelectrophoresed before and after

purification on the Amberlite column. This is accomplished by applying approximately 50 µl samples of the test mixtures to 1 x 24 inch strips of Whatman 3 MC chromatography paper. These are run in 0.05 M barbitone buffer in a chromatoelectrophoresis tank for 40 minutes at 600 volts. The strips are then dried and scanned for radioactivity using a Tracerlab 4π scanner.

Damaged hormone and free iodine migrate at different rates while undamaged peptide remains at the point of application. Damaged calcitonin, incurred during the labelling procedure, appears as a 'shoulder' adjacent to the peak of intact hormone at the origin (Figure 2). The percentage of labelled and damaged hormone to free iodide is calculated using an integrator on the strip scanner. If all the iodine were incorporated, the specific activity would be 250 µCi/µg of hormone. If, however, 20% of the iodine is shown in the chromatoelectrophoresis strip not to be incorporated, the specific activity would be 20% less or 200 µCi/µg. By the labelling technique described, between 80-100% of the radioisotope used was regularly incorporated into the calcitonin molecule.

A second evaluation of the labelled hormone was made by demonstrating that the labelling procedure had not altered the ability of the hormone to bind to excess antibody. An aliquot of the purified labelled hormone was diluted in phosphate buffer 0.1 M, pH 7.5, containing 0.2% bovine serum albumin, to give 200 cps in a 50 µl sample. Fifty microlitre amounts were added to 0.2 ml of a 1:80 dilution of antibody in the same buffer or to 0.2 ml of buffer alone, both in duplicate. Following a 30 minute incubation at 4°C, bound and free hormone in each tube were separated using dextran-coated charcoal (see next section) and

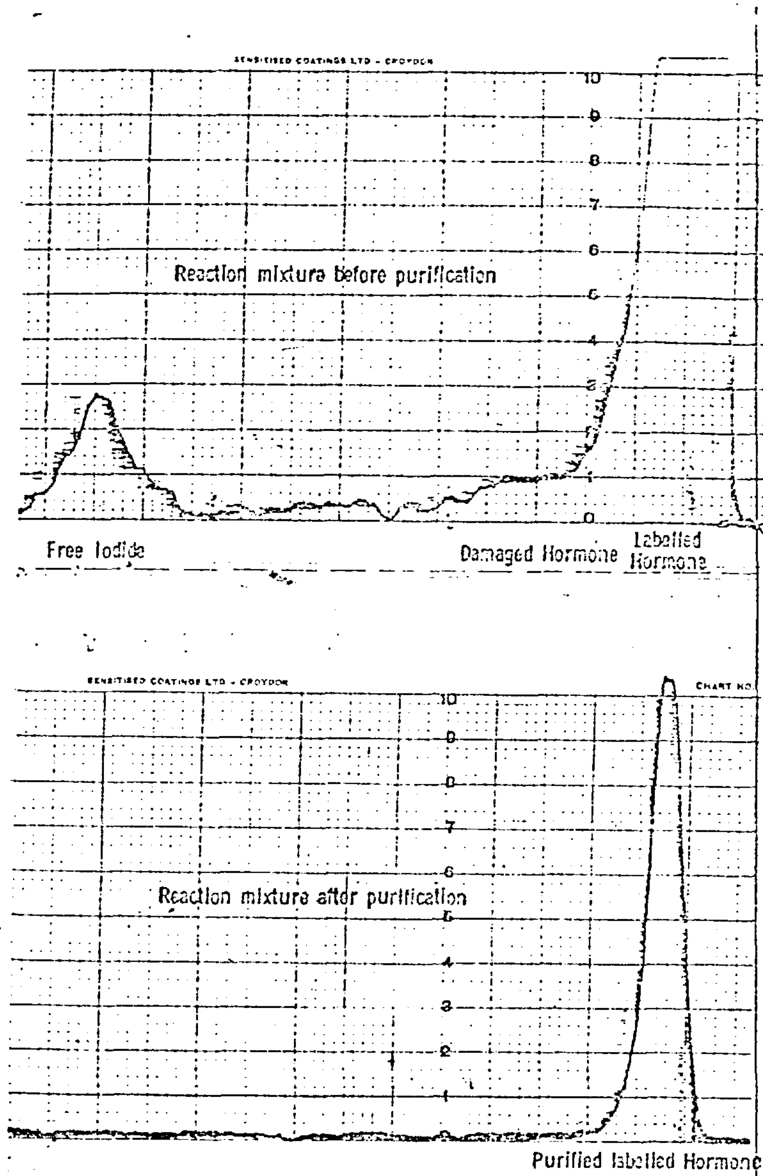

Figure 2. Comparison of labelled hormone before and after purification with Amberlite CG 400 as evaluated by paper chromatoelectrophoresis. All detectable free iodide was removed.

the percentage of label bound to the antibody calculated. During this period, 70-80% of the labelled hormone should bind to antibody. If binding was less than 70%, the label was discarded and a new preparation made. Labelled calcitonin was subsequently diluted in assay buffer to give 100 cps per 50 µl aliquot and this solution was stored in 10 ml aliquots at -20°C until used.

Since 125-Iodine has a longer half life than 131-Iodine, the former was used with calcitonin. Labelled hormone was stored for periods up to four weeks without observable deterioration.

METHODS OF SEPARATION

Two separation methods were investigated: chromatoelectrophoresis and dextran-coated charcoal.

Chromatoelectrophoresis.

The first method to be investigated was chromatoelectrophoresis. In this method the incubation mixture is applied to a strip of Whatman 3MC chromatography paper. When a high voltage is applied, free hormone remains at the point of origin while bound and damaged hormone migrate. The ratio of bound to free hormone is calculated by measuring the radioactivity of each fraction. Using this technique, hormone damaged during the incubation migrates from the origin, but more slowly than hormone bound to antibody. Separation from intact hormone, however, was found to be incomplete. For this reason and because the technique was time-consuming, chromatoelectrophoresis was not subsequently used and the following method was adopted in its stead.

Dextran-coated charcoal.

The method used was that of Herbert, Lau, Gottlieb and Bleicher (1965).

Most proteins and peptides bind to charcoal. By coating charcoal particles with dextran of a molecular size intermediate between that of the free hormone and the antibody-bound hormone, a "molecular sieve" is produced through which only the free hormone passes. Centrifugation of the coated-charcoal with its absorbed free hormone allows separation of free hormone from antibody-bound hormone.

This technique has the advantage of allowing large numbers of incubation mixtures to be separated at one time. Furthermore, the results are highly reproducible if times of mixing and centrifuging are well controlled. Intact labelled hormone is completely bound to the charcoal but free iodide or damaged peptide remains with antibody-bound hormone in the supernatant. For this reason, it is necessary to calculate the radioactivity in the supernatant of control tubes without antibody.

Dextran-coated charcoal is prepared in the following way. Two grams of charcoal (Norit SX.1., Haller and Phillips, Ltd.) are placed in a 100 ml cylinder and 100 mls distilled water added. The mixture is shaken and allowed to settle. The supernatant, which includes charcoal 'fines', is aspirated and 125 mg of Dextran T.40 (Sephadex) added to the slurry. The volume is adjusted to 100 ml with assay buffer.

Two hundred microlitres of the suspension are added to all

tubes containing incubation mixtures and tubes are well mixed. Free hormone adsorbed to charcoal is separated within five minutes to ensure standard and reproducible conditions.

Separation by this procedure is affected by protein. In tubes containing buffers and standards alone, the protein concentration does not significantly vary. However, tubes containing different dilutions of test plasmas have varying protein concentrations. The presence of 20 µl of plasma in a 300 µl reaction system did not affect separation of bound and free calcitonin. Therefore this amount was not exceeded.

SELECTION OF BUFFER

To obtain optimum assay conditions several factors required consideration. These included the choice of buffer for use in the assay and the means of separating bound from free hormone.

A phosphate buffer at neutral pH was chosen for preliminary work for two reasons. First, calcitonin was known to be more stable at acid pH. Second, antigen-antibody binding is reversed at acid pH. As a compromise, a neutral buffer was chosen. In addition, in order to prevent absorption of the peptide hormone to tube walls, 0.2% bovine serum albumin was added to all buffer solutions (Parsons, 1968).

Having selected a separation technique, other assay buffers were investigated. A series with varying pH were prepared and a 1:80 dilution of antiserum made in each. The buffers examined were 0.05 M acetate at pH 4.0, 4.6 and 5.0, and 0.1 M phosphate buffer at pH 6.0 and 7.5, all containing 0.2% bovine serum

albumin. No alkaline buffers were tested since human calcitonin was then thought to be unstable under alkaline conditions. Aliquots of freshly prepared ^{125}I-labelled hormone were diluted to 200 cps in 50 µl of each buffer.

Five sets of six tubes (Luckham, LP.3) each were numbered. Two hundred microlitres of the relevant buffer were pipetted into the first three tubes of a set and 200 µl of the diluted antibody in the remaining three tubes. Fifty microlitres of labelled hormone, diluted in the relevant buffer, were added to all tubes. The reactants were incubated at 4°C for 30 minutes. Following incubation, antibody bound and free hormone were separated by dextran-coated charcoal prepared in the relevant buffer system.

The pH of the incubation mixture had a marked effect on binding of the labelled hormone to antibody. Binding at pH 4.0 was 40% less than at pH 7.5 (Figure 3). The absorption of the free hormone to charcoal, observed in the tubes without added antiserum, was also affected by the pH change. Twenty per cent of the labelled hormone was not adsorbed to charcoal at pH 4.0 in contrast to 10% at pH 7.5.

The results of these studies on the effects of pH on the antibody-antigen binding and on the charcoal separation indicated that of the series studied, a buffer with a pH of 7.5 was optimal.

To confirm this finding, antibody dilution curves with the acetate buffer pH 4.6 or the phosphate buffer pH 7.5 were set up as follows. Ten tubes (Luckam, LP.3) in a rack were numbered 1-10. Into each was pipetted 200 µl of assay buffer using a

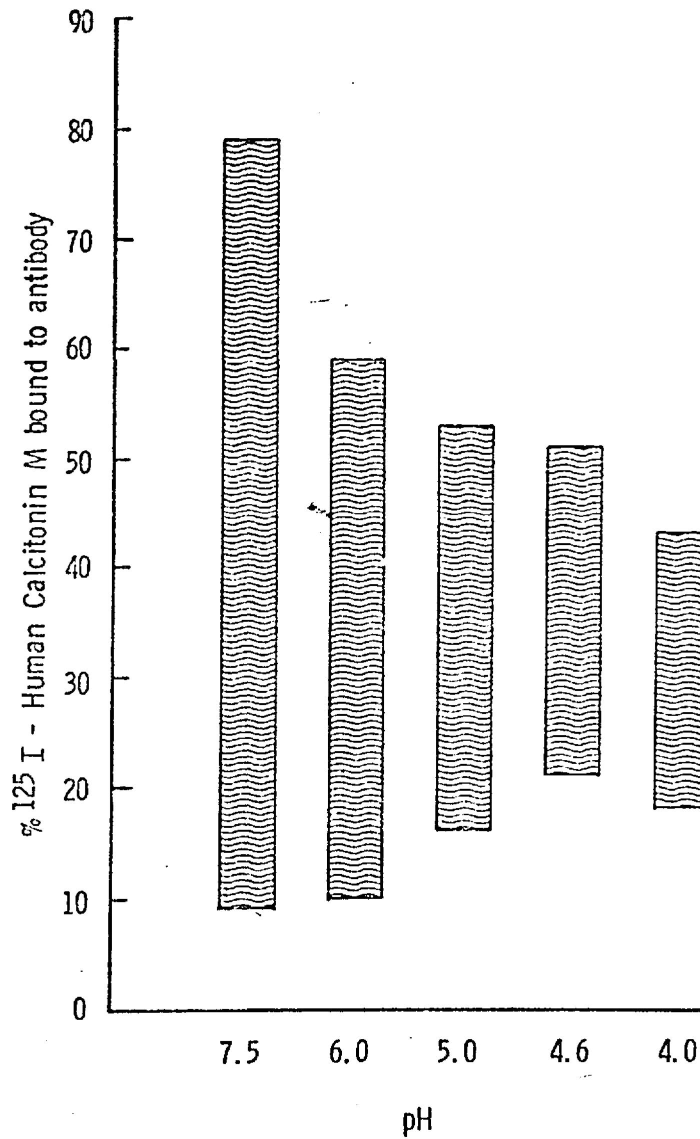

Figure 3. Effect of pH on binding of labelled hormone to antibody. Binding decreases with increasing hydrogen ion concentration and adsorption of labelled hormone to dextran-coated charcoal is likewise diminished.

constriction pipette. With the same pipette, 200 µl of a 1:80 dilution of antiserum in the same buffer was added to tube 3. This tube was mixed by mechanical vibration and 200 µl of its contents pipetted into tube 4. This double diluting procedure was continued until tube 10, when the excess 200 µl was discarded. Fifty microlitres of the labelled hormone diluted in the same buffer to give 100 cps were then added to all tubes. These were mixed and incubated for 18 hours before separation with dextran-coated charcoal. All dilution curves were set up in duplicate.

There was less binding of labelled calcitonin with each dilution of antiserum at pH 4.6 than at pH 7.5 (Figure 4). The results of these two experiments confirmed that binding of the antiserum to ^{125}I-human calcitonin was more satisfactory at neutral pH. For this reason 0.1 M phosphate buffer at pH 7.5 was used in subsequent investigations.

Using the antibody dilution curve derived from studies using 0.1 M phosphate buffer pH 7.5, the amount of antiserum which bound between 50-60% of the labelled hormone was estimated. This was found to be a dilution of 1:3000 in the final incubation volume. This amount was subsequently used in establishing standard curves.

STANDARD CURVES

A standard solution of calcitonin for assay was prepared by adding 10 µl of the standard solution in formic acid (1 µg/µl) to 0.1 M phosphate buffer, pH 7.5, containing 0.2% bovine serum albumin in a 100 ml calibrated flask and adjusting the volume to 100 ml. This solution of 100 ng/ml was aliquoted into 2 ml

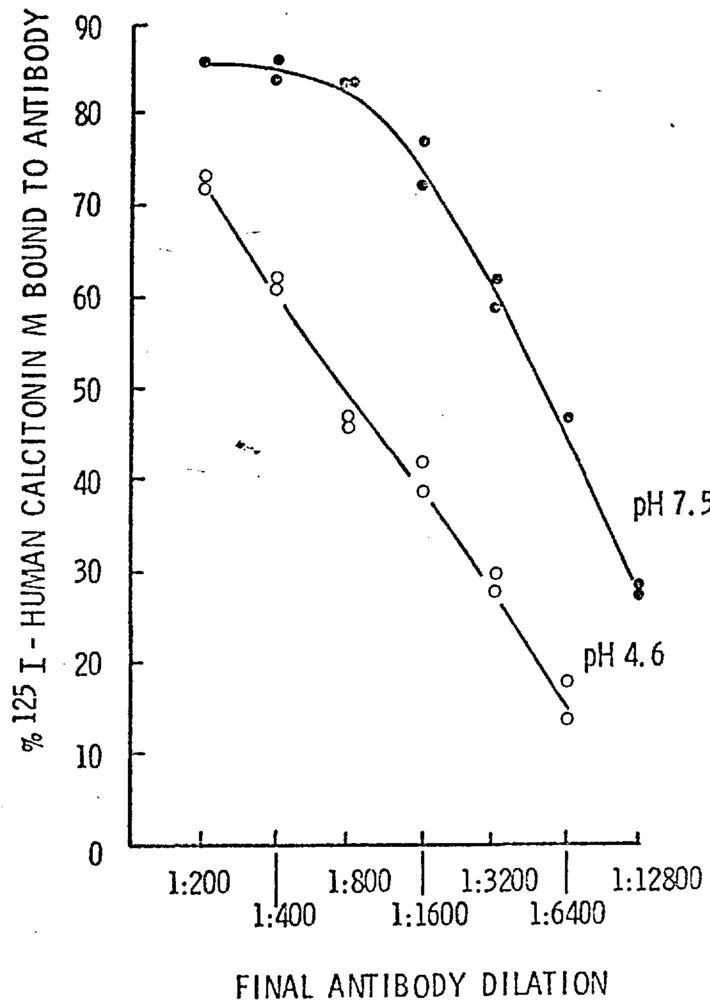

Figure 4. Antibody dilution curves in pH 4.6 and pH 7.5 buffer systems. The binding of labelled hormone to antibody was less at the more acid pH.

amounts and stored at -20°C. One aliquot was thawed for each set of standard curves, and the remainder of the solution was then discarded.

Standard curves were prepared as follows (Figure 5). Twelve tubes (Luckham, LP.3) were numbered 1 to 12 and placed in a rack. Two hundred microlitres of assay buffer were pipetted into each tube. A similar volume of the standard solution of human calcitonin (100 ng/ml) was added to tube 3, the contents mixed and 200 µl transferred to tube 4. This procedure of double diluting was repeated to tube 10 and the remaining 200 µl from this tube discarded. Tube 3 now contained 10 ng of standard calcitonin and tube 10 contained 80 pg. This procedure was done in duplicate. Fifty microlitres of ^{125}I-labelled calcitonin containing 100 cps were added to all tubes. Tubes 1 and 2 in each set of twelve were capped, and 50 µl of antiserum diluted to 1:500 were added to the remaining tubes. The final antiserum dilution was therefore 1:3000 in an incubation volume of 300 µl. All tubes were mixed thoroughly by mechanical vibration, and incubated at 4°C for 18 hours. Following incubation, antibody-bound and free hormone were separated with dextran-coated charcoal, and the charcoal precipitate containing adsorbed free hormone counted in a Packard Autogamma counter. The percentage of labelled hormone bound to antibody was calculated for each dilution of the calcitonin standard as follows:

$$\% \text{ Bound} = 100 - \left[\frac{\text{Free Counts}}{\text{Total Counts}} \times 100\right]$$

and the values obtained plotted as a standard curve (Figure 6). Tubes 1 and 2 indicated the amount of labelled hormone not binding to charcoal in a buffer solution alone and tubes 11 and

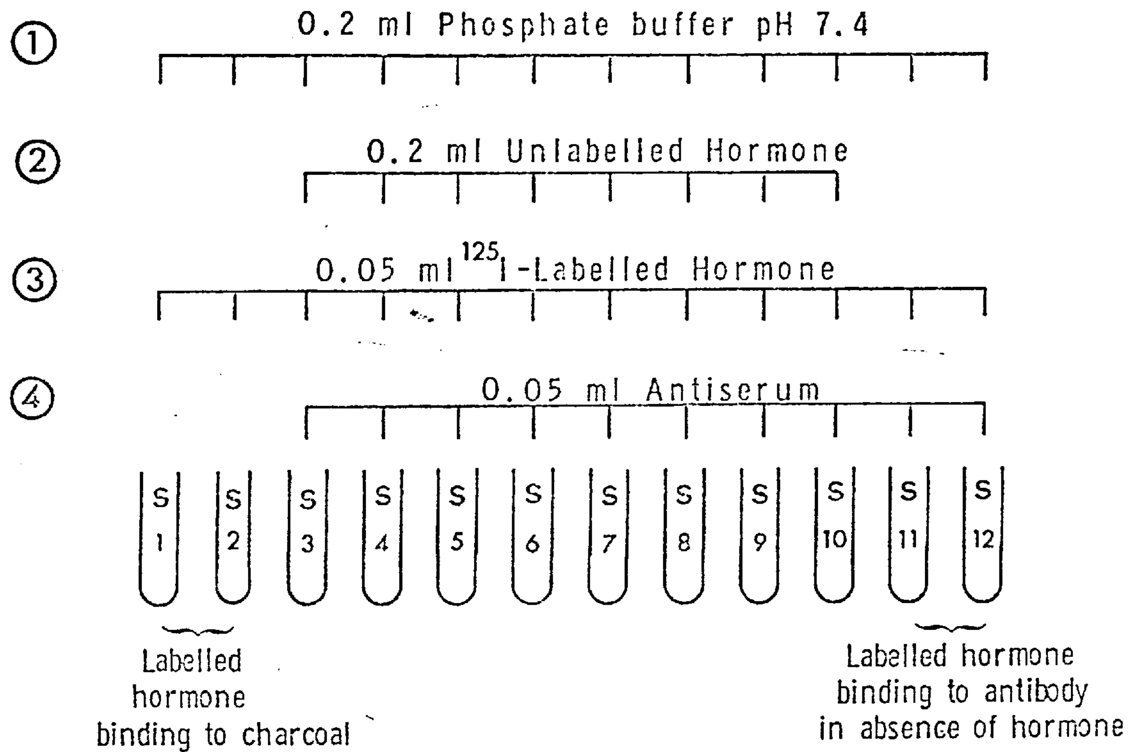

Figure 5. Protocol used to establish a standard curve. Into tubes 3-10 are added doubling dilutions of a standard preparation of human calcitonin over a range of 10-0.08 nanograms.

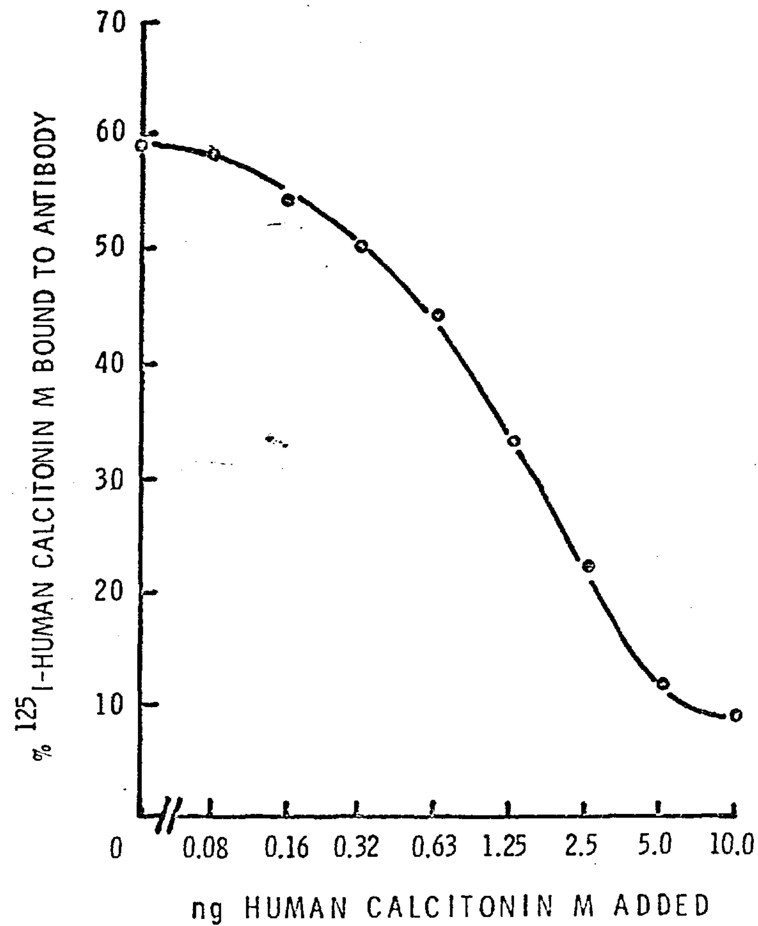

Figure 6. Standard curve for human calcitonin using an 18 hour incubation. The range of detection is 100 pg to 10 ng. Approximately a 10% change in binding of labelled hormone to antibody for each doubling dilution of standard hormone was found.

12 (containing only labelled hormone and antibody), the amount of labelled hormone bound to the fixed amount of antibody in the absence of any added standard hormone.

With this standard curve, the assay obtained had a detection range 100 pg to 10 ng, with a 10% change in binding of labelled hormone to antibody for each doubling dilution of standard hormone.

ALBUMIN AND NEOMYCIN

Preliminary studies indicated that the sensitivity of the assay decreased with increasing time of storage of labelled calcitonin. This was thought to be due to deterioration of the labelled hormone.

The buffer in which the labelled hormone was stored and in which the assay was carried out contained 0.2% bovine serum albumin to prevent adsorption of the hormone to the incubation tubes (Parsons, 1968). Since bovine serum albumin damages other labelled hormones during incubation (Greenwood, 1969; Herbert, Lau, Gottlieb and Bleicher, 1965), an experiment was designed to assess whether substitution with human serum albumin might prevent destruction of the labelled calcitonin.

Aliquots of freshly iodinated hormone were diluted in 0.1 M phosphate buffer, pH 7.5, containing either 0.2% human serum albumin or 0.2% bovine serum albumin (crystalline, Armour) to give 100 cps in a 50 µl sample. Half of each dilution was stored at room temperature overnight and the remainder at $-4^{O}C$. Eighteen hours later two fresh aliquots of labelled hormone were

diluted to give the same number of counts per second in 50 μl with a similar volume of 0.1 M phosphate buffer pH 7.5, containing either no protein or 0.2% human serum albumin. These six preparations were added as labelled hormone to tubes containing increasing dilutions of antiserum as previously described. Following incubation overnight, antibody-bound and free hormone were separated by dextran-coated charcoal made up in phosphate buffer with no protein and the binding of labelled hormone at each antibody dilution calculated. The results are summarised in Figure 7.

Fifteen percent more labelled hormone, stored in phosphate buffer with human serum albumin, bound to the antiserum than labelled hormone stored in buffer with bovine serum albumin. Labelled hormone in a buffer solution containing no added protein also showed reduced binding. This may have been due to competitive binding to the surfaces of the incubation tubes. Human serum albumin was subsequently used in all assay buffer solutions to prevent adsorption to glassware.

To prevent bacterial growth, the addition of 0.02% Neomycin sulphate to the buffer was also tested and found not to affect binding of labelled calcitonin to antibody. It was subsequently added routinely to all the phosphate buffers.

The final buffer chosen for the assay was therefore 0.1 M phosphate buffer, pH 7.5, containing 0.2% human serum albumin and 0.02% Neomycin sulphate. All labelled calcitonin was stored in this buffer and proved satisfactory for use for four or more weeks.

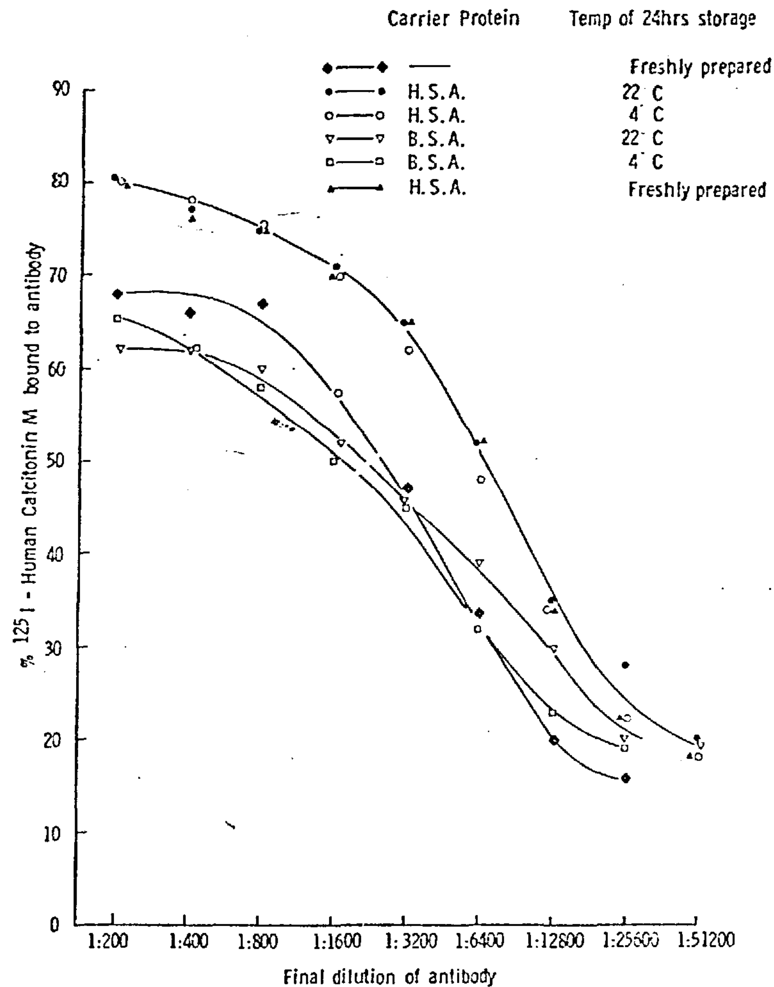

Figure 7. Comparison of binding of labelled hormone in buffers containing either bovine serum albumin (B.S.A.) or human serum albumin (H.S.A.). Greater binding was achieved with labelled hormone stored in the presence of human serum albumin at both 4° and 22° than in the presence of bovine serum albumin at either temperature, and this binding exceeded that found in the absence of any carrier protein.

These investigations led to the development of an assay which was capable of measuring between 100 pg and 10 ng of calcitonin. This assay was used for studies described in the following two chapters. Because of the need to exploit the assay immediately, further modifications to improve its limits of detection were deferred until a later time. These will be presented in Chapter VI.

Chapter IV

SPECIFICITY STUDIES

Summary:

Antiserum to human calcitonin bound not only the monomer but also both the dimer and calcitonin sulphoxide. It did not cross-react with several other peptide hormones and was relatively specific for the human hormone. An extract of normal thyroid tissue displaced labelled human calcitonin M, presumptive evidence that calcitonin M is immunologically identical to the naturally occurring hormone. Two antigenic determinants were identified. Subsequent immunochemical studies suggest that the molecule is not a random coil but has a preferred conformation, bringing the amino and carboxyl termini into close proximity.

In this chapter are described studies on the specificity of the antiserum, evidence that calcitonin is present in normal thyroid tissue, the antigenic determinants of the hormone and a tentative model of its structure deduced from immunochemical studies. The first of these to be discussed is the specificity of the antiserum.

CALCITONINS OF OTHER SPECIES

Calcitonins from five different species have been isolated and characterised (Kahnt, Riniker, MacIntyre and Neher, 1968; Riniker, Neher, Maier, Kahnt, Byfield, Gudmundsson, Galante and MacIntyre, 1968; Brewer and Ronan, 1969; Keutmann, Parsons, Potts and Schleuter, 1969; Potts, Niall, Keutmann, Deftos and Parsons, 1970). All are single chain polypeptides, composed of thirty-two amino acids, containing a 1-7 disulphide bridge, and terminating in a proline-amide. In addition, all have calcium-lowering activity, and some may affect renal transport of ions (Williams, Matthews, Moseley and MacIntyre, 1971).

It was of interest to determine whether an antibody to human calcitonin would cross react with calcitonin from any other species, in which case it might be of use either in the immunoassay of calcitonins from other species or for specific immunofluorescent localisation of calcitonin-containing cells in tissues other than those from man.

Materials and methods.

Calcitonins from four species were investigated. The highest concentration of each peptide assayed, its purity and biological activity are indicated in Table 1.

Pure porcine and salmon calcitonins and an extract of chicken calcitonin were dissolved in 0.1 M formic acid and diluted in phosphate assay buffer. Doubling dilutions of these were assayed and their displacement curves compared to that of the human hormone. In addition, a crude extract of rat calcitonin, estimated to contain less than 1% pure hormone, was also studied. Three milligrams of this material were dissolved in 200 µl of formic acid and 600 µl of phosphate assay buffer added. Two hundred microlitres were tested at five doubling dilutions.

Labelled human calcitonin (50 µl) and antiserum to the human hormone (50 µl of 1:500) were added to all tubes. Following overnight incubation at $4^{o}C$, antibody bound and free labelled hormone were separated as described in Chapter III. The radioactivity of free hormone was counted and inhibition of binding of labelled human hormone to human antibody produced by calcitonins from the other species was calculated.

Species	Amount Tested μg	Estimated percent purity	Source	Biological activity MRC units/mg
Porcine	1	100	Ciba-Geigy	100
Salmon	50	100	Potts	4,000
Chicken	2.0	80	Copp	-
Rat	750	1	Byfield	-

Table 1. Calcitonins from different species tested in the human calcitonin assay system.

Results and discussion.

Pig and chicken calcitonins, at concentrations one hundred and two hundred times greater respectively than human calcitonin did not displace labelled human hormone from antibody. Salmon calcitonin at a concentration 5,000-fold greater than human hormone displaced only 10% of the labelled hormone.

The extract of rat thyroid which contained less than 1% biologically active calcitonin, fully inhibited binding of the labelled human calcitonin to antibody. This finding may indicate a similarity in sequence between rat and human calcitonins, but not complete identity since the slopes of the displacement curves were not the same (Figure 1).

OTHER POLYPEPTIDE HORMONES

Other polypeptide hormones were examined for their ability to compete with ^{125}I-human calcitonin for binding sites on the human calcitonin antibody.

Materials and methods.

The peptides tested were parathyroid hormone, oxytocin, vasopressin, bradykinin and beta 1-24 adrenocorticotrophin.

Between 500 ng and 1 µg of each peptide was diluted in 200 µl of assay buffer and four doubling dilutions made from this concentration. Labelled hormone and antibody, as described in the preceding study, were added to all tubes. The contents were incubated overnight at 4°C and the amount of binding of labelled

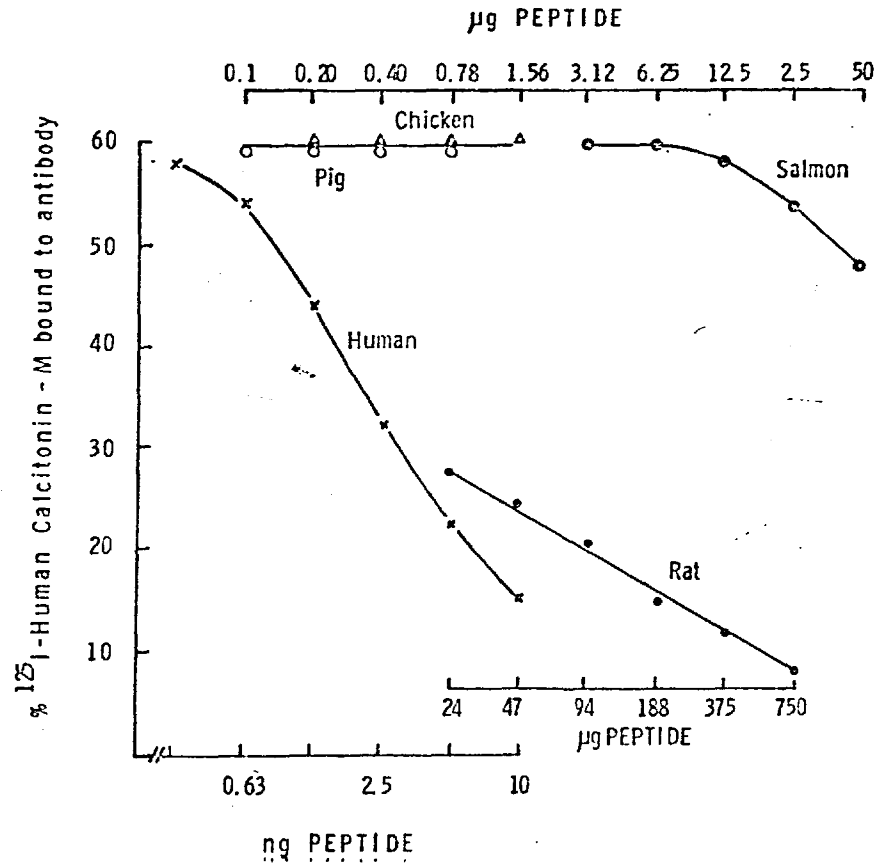

Figure 1. Inhibition of binding of labelled human calcitonin to antiserum by calcitonins from other species. Pig and chicken in the amounts tested showed no inhibition, salmon showed 10% inhibition at high concentration, and an impure extract of rat hormone completely inhibited all binding of labelled human hormone to antibody.

hormone to antibody was calculated. In these and all subsequent investigations, results were compared with standard curves for human calcitonin run simultaneously.

Results and discussion.

At the lowest dilutions tested, no peptide other than calcitonin displaced labelled human calcitonin from antibody (Figure 2). The studies carried out on each of the preceding polypeptide hormones have special relevance.

In animals, calcitonin levels measured by radioimmunoassay are inversely proportional to levels of parathyroid hormone (Arnaud, Littledike and Tsao, 1968; Potts, 1970). Since parathyroid hormone does not cross-react with this calcitonin antiserum, the conclusion is drawn that this hormone could not be responsible for spurious results due to competition for non-specific binding sites.

Oxytocin, vasopressin and calcitonin affect membrane permeability (Leaf, 1967; Tenenhouse and Rasmussen, 1968). Each contain a disulphide bond. The findings suggest that this area of molecular similarity is not responsible for the binding of the human hormone to its specific antibody.

Bradykinin is a peptide secreted by carcinoid tumours (Oates, Pettinger and Doctor, 1966). Since carcinoid tumours may contain calcitonin-like immunological activity (Chapter V), the results indicate that bradykinin is not responsible for the displacement of labelled human calcitonin observed.

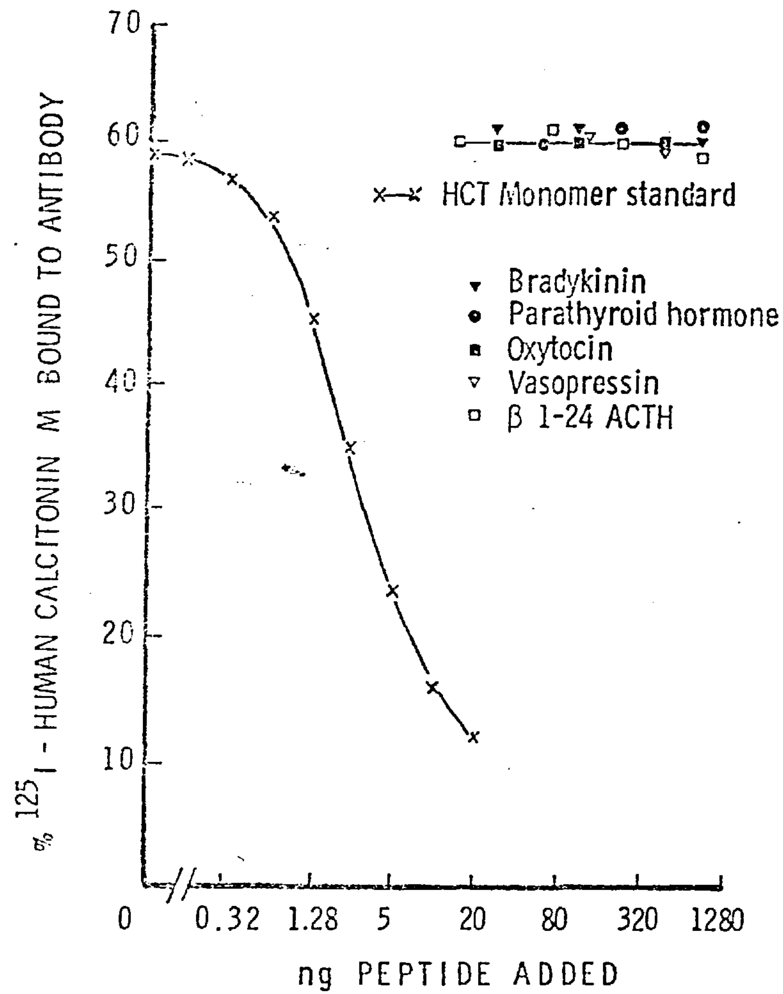

Figure 2. Specificity of calcitonin antiserum. Even at concentrations 100-fold greater than that which produced displacement with human calcitonin, other polypeptide hormones did not affect binding of labelled hormone to antibodies present in the antiserum.

DERIVATIVES OF HUMAN HORMONE

The following studies were designed to assess the immunological activity of the dimeric form of the human hormone and human calcitonin M sulphoxide.

Calcitonin extracted from tumour tissue of patients with medullary carcinoma of the thyroid is found in two major forms: the monomer, calcitonin M, and its antiparallel dimer, calcitonin D (Riniker, Neher, Maier, Kahnt, Byfield, Gudmundsson, Galante and MacIntyre, 1968). In addition, calcitonin with the methionine^{8} residue oxidised has also been found in some extracts. As these modifications of the hormone may be present in plasma, it was necessary to determine the relative capacity of the antibody to bind with them.

Materials and methods.

Ten micrograms of calcitonin dimer and 10 μg of synthetic human calcitonin sulphoxide were each dissolved in 10 μl of 0.1 M formic acid and diluted to 100 ng/ml in assay buffer. Ten doubling dilutions, in duplicate, of the two solutions were made and assayed.

Results and discussion.

The displacement curve produced by the dimeric form of calcitonin was not significantly different from that produced by the monomer (Figure 3). This finding indicates either that the antibody binds the anti-parallel dimer, or that, under the conditions of the assay, the dimer is converted to the monomer.

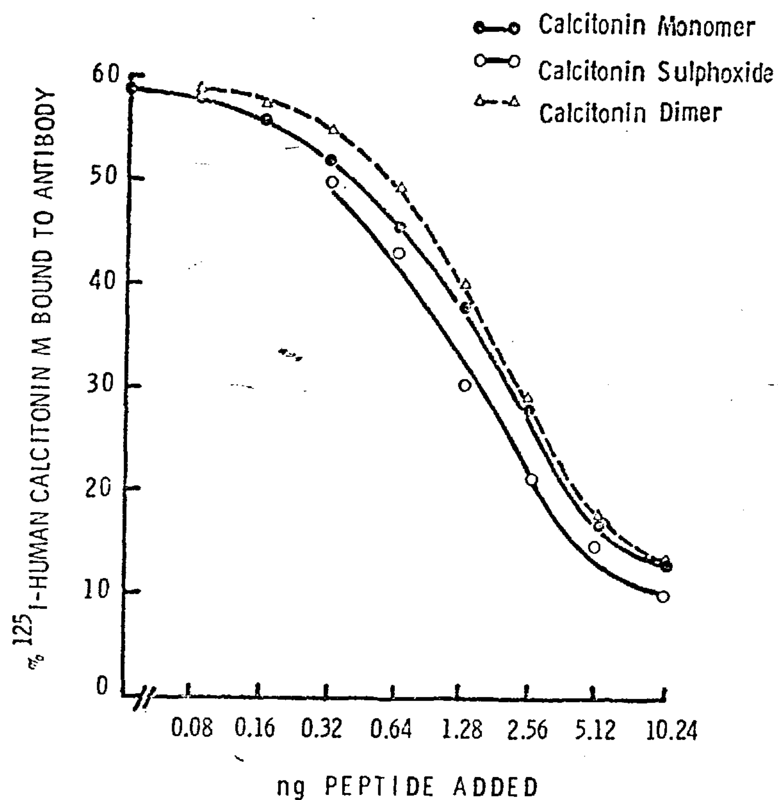

Figure 3. Comparison of inhibition curves for calcitonin monomer, dimer and sulphoxide forms. The monomer and dimer produced similar displacement in the assay while the sulphoxide displaced 25% more labelled monomer from the antibody.

The dimer, which has no biological activity, is stable in 0.1 M solutions of formic acid at pH 3.0 (Byfield, 1970). There is no evidence, however, of its stability in phosphate buffer, since the phosphate in the buffer interferes with the bioassay by producing hypocalcaemia.

Displacement curves produced by the sulphoxide indicate that the binding affinity to antibody was increased by oxidation (Figure 3). More vigorous oxidation of this molecule with performic acid breaks the 1-7 disulphide bridge, and displacement curves of this product were identical with that of the sulphoxide. The results are consistent with the conclusion that the increased binding of the oxidised peptide was due to an effect at the methionine residue, and not to the disulphide bond.

Both calcitonin D (MacIntyre, 1970) and calcitonin sulphoxide (Riniker, Neher, Maier, Kahnt, Byfield, Gudmundsson, Galante and MacIntyre, 1968) are biologically inactive, but as has just been shown, both are immunologically active. The immunoassay may therefore measure in plasma hormone which is biologically inert. This, however, appears unlikely, since the results obtained by bioassay and immunoassay are in good agreement (Chapter V).

EXTRACTS OF NORMAL THYROID

The antibody to calcitonin M was raised against an extract of medullary carcinoma tumour tissue. This antiserum bound pure hormone isolated from similar tissue. There was, however, no evidence that this calcitonin was the naturally occurring hormone. Since radioimmunoassay is likely to distinguish minor alterations

in either sequence or structure, it was used to test for immunological similarity between the tumour-derived peptide and the hormone extracted from normal thyroid tissue.

Materials and methods.

Samples of normal appearing non-malignant human thyroid tissue were obtained at operation from three patients with nodular non-toxic goitre. Each was extracted with butanol/acetic acid/water (150/15/42 by vol.) (Neher, Riniker, Zuber, Rittel and Kahnt, 1968). Following lyophilisation, the dried powder was dissolved in 0.1 M formic acid, diluted with buffer and assayed.

Results and discussion.

The displacement curve produced by normal tissue extract was parallel to that of the calcitonin standard (Figure 4). This finding indicates that the two peptides are immunologically similar. Since insufficient normal human thyroid was available to permit isolation of pure natural hormone, known amounts could not be compared to confirm immunological identity. However, both normal thyroid and medullary carcinoma tissue contain biological activity (Gudmundsson, Galante, Horton, Matthews, Woodhouse, MacIntyre and Nagant de Deuxchaisnes, 1969). It is therefore a reasonable assumption that calcitonin M, isolated from neoplastic tissue is the naturally occurring hormone.

IMMUNOCHEMICAL STUDIES

If equimolar amounts of either a whole peptide or one of

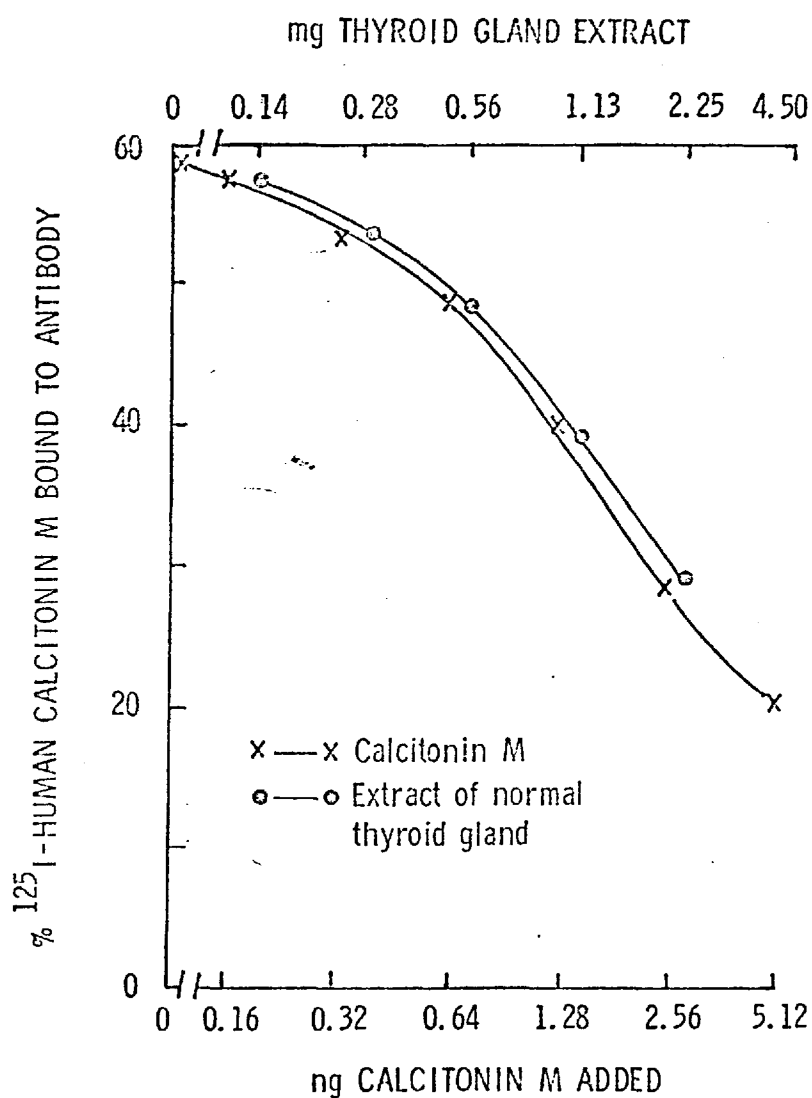

Figure 4. Comparison of the inhibition curves produced by human calcitonin extracted from a medullary carcinoma tumour, and by an extract from normal thyroid tissue. The curves are not appreciably different from each other, evidence that the two substances are immunologically similar.

its altered fragments displace all labelled hormone from antibody, then no antibody binding sites have been lost from the fragment, and its binding energy has not been reduced. If, however, complete displacement of the labelled hormone from antibody is only produced by increased concentrations of the altered hormone, then the binding energy has been reduced. When complete displacement of labelled hormone from antibody is never accomplished, irrespective of the concentration of the peptide fragment, the fragment must then have lost one of its antibody binding sites.

By comparing the competitive inhibition of binding of labelled hormone by peptides and closely related fragments, some conclusions on the relationship between the peptide structure and its immunological activity may be drawn.

The assay was used to study the antigenic determinants of the hormone. In these investigations, displacement of ^{125}I-labelled calcitonin from antibody by chemically modified calcitonin and fragments of the hormone were compared with the displacement by the whole peptide.

Materials and methods.

The chemical modifications carried out in the laboratory were as follows: (1) cyanoethylation at the terminal and ε-lysine^{18} amino groups and at the imidazole side chain on histidine^{20}, thereby removing the three positive charges on the calcitonin molecule, (2) oxidation of the molecule at the methionine^{8}, (3) oxidation of the molecule at the 1-7 disulphide bridge and the methionine^{8}.

The fragments studied were as follows:

H-1-18-OH and H-19-32-NH_2 prepared by tryptic digest, and not isolated from the resulting mixture.

H-1-12-OH and H-13-18-OH prepared by chymotryptic digest of the H-1-18-OH fragment, while in the tryptic digestion mixture.

H-11-28-OH, H-11-16-OH, H-17-28-OH, H-11-32-NH_2 and H-29-32-NH_2 were prepared by synthesis (Dr. Rittel, Ciba-Geigy, Basle).

H-9-32-NH_2, H-13-32-NH_2 and H-19-32-NH_2 were isolated by Dr. P.G.H. Byfield in the laboratory.

H-11-18-OH and H-19-28-OH were prepared but not separated by tryptic digestion of the synthetic fragment H-11-28-OH.

To permit quantitative comparison, weights of peptides were compared on a molar basis.

These peptides were compared with intact human calcitonin monomer for their affinity to bind to two antisera, Antiserum I and Antiserum II. Antiserum I was obtained eight months after immunisation with a 10% pure tumour extract of calcitonin. Antiserum II was obtained after further immunisation for nine months with the synthetic hormone (Chapter III).

Results.

The displacement curves produced with the intact hormone

when Antiserum I or Antiserum II were used were identical and the dilution of each antiserum required to produce 50% binding did not appreciably differ. However, the binding affinities of fragments to the two antisera were strikingly different.

(1) <u>Studies with Antiserum I.</u>

A displacement curve produced by calcitonin cyanoethylated at residues 1, 18 and 20 was identical to that of the intact hormone, indicating that the positive charges at these residues were not involved in the antigenic determinants of this antiserum (Figure 5).

Calcitonin altered by either oxidation of methionine^{8} alone, or methionine^{8} and the 1-7 disulphide bridge, displaced ^{125}I-labelled calcitonin from antibody more readily than the intact peptide (Figure 5). This finding suggests that oxidation of methionine^{8} is responsible for the increased binding, and not oxidation of the 1-7 disulphide ring.

During tryptic digestion, the hormone was cleaved at lysine^{18} and the mixture of peptides in the digest displaced less ^{125}I-labelled hormone from antibody than did the intact hormone. A chymotryptic impurity in the trypsin produced a further slow cleavage at tyrosine^{12}. This cleavage of H-1-18-OH further decreased the binding of the digest mixture with the antibody (Figure 6). The findings suggest that the binding energy of the antigenic sites is related to the integrity of the bonds 12-13 and 18-19.

That residues between tyrosine^{12} and phenylalanine^{19} are

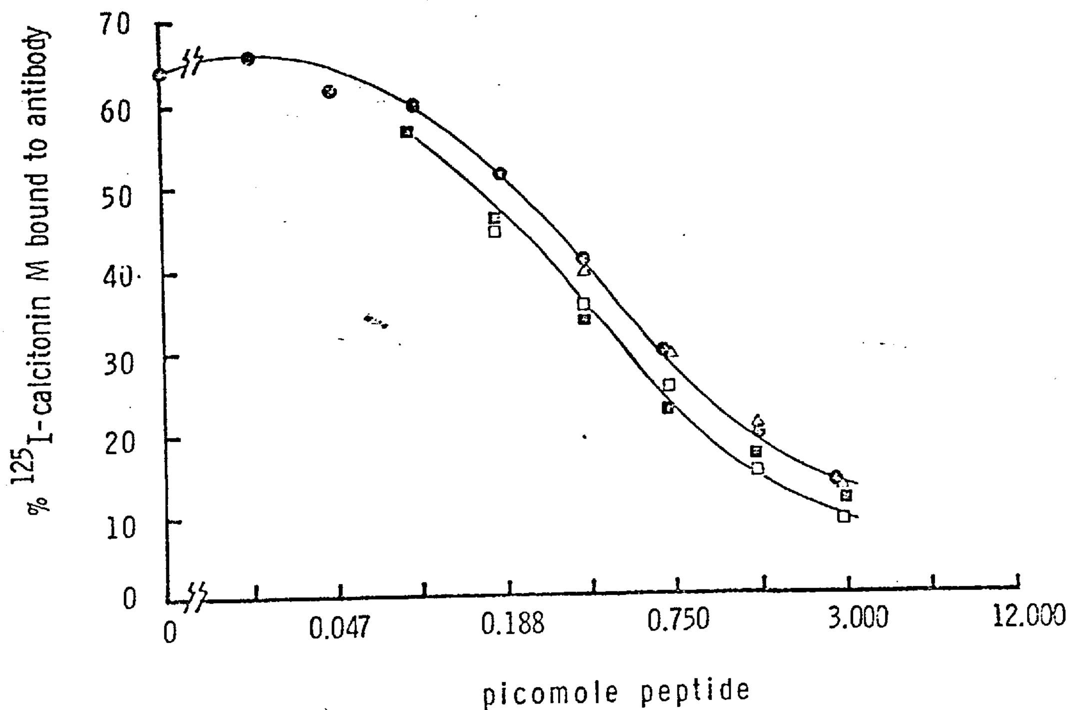

Figure 5. Displacement of labelled calcitonin monomer from antibody by cyanoethylated calcitonin (Δ). There was no difference in binding between the monomer (⊙) and this altered peptide, in contrast to the increased binding observed with calcitonin oxidised at the methionine^{8} alone (□), or at the methionine^{8} and the 1-7 disulphide bridge (⊡).

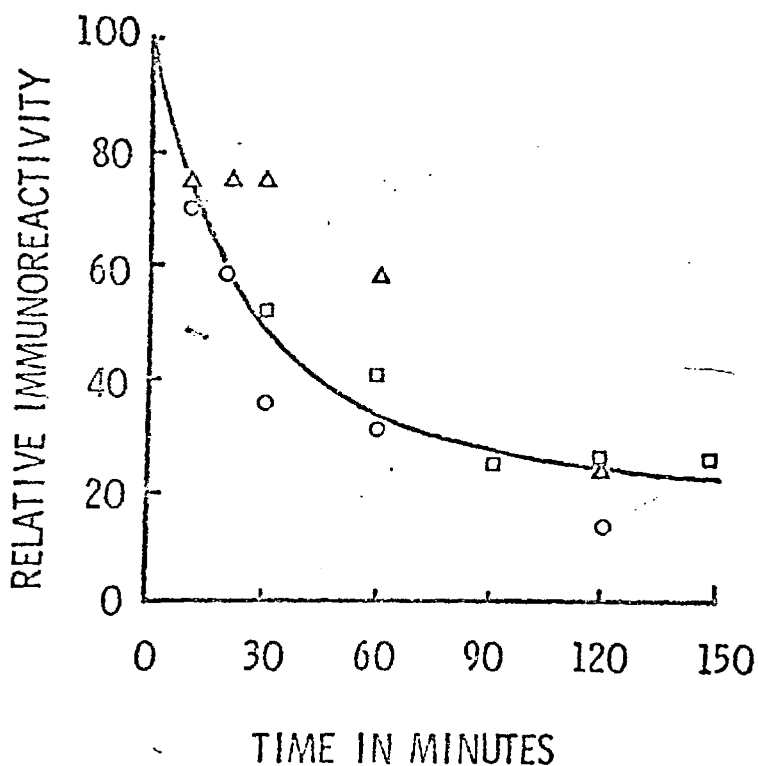

Figure 6. The change in immunoreactivity, due to tryptic digestion for 150 minutes, of calcitonin M. During the first 20-30 minutes of the digestion, chromatographic analysis indicated the presence of two peptides formed by cleavage at lysine[18]. A sharp fall in immunoreactivity was found in samples taken during this time. The subsequent slower decrease in immunoreactivity is related to the breaking of the bond between residues 12 and 13 by the chymotrypsin contaminant, as demonstrated again by thin layer chromatography.

involved in binding of the calcitonin molecule to Antiserum I, is supported by the following work using synthetic fragments H-11-28-OH, H-11-16-OH and H-17-28-OH. The peptide H-11-28-OH was reactive in the assay but did not fully inhibit binding of tracer hormone to antibody (Figure 7). In contrast, shorter peptides, H-11-16-OH and H-17-28-OH were unreactive at the concentrations used, suggesting that the antigenic site was located across residues 16 and 17. Tryptic fragmentation of H-11-28-OH to give H-11-18-OH and H-19-28-OH did not reduce the reactivity. The antigenic site has therefore localised within the sequence H-11-18-OH. Since cyanoethylation of the lysine[18] did not affect binding, the antigenic site may not require this residue, and the immunodominant group may be asparagine[17].

Peptide H-11-32-NH_2 inhibited all binding of labelled hormone to the antibody and therefore contained all the antigenic sites. As peptide H-11-28-OH did not give total inhibition, the residues H-29-32-NH_2 must be involved in a further site adjacent to residues 28 and 29, since the tetra peptide H-29-32-NH_2 is not active (Figure 7).

Since the peptide H-11-32-NH_2 produced displacement similar to that of the whole hormone, the antigenic sites of Antiserum I appear to be independent of the N-terminal region, although some distortion does occur if the methionine[8] is oxidised.

(2) Studies with Antiserum II.

Studies of the binding of fragments with Antiserum II indicated that the antibody population had changed during the course of immunisation. The evidence is two-fold. First,

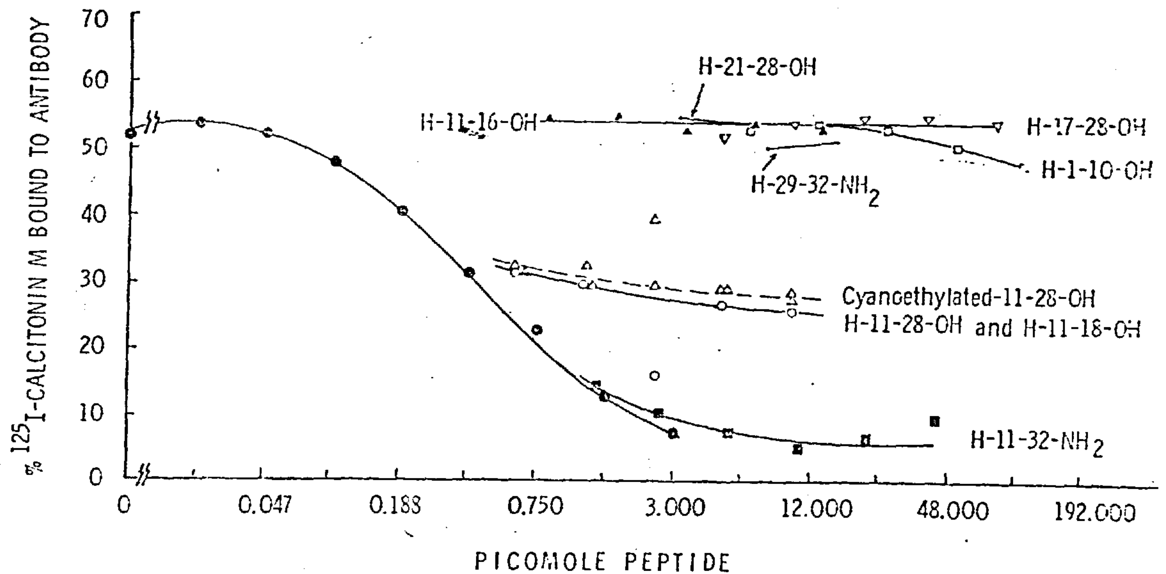

Figure 7. Demonstration of the importance of the intact H-16-17-OH bond in binding of human calcitonin to Antiserum I. Some inhibition of binding of labelled hormone to antibody was shown by the peptide H-11-28-OH with the bond intact, but not by the two fragments, H-11-16-OH and H-17-28-OH, incurred when the bond is broken. Although the peptide H-29-32-NH_2 does not itself displace tracer hormone from antibody, it is involved in the antigenic site as seen from the fact that peptide H-11-32-NH_2 inhibits all binding of labelled hormone to antibody, while peptide H-11-28 does not.

peptide H-11-28-OH bound less to similar dilutions of Antiserum II than Antiserum I (Figure 8). The antibodies in the second antiserum directed to sites on this peptide must therefore have been reduced. Second, cleavage at lysine^{18} with trypsin reduced the total antibody binding of the fragments less with Antiserum II than with I.

Fragments H-9-32-NH_2, H-13-32-NH_2 and H-19-32-NH_2 fully inhibited binding of labelled hormone to Antiserum II but H-11-28-OH showed little inhibition. The major antigenic sites must therefore be contained in the segment H-19-32-NH_2 (Figure 9). Because little displacement was produced by H-11-28-OH, the sequence residues 29-32 must be involved in the antigenic site, although the isolated tetrapeptide is not itself active.

Although peptides H-9-32-NH_2, H-13-32-NH_2 and H-19-32-NH_2 completely inhibited binding of labelled calcitonin to antibody, much greater amounts of these peptides are needed than of the intact hormone. The common missing residues 1-8 must therefore have some effect on the binding energy. A possible explanation is that methionine^{8} interacts with some other part of the molecule, because oxidation of this residue increased the binding of the altered hormone to antibody.

Discussion.

In summary, Antisera I and II contain antibodies to two different sites on the calcitonin molecule. Antiserum I reacts to two principal sites, one within the sequence 11-18 and the other at the C-terminus end on either side of residue 28. Binding of the hormone to this antiserum was increased by

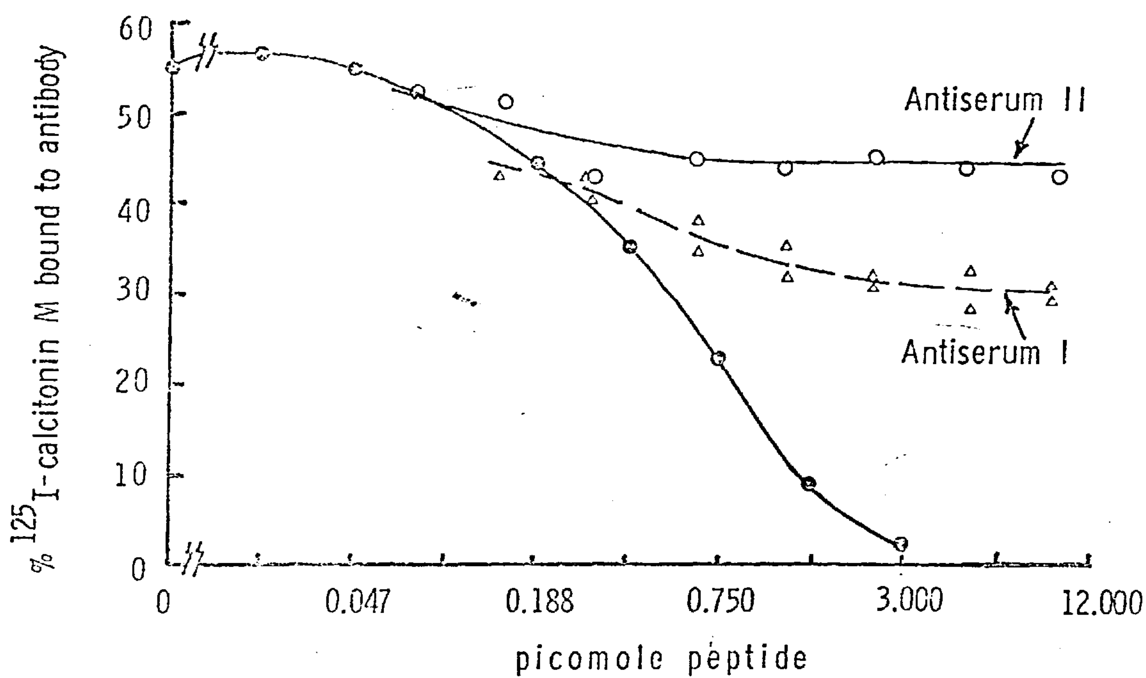

Figure 8. Comparison of binding of peptide H-11-28-OH with equal dilutions of Antisera I and II. The peptide bound less with Antiserum II, indicating that the site recognised in the sequence 11-28 by Antiserum I is very much less important to Antiserum II.

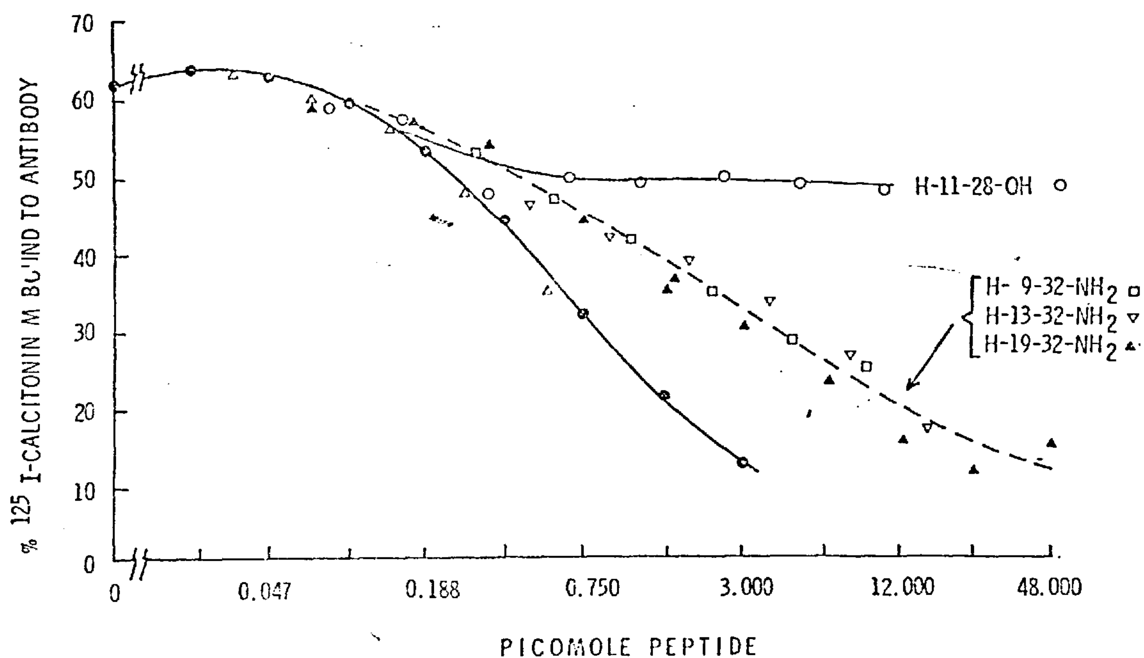

Figure 9. Complete inhibition of binding of labelled calcitonin monomer to Antiserum II is achieved by fragments $H-9-32-NH_2$, $H-13-32-NH_2$ and $H-19-32-NH_2$, indicating that almost all the antigenic sites are present on the common sequence $H-19-32-NH_2$. In contrast, peptide H-11-28-OH showed little inhibition, demonstrating that the sequence $H-29-32-NH_2$ must be involved in the antigenic site.

oxidation of the methionine[8]. In contrast, Antiserum II reacted mainly with one antigenic site around residue 28, which was dependent on some action of the N-terminal region.

Antisera raised against a single antigen may contain several antibodies which bind to different sites on the antigen. There are two types of sites against which antibodies may develop: conformational and sequential (Goodman, 1969). In conformational sites, the protein chain is folded to bring widely separated residues together, and antibodies are developed to the total conformation. These antibodies will not bind to the antigen if it has lost its three dimensional structure, even if the molecule is otherwise intact (Brown, 1963).

A sequential site is one composed of a linear sequence of amino acids. If one of the residues in this sequence is removed, binding may not be prevented, although it may be reduced. This type of antigenic site may be isolated from its parent protein and still bind to antibody, in contrast to a conformational site which will not bind if the tertiary structure of the antigenic determinant is altered (Crumpton, 1968).

TENTATIVE MODEL

From the results presented, the antigenic sites of calcitonin would seem to be principally sequential. However, some conformational limitations appear to be imposed: one at the N-terminal end, possibly due to the methionine[8], and a second in the middle of the molecule at lysine[18], since cleavage at this point results in reduced binding energy, although the residue is possibly not included in the antigenic site. The presence of

conformational determinants indicate that calcitonin is not a random coil but has a preferred shape determined by intra-molecular interactions.

Since there is some form of interaction between residue 8 and the carboxyl terminus of the molecule, the amino and carboxyl termini may be held in close proximity. This has also been postulated from evidence from studies using antisera to porcine calcitonin (Lequin, Hackeng and Schopman, 1969; Tashjian, Bell and Levine, 1970) and human calcitonin (Dietrich and Rittel, 1970). The presence of conformational strictures at the methionine^{8} and lysine^{18} residues, together with evidence that linear antigenic sites tend to be on easily accessible regions of the molecule (Arnon and Sela, 1969; Crumpton, Law and Strong, 1970), suggest that there is a 'bend' in the peptide chain. On the basis of these conclusions, a tentative model for the human calcitonin is proposed (Figure 10). In this model the disulphide bond and residues 1-7 have no interaction with the rest of the molecule. Evidence for a flexible bond at the disulphide bridge (Brewer, 1970) supports this view.

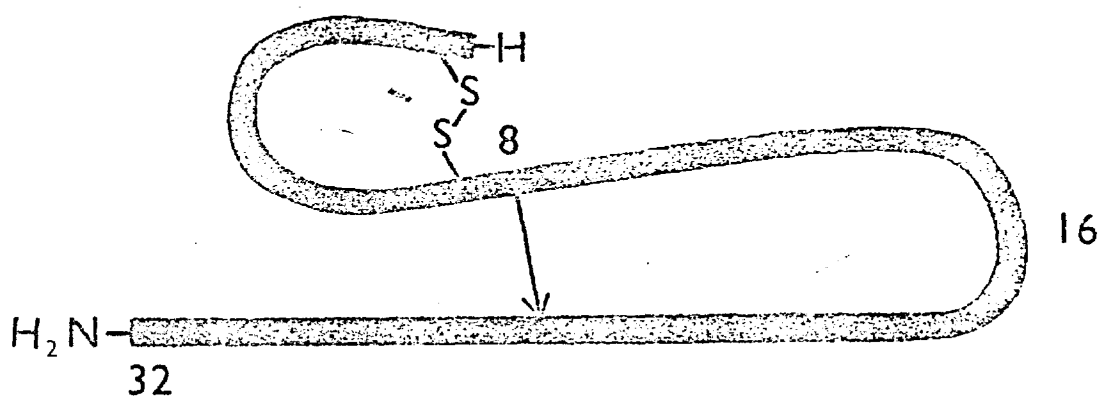

Figure 10. A tentative model for human calcitonin. There is no interaction of the 1-7 disulphide ring with the rest of the molecule. A fold in the molecule around residues H-13-18-OH may bring the C-terminal end into closer proximity with the rest of the peptide chain, perhaps allowing some interaction between methionine[8] and residues near the C-terminus.

Chapter V

CLINICAL INVESTIGATIONS

Summary:

Plasma from 17 out of 22 patients with histological evidence of medullary carcinoma of the thyroid had detectable levels of calcitonin. No diurnal variation was observed. Levels of the hormone were raised both by calcium infusion and by injection of glucagon. At operation, levels rose during manipulation of the thyroid and fell to undetectable limits when the tumour was removed. An extract of a tumour from a patient with carcinoid syndrome appeared immunologically active suggesting that a substance similar to or identical to calcitonin was present. It could not be confirmed that calcitonin is secreted by the adrenal glands in health or disease.

The assay described in the preceding chapter was unable to detect circulating levels of hormone in normal patients. For this reason it was applied to investigate those conditions in which high levels might be found. The results are separately described under the following headings: Medullary Carcinoma, Carcinoid Syndrome, Adrenal Gland and Other Conditions.

In these studies, blood specimens from patients were collected in chilled heparinised tubes, centrifuged at 4° and the plasma kept frozen until studied. Plasmas were diluted 1:5 with 0.1 M phosphate buffer pH 7.5, containing 0.2% human serum albumin and 0.02% Neomycin sulphate. Two hundred microlitres of this diluted plasma were added to each of two tubes containing 200 µl of buffer and three doubling dilutions were made from each. Labelled hormone was added to all tubes and antibody to one set of the doubling dilutions. The remaining four tubes served as controls to study whether or not plasma interfered with separation by charcoal.

MEDULLARY CARCINOMA

Medullary carcinoma of the thyroid is a calcitonin-

secreting tumour. Calcium-lowering activity in tumour tissue from patients with this disease has been shown by a number of investigators (Meyer and Abdel Bari, 1968; Melvin and Tashjian, 1968; Tashjian and Melvin, 1968; Milhaud, Tubiana, Parmentier and Coutris, 1968; Cunliffe, Black, Hall, Johnston, Hudgson, Shuster, Gudmundsson, Joplin, Williams, Woodhouse, Galante and MacIntyre, 1968; Tubiana, Milhaud, Coutris, Lacour, Parmentier and Bok, 1968; Dube, Bell and Aliapoulios, 1969). The cells containing the hormone have been localised using immunofluorescent techniques (Kalina, Foster, Clark and Pearse, 1970). The hormone has been isolated in its pure form (Neher, Riniker, Maier, Byfield, Gudmundsson and MacIntyre, 1968) and its sequence has been determined (Riniker, Neher, Maier, Kahnt, Byfield, Gudmundsson, Galante and MacIntyre, 1968) and confirmed by its total synthesis (Sieber, Brugger, Kramer, Riniker and Rittel, 1968).

Levels in patients.

Major teaching centres in the British Isles were asked to send frozen plasma samples from patients suspected of having medullary carcinoma. Including patients admitted to Hammersmith Hospital, plasma samples were obtained from thirty-five subjects. Of these, twenty-two subsequently had a diagnosis of medullary carcinoma confirmed histologically. Of the remaining, twelve had non-toxic thyroid goitre and one papillary carcinoma of the thyroid (Table 1).

Of the twenty-two patients with medullary carcinoma, seventeen had detectable levels of calcitonin and five did not (Figure 1). The levels measured varied from 8 to 2000 ng per ml. The values for most samples ranged between 15 and 200 ng/ml.

Table 1. List of diseases in which plasma has been assayed for calcitonin

CONFIRMED DIAGNOSIS	CALCITONIN ASSAY	
	ACTIVITY DETECTED	NO ACTIVITY DETECTED
Medullary carcinoma of the thyroid	17	5
Papillary carcinoma of the thyroid	-	1
Thyroid goitre	-	12
Carcinoid	4	12
Cushing's syndrome	-	5
Carcinoma of the breast (adrenal vein blood)	-	1
Phaeochromacytoma	-	4
Acromegaly (with pituitary implant)	-	1
Pseudohypoparathyroidism	-	1

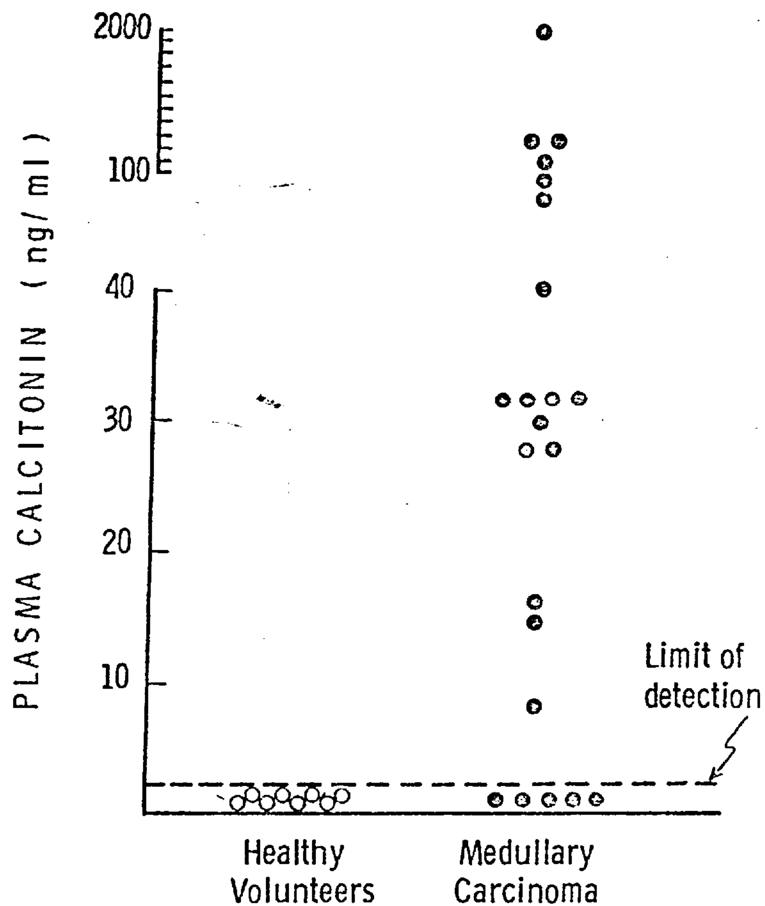

Figure 1. Levels of calcitonin in plasma samples from patients with medullary carcinoma of the thyroid. Compared to normal plasmas with undetectable levels, 17 out of 22 patients with the tumour had elevated levels ranging from 8-2000 ng/ml.

Of the five patients who had the disease, but in whom levels could not be detected, two had previously undergone total thyroidectomy and were free of symptoms at the time their plasma was assayed. The remaining three patients had levels which clearly could not be detected with our assay.

Plasma from normal adult subjects was treated in a similar way as a control for each assay. This addition of plasma, which was 20 μl in the tube containing the greatest concentration, had no effect on antibody binding or on charcoal separation.

Immunological similarity.

It might be argued that a substance other than calcitonin circulates in the plasma of patients with medullary carcinoma and interferes with the radioimmunoassay to produce spurious results. This, however, is unlikely for the following reason. Plasma from the patient with the highest levels of calcitonin produced a displacement curve identical to that found when pure hormone was used (Figure 2). It can therefore be concluded that the substance present in plasma must be either immunologically similar or identical to that of the native hormone.

Diurnal variation.

Two patients with medullary carcinoma of the thyroid were studied. These patients received normal hospital meals three times a day and blood samples for assay were drawn every six hours during a twenty-four hour period. No sample was taken immediately following a meal. The findings in one of these is shown in Figure 3. In neither this nor in the other patient

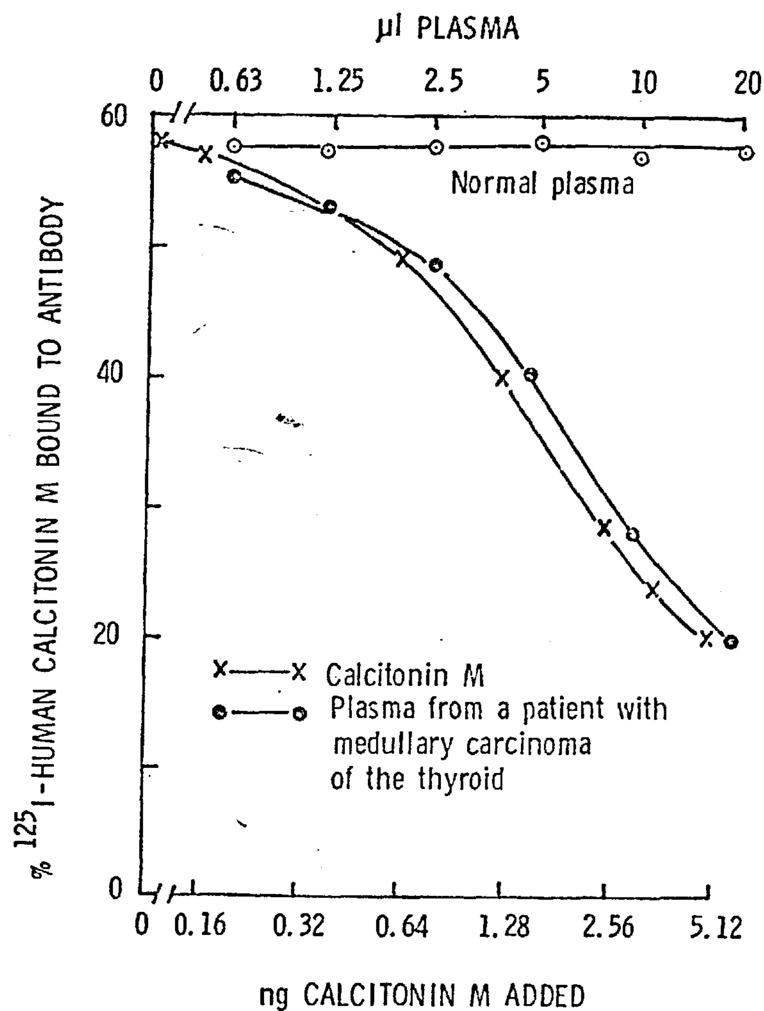

Figure 2. Comparison of displacement curves produced with plasma from a patient with medullary carcinoma to that produced with human standard calcitonin. Similar slopes suggest that a substance immunologically similar or identical to calcitonin circulates in blood of patients with this condition.

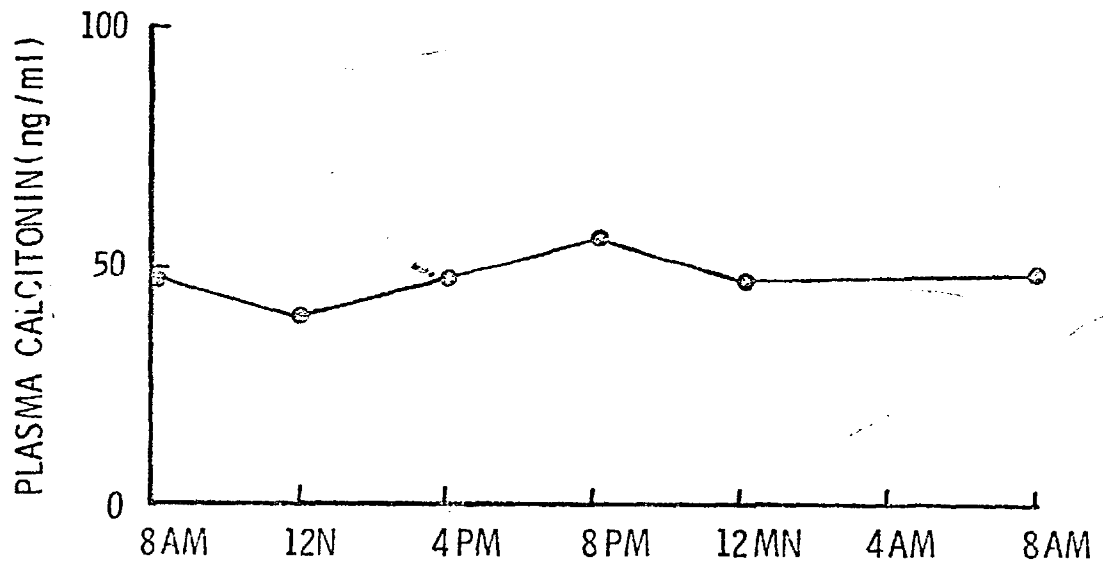

Figure 3. Calcitonin levels in plasma samples obtained from a patient with medullary carcinoma of the thyroid at 4-hourly intervals over 24 hours. No diurnal variation is noticeable.

was there any marked change in calcitonin levels. These findings are in agreement with those of Tashjian and Melvin (1968) who similarly were unable to demonstrate any diurnal variation.

Effect of calcium infusion.

In normal healthy subjects, infusions of calcium stimulate the secretion or release of the calcitonin (Gudmundsson, Galante, Horton, Matthews, Woodhouse and MacIntyre, 1970). In order to examine whether patients with medullary carcinoma of the thyroid had autonomously functioning tumours, three patients with this disease were studied. Following an overnight fast, calcium as calcium gluconate was given in a dose of 15 mg/kg of body weight during a 4 hour infusion and plasma samples collected before, during and after the infusion.

In each of the three patients investigated, a rise in calcitonin was observed (Figure 4). Two to three-fold increases in the plasma calcitonin levels were measured. The data is interpreted as evidence that the secretion of the hormone is dependent upon the circulating plasma calcium level. These results are in good agreement with those of others (Melvin, Voelkel and Tashjian, 1970).

Effect of glucagon.

Glucagon produces hypocalcaemia in dog (Paloyan, Paloyan and Harper, 1967), man (Paloyan, 1967; Birge and Avioli, 1969) and rat (Morain and Aliapoulios, 1968). Glucagon infusions in the pig induced the appearance of a hypocalcaemic factor in

plasma which is bio-assayable in the rat (Care, Bates and Gitelman, 1969). The hypocalcaemia produced may be due to the stimulation of calcitonin secretion, or to glucagon itself, as the latter also inhibits bone resorption.

If medullary carcinoma tumours are not completely autonomous, as suggested by the preceding study, glucagon may cause the release of calcitonin from them. To test whether or not this was so, one patient was studied. Following an overnight fast, glucagon was administered in a dose of 0.01 mg/kg of body weight and plasma samples were obtained during the next hour.

There was a two-fold rise in the level of calcitonin following administration of glucagon (Figure 4). This rise, however, was only transitory, the increase occurring within the first five minutes.

The rise produced in this patient with glucagon was not as great nor as prolonged as that produced by calcium infusion. Independently, Potts, Niall and Deftos (in press) and Melvin, Voelkel and Tashjian (1970) found that compared to calcium, the response to glucagon was not only smaller but less reliable. Calcium infusion would therefore appear to be a more satisfactory provocative test to demonstrate a calcitonin-secreting tumour.

<u>Levels of hormone in patients at operation for medullary carcinoma of the thyroid</u>.

In two patients, calcitonin in plasma was assayed before, during and after operation, and from the thyroid vein during surgery.

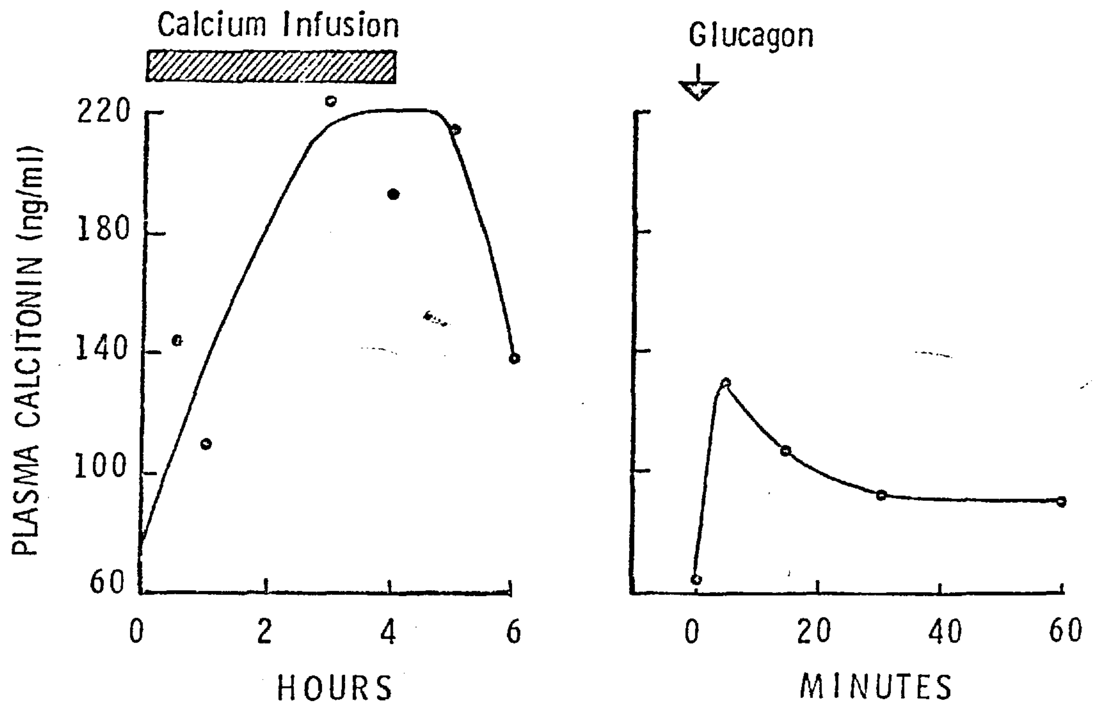

Figure 4. Responses in circulating plasma calcitonin levels after administration of calcium infusion or glucagon injection. The responses to calcium were greater and more prolonged than that to glucagon.

Plasma from thyroid venous blood obtained during operation contained ten to thirty times more hormone than did peripheral samples. In one patient, thyroid venous plasma contained 160 ng/ml whereas preoperatively the level had been only 16 ng/ml. In the other patient, 450 ng/ml were found in thyroid venous plasma, and 16 ng/ml in peripheral plasma obtained at the same time.

Mobilisation of the thyroid increased the level of circulating hormone. In one patient values rose from 16 to 100 ng/ml. In the second patient, partial mobilisation produced a rise from 15 to 18 ng/ml.

The results before, during and after surgery are summarised in Table 2. In one patient calcitonin could not be detected in plasma one hour after removal of the tumour. In the second patient, elevated levels were still present five hours after surgery, confirming the surgeon's impression that not all the tumour had been removed. Demonstration that high levels of hormone were present in the thyroid venous blood and that, with removal of the tumour, levels previously detected could not be measured are convincing proof that the thyroid tumour itself was secreting calcitonin.

<u>Correlation of bioassay with immunoassay</u>.

Three plasma samples from one patient and one sample from a second patient were assayed by both radioimmunoassay and bioassay.

There was good correlation between both methods (Figure 5). This finding supports the conclusion that immunologically detectable activity accounts for all biological activity in this condition.

Table 2. Calcitonin plasma levels observed at operation in two patients with medullary carcinoma of the thyroid.

Patient	Time of Sampling	Calcitonin ng/ml*
J.A.	Pre-operative	16
	Thyroid venous blood, during operation	160
	During operation	32
	During operation	100
	At closure	50
	20 min. after closure	50
	40 min. after closure	50
	2 hr. after closure	45
	4 hr. after closure	28
R.C.	Pre-operative	15
	After partial mobilisation of the thyroid at operation	18
	Thyroid venous blood at operation	450
	Simultaneously collected peripheral blood at operation	16
	10 min. post-operative	15
	1 hr. post-operative	Not detectable

* Normal levels are less than 500 pg/ml.

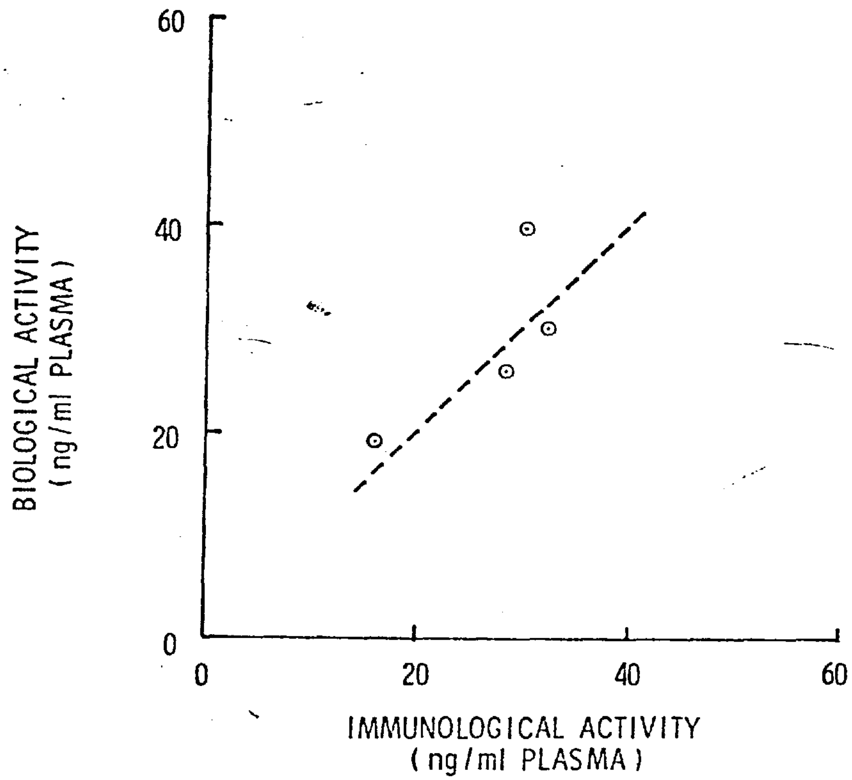

Figure 5. Correlation of plasma calcitonin values in patients with medullary carcinoma, as measured by immuno- and bio-assays.

CARCINOID SYNDROME

Tumours producing the carcinoid syndrome have an affinity for silver salts like cells in the thyroid known to contain calcitonin (Pearse, 1966). Carcinoid tumours may occur in the gastrointestinal tract, bronchus, gall bladder, ducts of the pancreas and rectum. The tumours are associated with the following clinical features: flushes, bronchial constriction, lesions of the valves of the heart, and diarrhoea. They are known to secrete a number of substances including serotinin, histamine, catecholamines, prostaglandin, vasoactive peptides and other peptides including adrenocorticotropic hormone and melanocyte stimulating hormone (Graham-Smith, 1968).

Because these tumours histologically and cytochemically closely resemble C cell tumours of the thyroid, it was decided to investigate whether or not patients with this condition might have elevated levels of calcitonin.

Plasma from sixteen patients with carcinoid syndrome were assayed. Of these, four questionably displaced labelled hormone at the lowest dilution (1:10).

It was not, however, possible to assess whether the displacement curves were similar to that of a standard calcitonin preparation since insufficient immunoactive material was present in the plasma to test. For this reason, tumour tissue from two patients was obtained. The tumour from one was collected at operation; the other, a liver containing metastases, was obtained at post-mortem. Both were defatted with ether, dried, extracted with butanol:acetic acid:water (150/15/42 by volume),

and the extract dissolved in 0.1 M formic acid (Neher, Riniker, Zuber, Rittel and Kahnt, 1968). The extract prepared from tissue obtained from the patient at operation produced no displacement of ^{125}I-labelled calcitonin. However, the second extract from tissue obtained at post-mortem did. The apparent potency of the preparation, as assayed against human calcitonin standard, was 6.5 ng/mg of extract (Figure 6).

Since this observation, Milhaud has observed calcium-lowering activity in plasma of patients with the carcinoid syndrome as assayed in the rat (Milhaud, Calmettes, Raymond, Bignon and Moukhtar, 1970). In some of these in whom operative tissue was available, the cytochemical findings suggest that the predominant cell type resembled those found in C cell adenomata.

ADRENAL GLAND

Following adrenalectomy in dogs, cats and rabbits, hypercalcaemia frequently occurs (Jowsey and Simons, 1968). Likewise in man, elevated serum calcium levels occur following partial removal of the adrenal for various diseases if no replacement therapy is given (Leeksma, De Graaf and DeCock, 1957). These findings led Kaplan and his colleagues to investigate whether or not calcitonin might be produced by this gland (Kaplan, Peskin and Arnaud, 1969). They demonstrated that a hypocalcaemic agent was present in the adrenal glands of the pig. They were able to show that this substance circulated in the blood, produced hypocalcaemia when injected into rats and appeared to be indistinguishable from porcine calcitonin in a radioimmunoassay system. To determine whether or not this might be true in man, the following studies were undertaken.

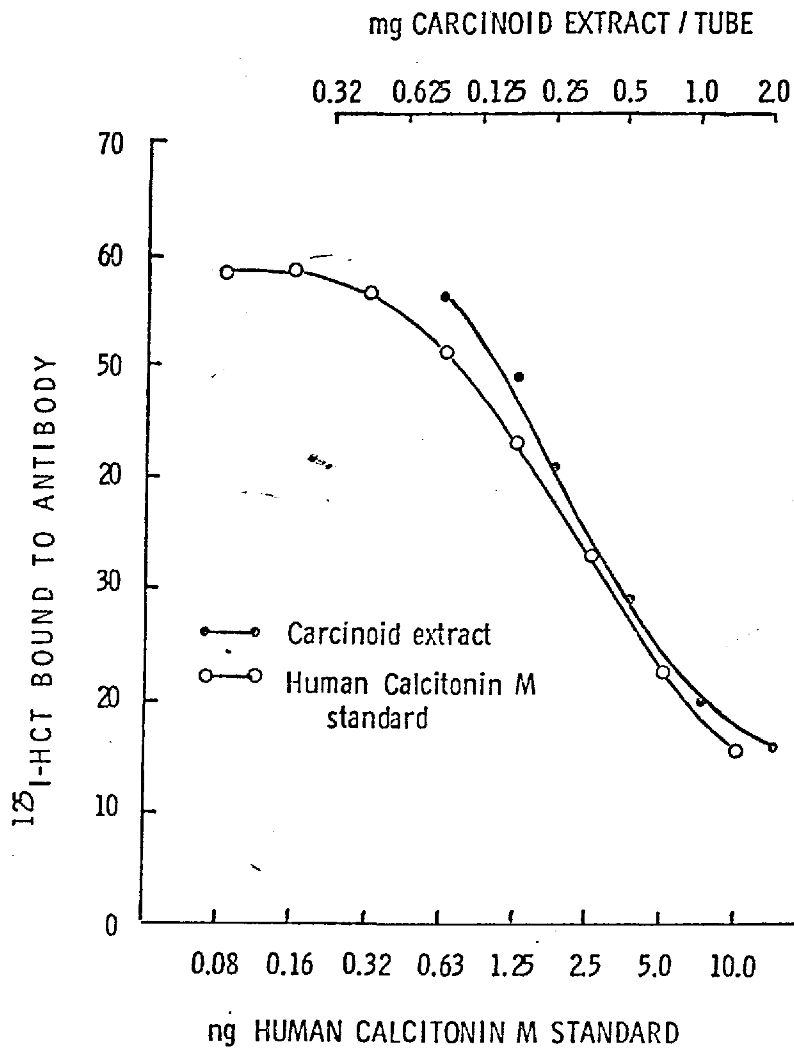

Figure 6. Comparison of inhibition curves obtained with human calcitonin M and an extract of carcinoid tumour metastases. As there is no significant difference in the slope of the two curves the carcinoid extract must therefore contain a substance immunologically similar or identical to human calcitonin M.

Adrenal glands were collected at operation from a patient with phaeochromocytoma, and at post-mortem from one patient with an adrenal tumour and from one normal autopsy. This last gland was divided into cortex and medulla. Subsequently, extracts were prepared from these as described in the preceding section.

The results of radioimmunoassay of these extracts are summarised in Figure 7. In the extracts from the adrenal tumour and from the patient with phaeochromocytoma, there was sufficient displacement of labelled hormone to suggest that a calcitonin-like substance might be present. However, it could not be discounted that the findings were due to non-specific interference since large amounts of protein were present in the extract.

In addition, plasma samples from five patients with Cushings disease were assayed. Two of these were studied before and after a calcium infusion. Likewise, blood samples were obtained from the adrenal and renal veins of two patients undergoing adrenalectomy and oophorectomy for carcinoma of the breast. Finally, plasmas from four patients with phaeochromocytoma were assayed (one of these patients had a medullary carcinoma with undetectable levels of calcitonin). In one patient undergoing operation, samples were drawn both from the peripheral circulation and from a tumour vein. In none of the plasmas assayed was the level of calcitonin within the detectable limits of the assay.

In only one patient was there a suggestion of a calcitonin-like substance being elaborated by the adrenal. This patient had previously had a unilateral adrenalectomy for suspected, but unproven, Cushings disease. At the time when studied, he was undergoing surgery for the removal of the contralateral gland.

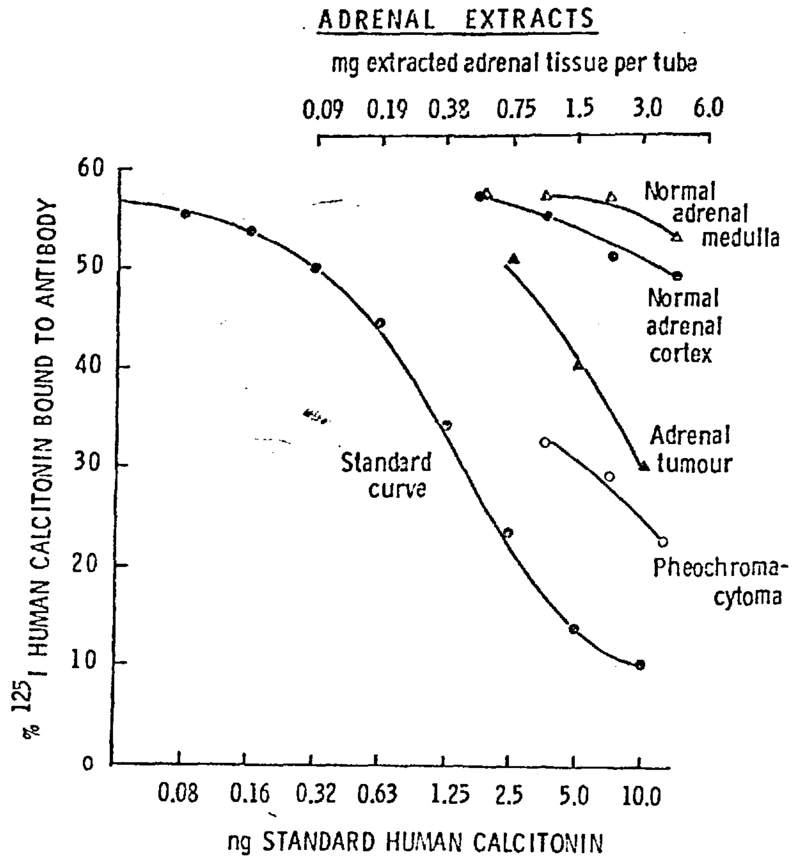

Figure 7. Comparison of displacement curves produced by human calcitonin M and various extracts of adrenal tissue. The findings indicate that a substance in the adrenal gland, immunologically similar to calcitonin M, may be present. Since the protein concentrations of the extracts were high, it cannot be ruled out that the apparent displacement observed is due to non-specific substances.

Blood was obtained from an adrenal vein and peripheral circulation at the same time. The plasma levels were 86 and 12 ng/ml respectively. Three days following adrenalectomy, the plasma level was 24 ng/ml. Since the diagnosis of the patient remains in doubt and since there was no evidence of a medullary carcinoma of the thyroid, the cause of the apparent displacement of labelled hormone is uncertain.

OTHER CONDITIONS

Plasma from one patient with acromegaly who had had pituitary ablation and from another with pseudohypoparathyroidism had no detectable calcitonin. The latter was assayed since Deftos and co-workers (Deftos, Bury, Mayer, Habener, Singer, Powell and Potts, in press) have found higher than normal values in this condition. In addition, in two healthy normal volunteers, plasma sample obtained during a calcium infusion (15 mg calcium as calcium gluconate per kilogram given over a 4 hour period) did not have detectable levels. Thyroid venous blood obtained from two patients undergoing surgery for non-toxic thyroid goitre had undetectable levels.

GENERAL DISCUSSION

Medullary carcinoma, carcinoid and tumours of the adrenal medulla have one feature in common. The cells which give rise to these neoplasms are cytologically and histochemically similar and may have a common origin.

It is now well accepted that the calcitonin-producing cells of the thyroid derive from the last branchial pouch (Carvalheira

and Pearse, 1968; Pearse, 1968). Recent evidence, however, suggests that these may originate initially from the neural crest. The evidence is two-fold. Cells from the neural crest take up 5-hydroxytryptamine, indicating that they may be the same or similar to cells known to secrete calcitonin (Pearse and Polak, 1971). Second, when neural crest tissue from Japanese quail is transplanted into a chicken embryo, surviving cells from the quail migrate to and become incorporated within the thyroid (Le Douarin and Le Lievre, 1970). This latter observation was made possible by the fact that the somatic cells of the quail are readily distinguishable by a heavily stained chromatin body within the nucleus. If these findings are true, it can be argued that all cells deriving from the neural crest may be capable of secreting calcitonin under appropriate conditions. Pearse has proposed such a hypothesis (Pearse and Polak, 1971). He has recognised that polypeptide secreting cells have many common features. These include a high content of fluorogenic amine, amino-acid decarboxylase, non-specific esterase or cholinesterase and high alpha-glycerophosphate menadione reductase as well as exhibiting masked metachromasia and avidity for amine precursors. All these characteristics are shared to a greater or less extent by a large group of endocrine polypeptide cells now referred to as the APUD series (Pearse, 1968). This unifying hypothesis makes more readily understandable how cells in the body, not normally thought to be associated with endocrine systems, may, under specialised conditions, secrete hormonal products and how tumours of these tissues can secrete more than one hormone.

Chapter VI

IMPROVEMENT IN THE ASSAY

Summary:

To improve the sensitivity of the assay for human calcitonin all the conditions of the assay were reinvestigated. Purification of the radioiodinated hormone, pH and molarity of the assay buffer, incubation time, late addition of labelled hormone, and effect of plasma proteins were studied and other antisera assessed. Late addition of labelled hormone improved sensitivity ten-fold and permitted detection of 16 picograms. However, the limits of detection were markedly decreased in the presence of plasma. Since not less than 500 picograms/ml could be measured in plasma, it is concluded that an extraction procedure will be required to measure normal levels in man.

The assay described in Chapter III, while applicable to the study of the immunogenic determinants of calcitonin and to the diagnosis of medullary carcinoma, was insufficiently sensitive to measure plasma levels in normal man. For this reason, a re-investigation of all the conditions of the assay was undertaken to improve the limits of detection. The following parameters were studied: purification of radioiodinated hormone, pH and molarity of the assay buffer, incubation time, late addition of labelled hormone, and effect of plasma proteins in both diluted and extracted plasma. In addition, other antisera were assessed.

Each of these studies will be separately described.

PURIFICATION OF IODINATED HORMONE

Purification of 125-iodinated calcitonin was originally achieved by passing the iodination reaction mixture through a column containing Amberlite CG 400 in the acetate form and eluting with 0.05 M acetate buffer pH 5.0. This procedure removed excess free iodide, but did not separate intact from

damaged labelled hormone. This labelled preparation was used in the assay procedure and bound 70-80% with excess antibody in 30 minutes.

In an attempt to increase the sensitivity of the assay, an alternative purification procedure was investigated.

Methods.

In this procedure a 12 x 1 cm column was packed with Bio Gel P10, 50—100 mesh (Bio Rad Laboratories, Ltd.) in phosphate buffer. After iodination by the method described in Chapter III, the reaction mixture was applied to the prepared column and eluted with phosphate buffer. Fifty fractions of 0.5 ml were collected and counted in a well counter. Total radioactivity per fraction was plotted and the binding with excess antibody was determined of aliquots, with equal amounts of radioactivity, from several tubes.

Results.

A definite peak of radioactivity was eluted from the column (Figure 1). Incubation of aliquots with equal radioactivity from different fractions, with excess antibody demonstrated that the binding capacity increased as the count rate increased. The amount of free iodide, which appeared as the fraction not bound to dextran-coated charcoal in control tubes, was high in the first fractions collected from the column, and thereafter insignificant.

The 70% binding of excess antibody with the best fractions

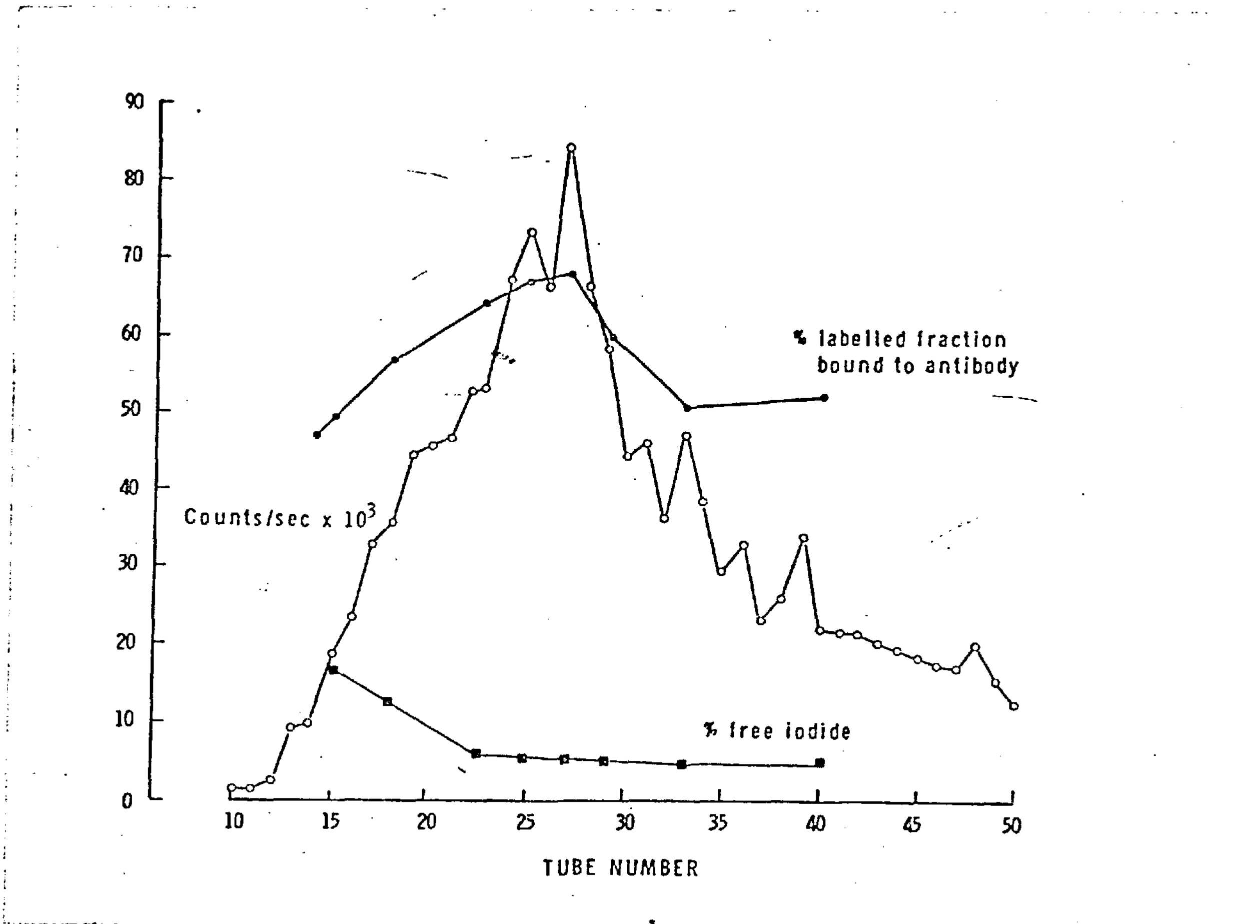

Figure 1. Elution profile of radioactive material from a Bio Gel P10 column. The percentage of labelled eluate binding to antibody varied across the profile, and was at a maximum between tubes 23 and 27.

collected from the column was not better than binding achieved with the Amberlite purified preparation. Subsequent experiments using the Bio Gel purified ^{125}I-calcitonin indicated that this preparation was not stable over four weeks of storage.

No useful increase in the purity of labelled calcitonin was obtained by using this different purification procedure, although it is recognised that the labelled hormone does contain impurities. This is seen from results presented later in this chapter which indicate that the antibody does not distinguish between 20 pg and 2 pg of labelled hormone, although it will distinguish between 20 pg and 10 pg of standard hormone.

A failure to purify a labelled preparation of another small polypeptide hormone has been observed (Chard, Kitau and Landon, 1970). The fragments of small peptide hormones which are formed during an iodination reaction may be similar in size to the native peptide. Separation of these fragments could be achieved by elaborate purification steps but the final yield of the pure hormone would be so low that no advantage would be gained.

pH OF BUFFER

The pH of 7.5 originally used was chosen for the buffer system following preliminary studies which evaluated binding of labelled hormone to antibody at pHs 4.0, 4.6, 5.0, 6.0, 7.0 and 7.5. The following studies were carried out to define more precisely the optimal pH.

Methods.

Antibody dilution curves were prepared in 0.1 M phosphate

buffers over a pH range of 7.0-8.0 at increments of 0.1. Both antiserum and labelled hormone were diluted in buffer at each pH and separation carried out, after an 18 hour incubation period, with 2% dextran-coated charcoal at the same pH.

Results.

Antibody bound optimally to calcitonin at a pH between 7.2 and 7.6 (Figure 2). The pH of 7.4, in the middle of the range, was chosen for all subsequent studies.

BUFFER SALT

To establish the optimum buffer for the assay, the following salts were studied: phosphate, borate, barbitone and Tris (Tris (hydroxy methyl) methylamine).

Methods.

Each buffer was tested at two molarities, 0.05 M and 0.1 M, excepting barbitone buffer which was insoluble at the higher concentration. Reactants for dilution curves were incubated for 18 hours in each buffer system and the binding of antibody to labelled hormone in each calculated.

Results.

The findings indicated that binding of labelled hormone to antibody was greatest in either phosphate or Tris buffer (Figure 3). For some inexplicable reason, binding of tracer hormone to antibody at the lowest antibody dilutions was decreased in Tris

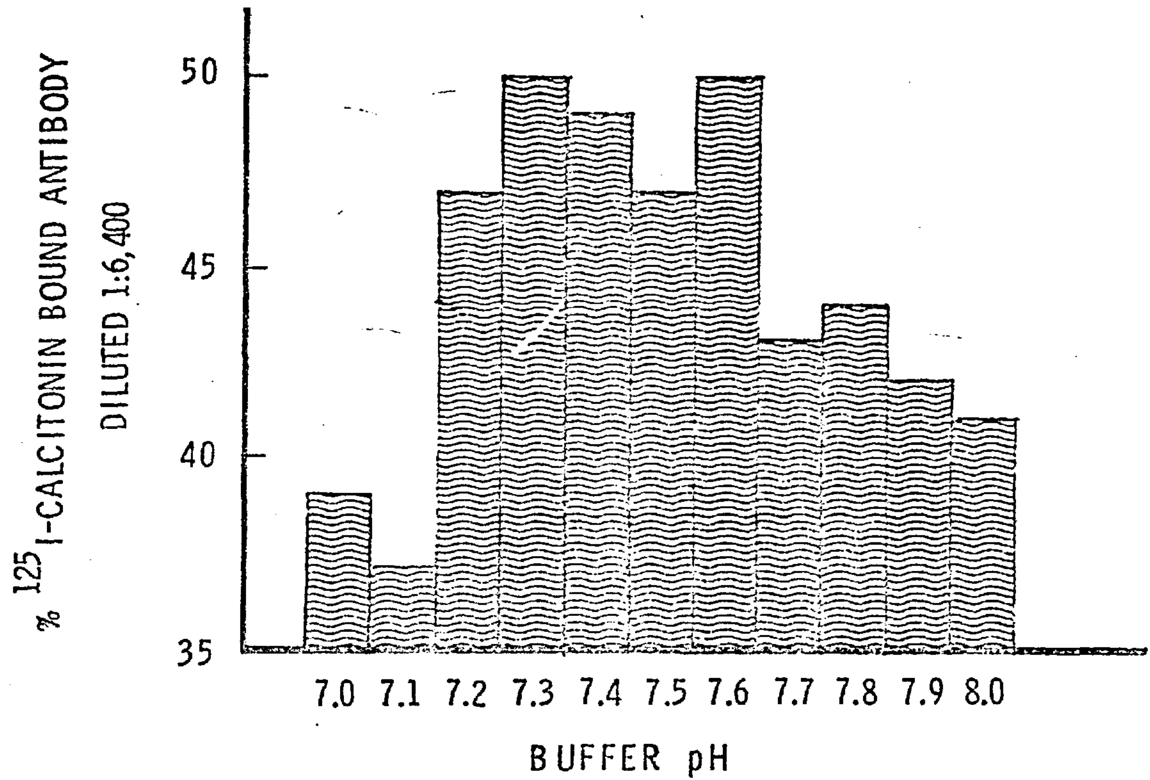

Figure 2. Variation in binding of labelled human calcitonin to antibody over a pH range of 7.0-8.0. Binding was maximal between pH 7.2 and pH 7.6.

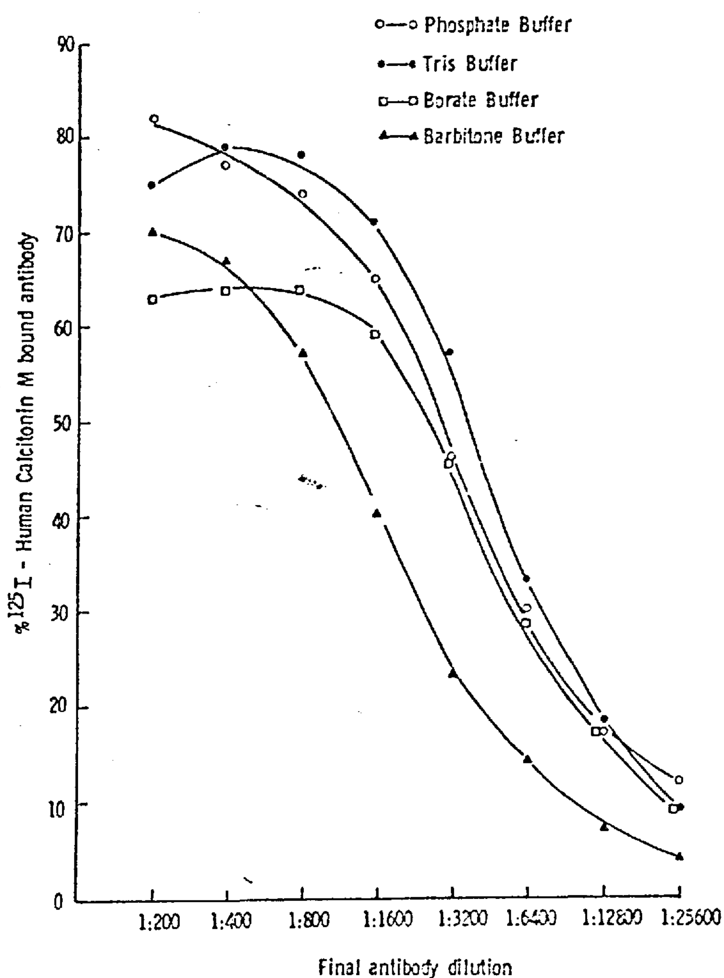

Figure 3. Variation of binding to antibody of labelled calcitonin in different buffer systems. Maximal binding was achieved in phosphate and Tris buffers. A decrease in binding of labelled hormone was observed at the highest concentrations of antibody in Tris buffer.

buffer, therefore phosphate buffer was selected for use in subsequent investigations.

No difference in antibody binding of ^{125}I-calcitonin with 0.05 or 0.1 M buffer was apparent (Figure 4). Phosphate buffer at a concentration of 0.05 M was therefore chosen because it is readily kept in solution at 4°C, the temperature at which incubations were carried out.

TIME OF INCUBATION

Initially an incubation time of 18 hours had been selected for the assay. In order to examine if more effective binding of hormone to antibody could be obtained with higher dilutions of antibody over a longer incubation period, the following studies were carried out.

Methods.

Fifty microlitres of labelled hormone were added to tubes containing antibody in 0.05 M phosphate buffer to give a range of dilutions from 1:800 to 1:20,480. These were mixed and incubated at 4°C. Duplicate sets of incubates were removed every day for ten days, antibody-bound and free labelled hormone in each tube separated and the percentage of labelled hormone bound to antibody was calculated.

Results.

The increase in binding of labelled hormone to antibody was directly related to the time of incubation. The greatest

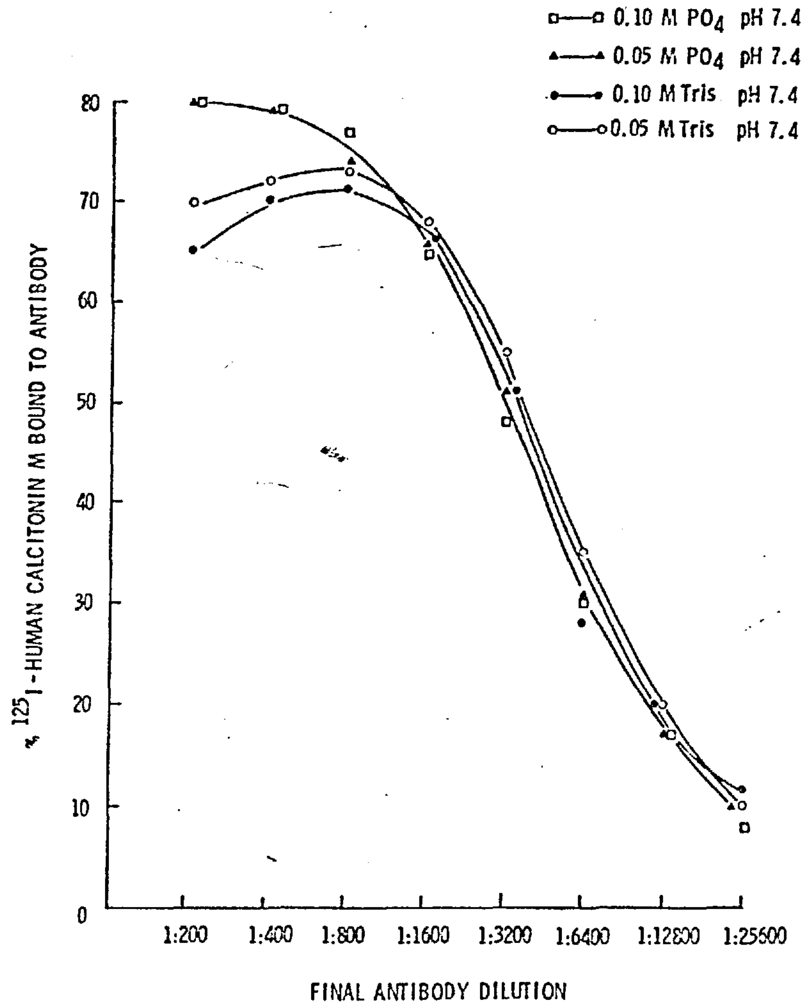

Figure 4. Effect of buffer molarity on antibody binding of labelled hormone. No significant difference due to buffer molarity was found. A decrease in binding at high concentrations of antibody in Tris buffer was again observed.

increase occurred in the first four days, although there was still evidence of increasing binding up to 10 days (Figure 5).

Binding of labelled hormone was between 40-50% at 4-7 days with antibody dilutions of 1:10,000-1:15,000 (Figure 6). This percentage of binding gives near optimal sensitivity and precision in the assay (Berson and Yalow, 1964; Ekins, 1969). Incubation for longer than seven days would be inconvenient and little advantage would be gained. These two antibody dilutions were subsequently used in the preparation of standard curves to assess which would be optimal. The results of these studies are presented in the secretion on late addition of labelled hormone.

AMOUNT OF LABELLED HORMONE

As the amount of antibody being used in the system is decreased, the amount of labelled hormone may have to be decreased. In the preceding studies, labelled hormone was calculated to be between 10 and 20 pg in the 50 µl amounts used and this gave a count rate of 100 cps per tube. The following study was carried out to investigate the effects of varying amounts of labelled hormone on antibody binding with varying times of incubation.

Methods.

Calcitonin M was labelled to a specific activity of 235 µCi/µg hormone. Using a well counter, calibrated for the number of counts per µCi of ^{125}I, labelled hormone was diluted to concentrations of 200 pg, 20 pg, 2 pg and 0.2 pg per 50 µl of assay buffer.

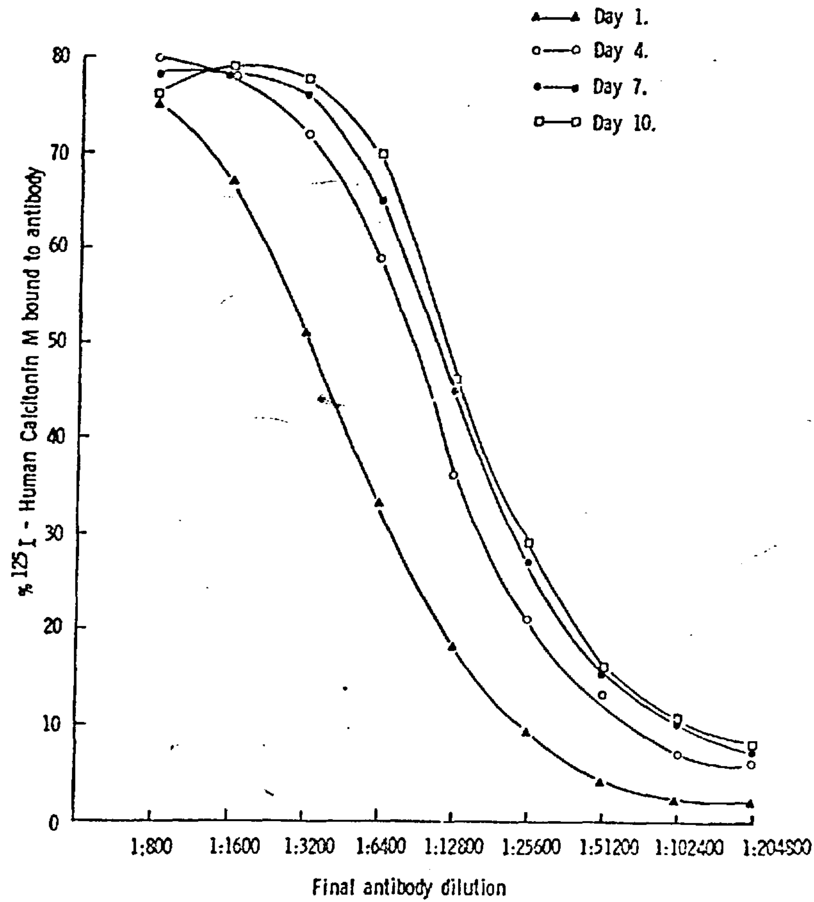

Figure 5. Dilution curves for labelled human calcitonin incubated with antibody for 1-10 days. Binding increased rapidly in the first four days but was not complete even at the 10th day.

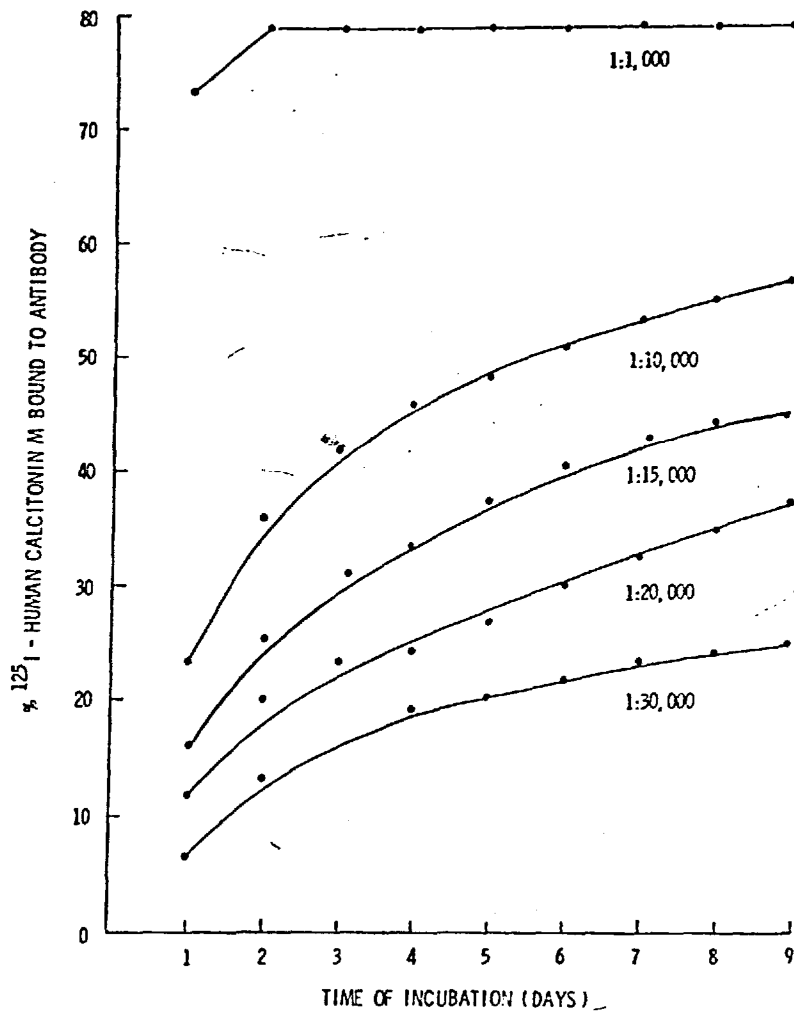

Figure 6. Binding of labelled calcitonin in varying dilutions of antiserum over 10 days. 40-50% labelled hormone was bound at dilutions of 1:10,000-1:15,000 over periods of 4-6 days.

Serial dilutions of antibody were prepared and the different amounts of labelled hormone added. At one, four or nine days, bound and free hormone in duplicate tubes at each concentration of antibody were separated by dextran-coated charcoal and the percentage of label bound to antibody was calculated.

Results.

The percentage of labelled hormone bound to antibody when 200 pg was used was significantly less after all incubation times than that when either 20 or 2 pg label was present (Figure 7) and the counting rate with 0.2 pg of tracer hormone was too low to be efficient.

The equal binding at any dilution of antibody when either 20 pg or 2 pg were added, indicated that the antibody did not distinguish between these amounts. It was concluded that no increase in sensitivity could be gained by the addition of less than 20 pg of labelled hormone to each assay tube.

LATE ADDITION OF LABELLED HORMONE

To investigate the optimal time of incubation for reactants in a standard curve, and to determine the effects of late addition of the tracer hormone to a pre-incubate of antibody and standard hormone, (Samols and Bilkus, 1964), the following studies were carried out.

From the results obtained with antibody dilution curves previously described, it was concluded that dilutions of antibody from 1:10,000 to 1:15,000 bound 40-50% of the labelled hormone

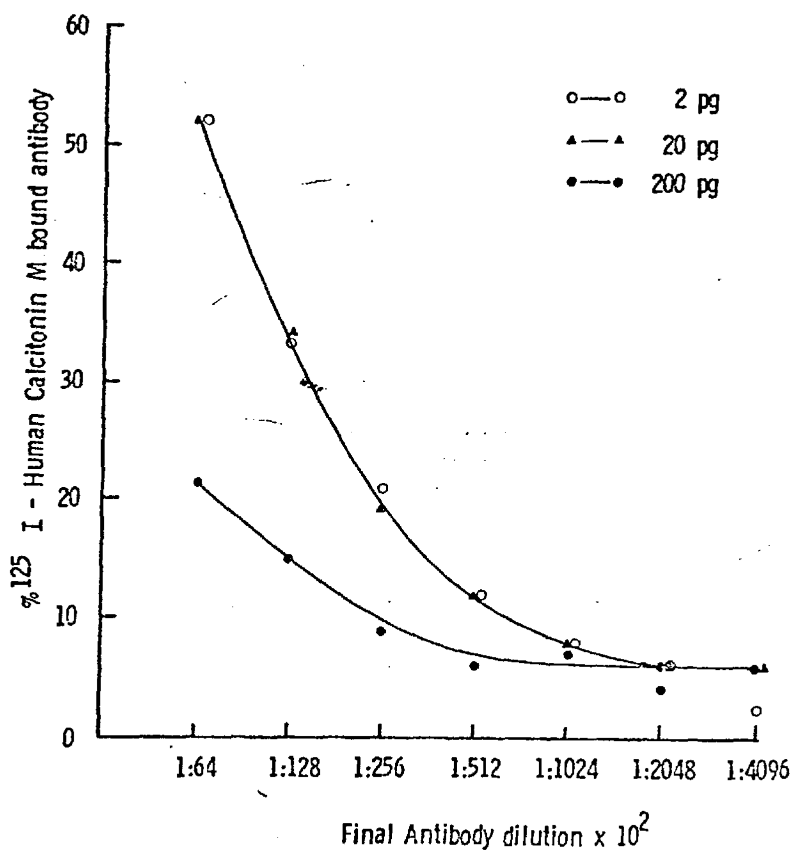

Figure 7. Binding of varying quantities of labelled hormone to antibody after a four day incubation. There was no difference in the binding of 2 or 20 pg amounts of labelled hormone, but significantly less binding of 200 pg amounts with equal dilutions of antibody.

in 4-6 days. The effect of these two antibody dilutions on standard curves was therefore tested in incubations carried out for four and six days.

Methods.

Two studies were undertaken. In the first, to tubes containing standard calcitonin in amounts varying from 2 ng to 16 pg in 200 µl, the following were added: 50 µl ^{125}I-labelled calcitonin (12 pg hormone) and 50 µl of antibody at a dilution of 1:1670 or 1:2500, giving final dilutions of antibody of 1:10,000 or 1:15,000 respectively in a volume of 300 µl.

Tubes containing a final dilution of antibody at 1:10,000 were incubated for four days before the antibody-bound and free hormone were separated and calculated. Tubes containing antibody at a dilution of 1:15,000 were incubated for six days before the reactants were separated.

In the second experiment run concurrently, the same dilutions of antibody were added to the standard solutions, but labelled hormone was omitted. These tubes were again incubated for four or six days. After this time, labelled hormone was added and the tubes re-incubated for a further number of days before separation of antibody-bound and free hormone.

Results.

The resulting standard curves are shown in Figure 8.

The sensitivity of the assay was increased ten-fold by using

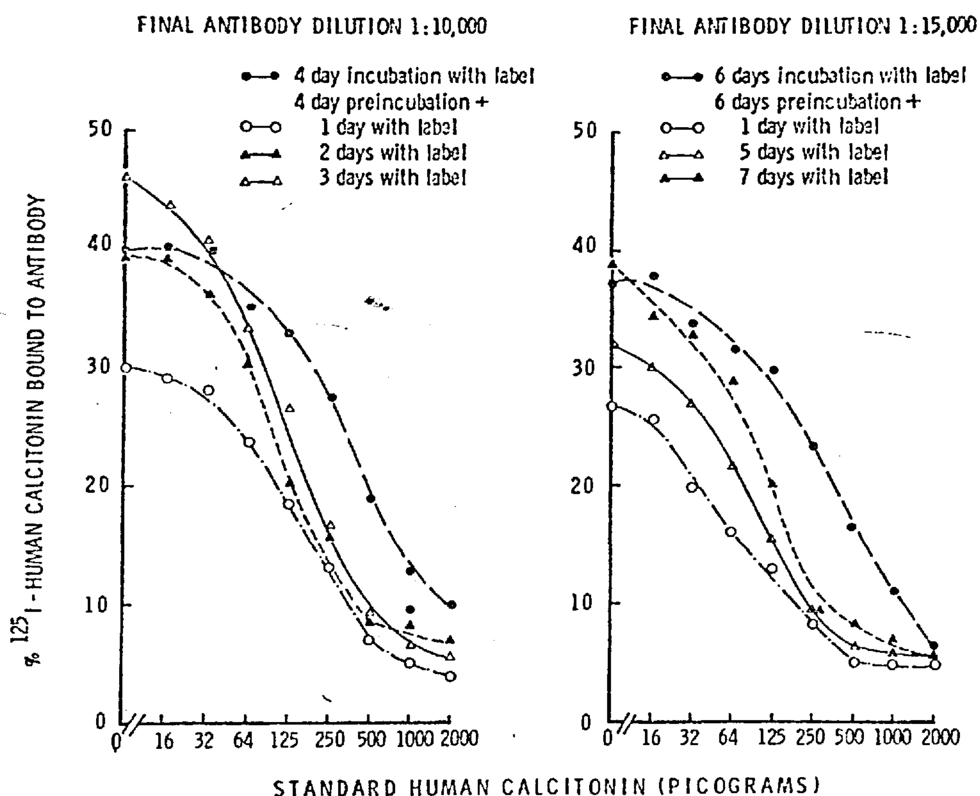

Figure 8. Comparison of standard curves prepared with antibody dilutions of 1:10,000 and 1:15,000 for 4 or 6 days respectively. Pre-incubation of standard hormone and antibody for these times, before the addition of labelled hormone for further periods of incubation, increased the detection limits of the assay. No increase in sensitivity was obtained using the greater dilution of the antibody.

greater dilutions of antibody with longer incubation times.

The detection limits of standard curves containing antibody at either 1:10,000 or 1:15,000 were not significantly different. The late addition of the labelled hormone after pre-incubation of standard and antibody, however, increased the detection limits in each study.

The binding of labelled hormone to antibody was greater at the lower dilution of antibody. Because this did not result in loss of sensitivity and because incubation times were shorter, this dilution was chosen for further work. The slightly higher binding allows greater changes in antibody binding over the range of standards, and the accuracy of the method increases as these changes become greater than the error in each measurement.

CALCITONIN-FREE PLASMA

The assay system described was carried out in phosphate buffer containing 0.2% human serum albumin. A system modified to include plasma was now investigated. Other workers have shown adverse effects of plasma in immunoassay systems, thought to be due to 'damage' by plasma constituents of labelled hormone during incubation (Hunter, 1971). As a consequence a proportion of the labelled hormone is prevented from reacting with the antibody. The resulting reduction in binding can be mistakenly interpreted for hormone in the plasma. In addition, plasma in the incubation system may also affect the separation of bound and free labelled hormone. This can be overcome in the dextran-coated charcoal separation technique by increasing the amount of charcoal per incubation tube.

Incubation damage to labelled hormone may be due to enzymes in the plasma which degrade the labelled hormone. Evidence that this is so has been shown by workers using a glucagon assay (Eisentraut, Whissen and Unger, 1968). Damage was effectively prevented by the addition of Trasylol, a peptidase inhibitor. Other agents used to prevent incubation damage in a porcine calcitonin assay include mercaptoethanol, 6-amino caproic acid and iodoacetamide (Yalow, 1969).

To assess the effect of plasma on binding, it was mandatory to work with material that was calcitonin-free. Since there is no condition in man in which there is known to be a complete absence of the hormone (Gudmundsson, Galante, Horton, Matthews, Woodhouse, MacIntyre and Nagant de Deuxchaisnes, 1970), attempts were made to remove calcitonin from normal plasma. Since dextran-coated charcoal in previous studies had been demonstrated to remove 95% of labelled hormone from incubation tubes, this method was tried for removal of endogenous calcitonin from normal plasma.

Methods.

Two plasmas were studied, one from a normal subject and one from a patient with medullary carcinoma with proven high levels of circulating calcitonin.

Dextran-coated charcoal, in a final concentration of 8% was added to 10 mls of each of these plasmas. The solutions were mixed vigorously for 5 minutes, centrifuged for 10 minutes and the plasma supernatants were assayed.

Results.

Measurement of the protein content of the normal plasma before and after the extraction procedure demonstrated that 10% of the protein had been removed. This loss appeared to be from all protein fractions, as an electrophoretic strip of the extracted plasma showed a normal profile. In addition to protein, all uric acid was removed and the concentration of other constituents slightly altered (Table 1). No detectable calcitonin was found in extracted plasma from the patient with medullary carcinoma either by bioassay or by immunoassay, using an 18 hour incubation assay.

While there is no proof that the extracted plasma was calcitonin-free, it is assumed that levels in the normal plasma would have been markedly reduced. This plasma was therefore used in subsequent studies.

EFFECT OF PLASMA

The following studies were carried out to investigate the effect of plasma on the sensitivity of the assay.

Methods.

Reagents for standard curves were set up to give a final incubation volume of 0.5 mls. In one set of duplicate standards the only protein present was 0.2% human serum albumin. In the others, 0.1 ml of plasma was added. Three plasmas were tested: normal plasma, charcoal-extracted normal plasma and charcoal-extracted medullary plasma.

Biochemical Parameter	Normal plasma (diluted)	Extracted normal plasma (diluted)	Extracted medullary carcinoma plasma (diluted)
Sodium mEq/litre	118	101	108
Potassium mEq/litre	2.0	1.8	1.6
Calcium mEq/litre	2.0	2.4	1.9
Urea mg/100 ml	20	20	20
Glucose mg/100 ml	40	30	30
Uric Acid mg/100 ml	2.0	0	0
Total protein mg/100 ml	3.9	3.6	3.2
Albumin mg/100 ml	2.5	2.2	2.0

Table 1. Effect of extraction of normal and medullary carcinoma plasma on several biochemical parameters. There was a decrease in several plasma constituents, and all uric acid was removed from the solutions.

Standard amounts of hormone in these differing solutions were incubated with antibody for four days. Labelled hormone was then added and the contents of the tubes incubated for a further three days. After separation of antibody-bound and free hormone, the percentages of binding were compared.

Results.

In the presence of buffer containing 0.2% human albumin, there was 55% binding of labelled hormone to antibody. Binding in the incubation tubes containing plasma was reduced to 42%, and the detection level of the assay was reduced 4-8 fold (Figure 9). This was interpreted as evidence of damage to labelled hormone, and was not thought to be due to calcitonin in the plasma as all three plasmas produced a fall in binding of the same magnitude.

ENZYME INHIBITORS

Damage during incubation is thought to be due principally to peptidases in the plasma which affect both labelled and standard hormones. Several peptidase inhibitors and other protective agents were therefore tested for their ability to prevent this deleterious effect.

Methods.

Two studies were carried out: one to assess the effects of several protective agents on binding of labelled hormone to antibody and another to measure the effect of the most appropriate of these on the sensitivity of the assay.

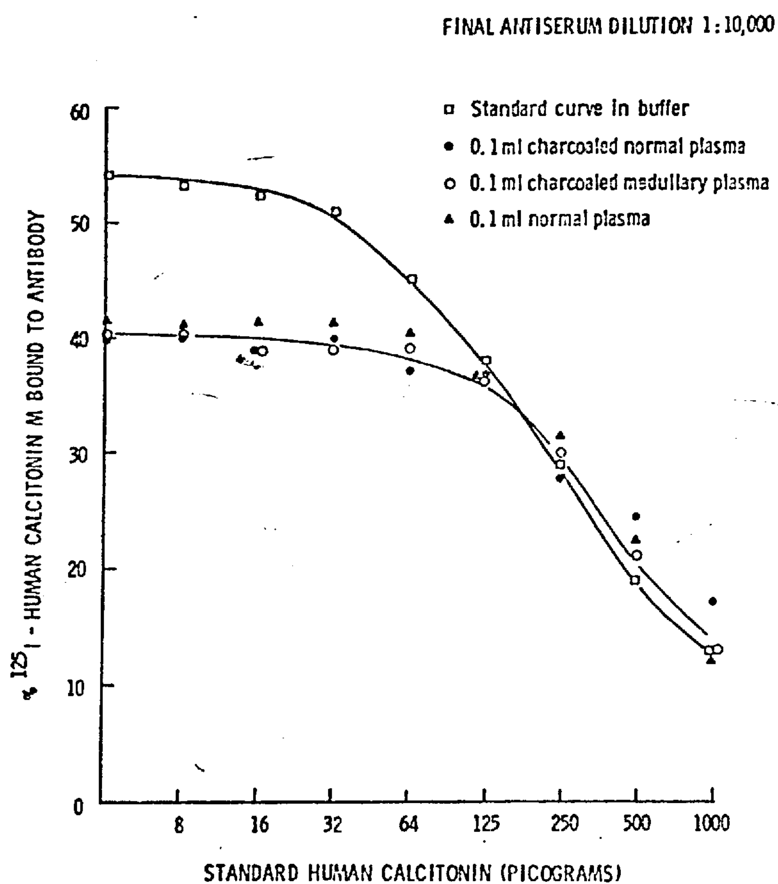

Figure 9. The effect of substituting 0.1 ml of plasma into an assay system containing 0.5 ml buffer. The detection limit of the assay is decreased. Pre-treatment of the plasma by extraction of hormone present with dextran-coated charcoal does not prevent the effect.

To study the effect of inhibitors on binding of ^{125}I-calcitonin to antibody, the following experiment was carried out. Plasma was added to two sets of tubes containing either buffer and labelled hormone, or antibody and labelled hormone, to give a final concentration of 20%. Control tubes with no plasma were also prepared. To one set of tubes were added appropriate concentrations of the following agents: Trasylol, ε-amino caproic acid, L-tyrosine, glycyl glycine, merthiolate, sodium azide, mercaptoethanol, glycine and B.A.E.E. (αN-benzoyl-L-argine ethyl ester). Incubation was carried out for a total of seven days and antibody bound and damaged hormone were separated by dextran-coated charcoal from free labelled hormone.

In a second study, the effect of Trasylol on the sensitivity of the standard curve was evaluated. Two hundred microlitre aliquots of standards in a range 8-1000 pg were pipetted into incubation tubes. To these were added diluted antibody. The volume was adjusted to 450 µl by adding 100 µl plasma and 100 µl buffer with or without 500 I.U. Trasylol. Tubes were incubated for four days, after which 50 µl of labelled calcitonin was added and incubation carried out for a further three days. After separation of bound and free hormone standard curves were compared.

Results.

All tubes containing plasma showed less binding of tracer to antibody than tubes with buffer alone.

Several of the protective agents produced more damage than the plasma alone and decreased the amount of bound labelled

hormone. None of the agents used gave any protection to the labelled calcitonin, and damage to the calcitonin molecule was not decreased, even in standard curves incubated with 500 I.U. Trasylol per tube.

ASSESSMENT OF OTHER ANTISERA

The antiserum, which was used in all the foregoing studies to improve the sensitivity of the assay was Antiserum II, obtained by bleeding the immunised rabbit seventeen months after the initial injection of human calcitonin.

Over a period of two years the animal was bled twelve times. The relative sensitivities of standard curves prepared with each of these antisera were now studied.

Methods.

The dilution of each antiserum needed to give 50% binding of labelled hormone in an 18 hour incubation period had been assessed on the day after collection. The dilution needed to give 50% binding over four days incubation was estimated from an 18 hour incubation knowing the increased dilution needed for Antiserum II.

Fifty microlitre aliquots of each diluted antiserum were added to sets of tubes containing 8-1000 pg of standard unlabelled hormone, and pre-incubated for four days. Labelled hormone was then added, and tubes incubated for a further three days. Bound and free hormone was separated, and the percentage of ^{125}I-calcitonin bound to antibody calculated for each tube.

Results.

The dilution of antiserum required to bind 50% of labelled calcitonin during 18 hours incubation varied greatly during the two years over which the sera were collected (Figure 10). Of the twelve antisera, only eight bound labelled hormone.

Standard curves using these eight antisera at dilutions giving about 50% binding in four days did not show much variation. The sensitivity tended to be greater in curves using the antiserum at the highest dilution.

Since Antiserum II which had previously been used had originally been chosen because it had the highest titre in the 18 hour incubation system, no increase in sensitivity would be achieved by using a different antiserum. Several of the other antisera could be used in the assay instead of Antiserum II without loss of sensitivity.

GENERAL COMMENTS

There are two principal approaches to improving the limits of detection of radioimmunoassays for small peptides in plasma. One is to use short-term incubation. This method does not give maximal sensitivity (Chard, Kitau and Landon, 1970) but interference from plasma is less likely. The assay is relatively accurate and not time-consuming. It can be exploited to measure high levels of hormone in plasma and lower levels when the hormone has been previously extracted and concentrated. However, extraction techniques introduce further errors and require elaborate controls to assess recovery.

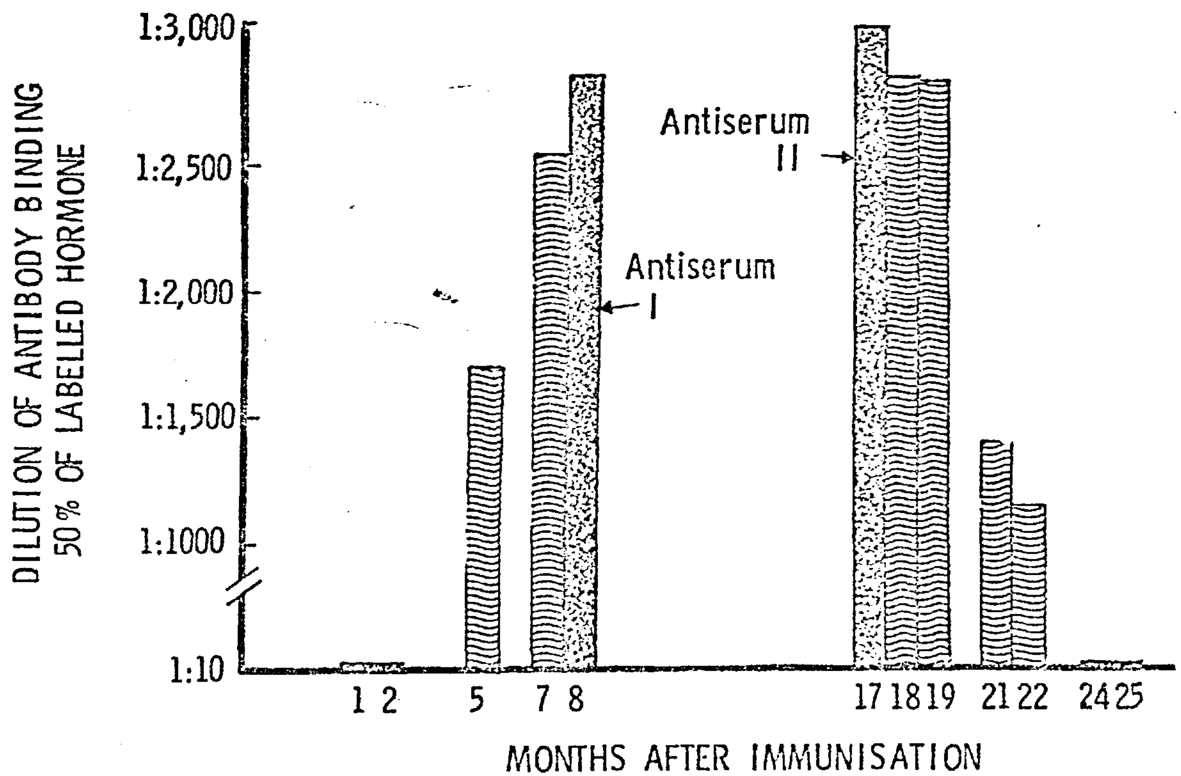

Figure 10. Comparison of binding of equal amounts of labelled calcitonin to antibodies in different antisera obtained from the same animal during the immunisation period. Binding increased during the first eight months, reached a plateau, and thereafter declined.

The second approach is to systematically examine each parameter of the assay and exploit the assay under the most favourable conditions. This frequently means that long-term incubation times with low concentrations of antibody are required and results are delayed.

In this chapter the latter approach was investigated. The sensitivity of the assay was increased 10-fold. Despite this improvement, levels in normal man could not be measured. We therefore conclude that an extraction procedure for calcitonin from plasma will be required. Work on this problem is in progress.

Chapter VII

METABOLIC FATE OF HUMAN CALCITONIN

Summary:

Uptake of radioactivity was demonstrated in the kidneys and liver of dogs injected with ^{131}I-labelled human calcitonin. To determine whether these organs played a role in the metabolism of the hormone, unlabelled calcitonin was infused into dogs. In each animal studied, simultaneous blood samples for radioimmunoassay were collected from the aorta and hepatic and renal veins. No significant calcitonin A-V difference across the liver at arterial levels above 90 ng/ml could be detected. Renal venous blood contained, however, only two-thirds as much calcitonin as arterial blood over a range of 70-400 ng/ml. From this it was calculated that the kidneys removed 8 per cent of the circulating hormone per minute. To assess whether this removal was due to degradation or excretion, urine was collected during and following infusion of unlabelled hormone. Since less than 0.3% of hormone infused could be detected in the urine, it was deduced that disappearance of the hormone via the kidney was most likely to be due to degradation. To confirm the role of the kidney, dogs were infused with ^{131}I-labelled or unlabelled human calcitonin. Blood was collected during and following infusion. The animals were then nephrectomised and studied under identical infusion conditions the following day. After removal of the kidneys, followed by a second infusion, the plasma calcitonin concentration was twice that previously observed. Two exponential components were observed in the disappearance curves of the unlabelled hormone. In both the intact and nephrectomised animals, the slower component had a half-time of disappearance of approximately 40 minutes. The rapid component had a half-time of disappearance of 3 minutes, and, following nephrectomy was decreased or abolished.

The studies presented in this chapter were undertaken to establish the fate of human calcitonin. For this purpose the distribution of the hormone, the effects of both kidney and liver on circulating levels, the excretion of the hormone in bile and urine, and its rate of disappearance from the blood were studied in dogs. Calcitonin, labelled with 131-Iodine, was used to localise those tissues which concentrated the hormone, and to study the rate of disappearance of the hormone from blood in intact, nephrectomised and hepatectomised animals, and its excretion in urine and bile. The radioimmunoassay described

in Chapters III and VI was used to study arteriovenous differences in the levels of hormone across liver and kidney, and to confirm the effect of nephrectomy on the disappearance of calcitonin from blood and its excretion in urine.

DISTRIBUTION OF ISOTOPICALLY LABELLED HORMONE

This study was carried out to investigate the distribution of radioactivity following a bolus injection of ^{131}I-labelled hormone. It was recognised that this approach would not give definite evidence of the presence of active hormone. However, it was expected to demonstrate which tissues might be involved in its metabolism.

Methods.

In a preliminary experiment a 23 lb young mongrel dog was starved overnight and given 1 ml of Lugol's solution by mouth. The iodine solution was administered to saturate iodine binding sites. The animal was placed on its stomach beneath a gamma camera and 600 μCi of ^{131}I-labelled human calcitonin (approximately 3 μg) was injected intravenously. The appearance of radioactivity in the region of the abdomen and pelvis was recorded photographically, using a Polaroid camera.

In definitive experiments, two young mongrel dogs, again starved overnight and weighing 16 and 17 lbs were studied. Each was given 1 ml of Lugol's solution orally and injected intravenously with 1 ml of saline containing 58 μCi of ^{203}Hg-labelled neohydrin to localise the kidneys. Four hours later the animals were anaesthetised with Nembutal (Abbott's Veterinary Nembutal),

and catheters were inserted into a vein in each forepaw. One catheter was subsequently used for the withdrawal of blood samples, the other for injections. To localise the liver, 300 µCi of $^{99}Tc^{m}$-sulphur colloid in 1 ml of normal saline was given intravenously. After 6 minutes the outline of the liver, kidneys and bladder were recorded at the appropriate energy levels for $^{99}Tc^{m}$ and ^{203}Hg respectively by the gamma camera. This information was stored on magnetic tape by a digital data acquisition system (Vernon and Glass, 1971). To make corrections for blood distribution, 150 µCi of ^{131}I-labelled polyvinyl pyrrolidone (PVP) was next injected and serial blood samples taken every 2 minutes for 20 minutes. Following this 600 µCi of ^{131}I-labelled calcitonin containing approximately 3 µg of hormone was injected intravenously and the disappearance of radioactivity recorded. Serial blood samples were collected to establish the disappearance of the radioactivity from blood. For this purpose, equal aliquots of each plasma sample were counted in a Packard automatic gamma counter.

The net uptake of radioactivity in liver, kidney and bladder regions, was calculated using the count rate recorded in these areas, previously outlined by the 203-mercury and 99^{m} technetium. A computer programme which operated on the magnetic tape data was used for this purpose. Areas adjacent to the kidneys were deliniated and the uptake in them was used to correct for tissue uptake of the radioactivity in the regions of interest. The contribution due to radioactivity in the blood was calculated from the ^{131}I-PVP level in each region and the measured disappearance of radioactivity from the blood. In this way the net uptake of 131-Iodine in liver, kidney and bladder following administration of labelled hormone was ascertained by subtracting

the activity in blood and tissue from the total radioactivity recorded in each organ.

Results.

The appearance of radioactivity following a bolus injection of ^{131}I-labelled human calcitonin is shown in Figure 1. These Polaroid photographs indicate that the radioisotope was concentrated in liver and kidney and was excreted into the urine within the bladder. Furthermore, these findings suggest that the uptake of radioactivity in both kidney and liver was cumulative.

Localisation of the kidney using ^{203}Hg-labelled neohydrin, the liver using ^{99}Tc^{m}-sulphur colloid, and the blood distribution using ^{131}I-labelled PVP are shown in Figures 2, 3 and 4 respectively. Figure 5 describes the disappearance of radioactivity from plasma following the administration of labelled human calcitonin. The net radioactivities in kidney, liver and bladder due to the labelled hormone injection, as calculated by computer, and corrected for blood distribution and tissue radioactivity, are also plotted in Figure 5.

LOCALISATION OF RADIOACTIVITY IN THE KIDNEY

The following studies were undertaken to determine where radioactivity accumulated within the kidney following a bolus injection of ^{131}I-labelled human calcitonin.

Methods.

Two studies were carried out. After the gamma camera study

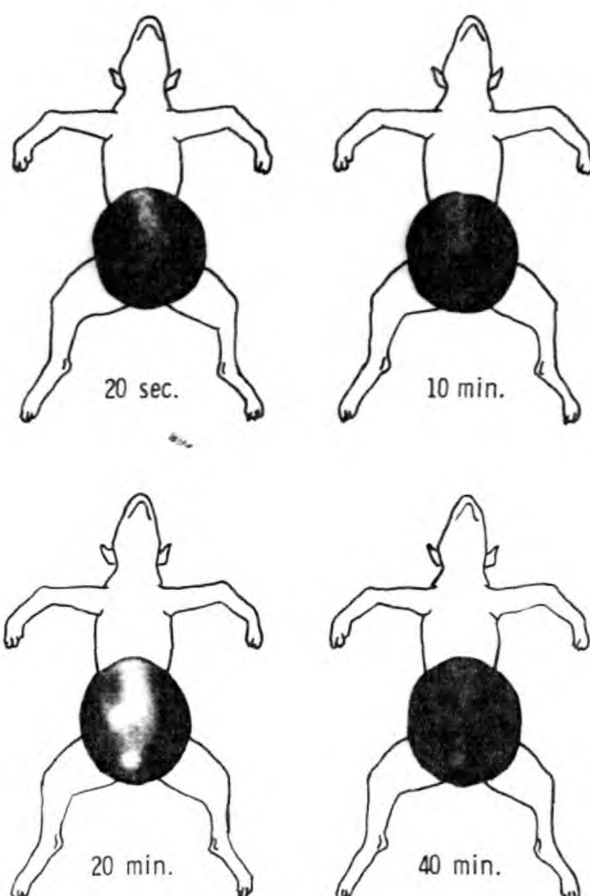

Figure 1. Serial Polaroid photographs of the appearance of radioactivity in the abdomen and pelvis following a bolus injection of ^{131}I-labelled human calcitonin in the dog. The findings suggest that radioactivity accumulated in liver, kidneys and bladder.

Figure 2. Computer printout of accumulation of ^{203}Hg-labelled neohydrin used to localise the kidneys in the dog.

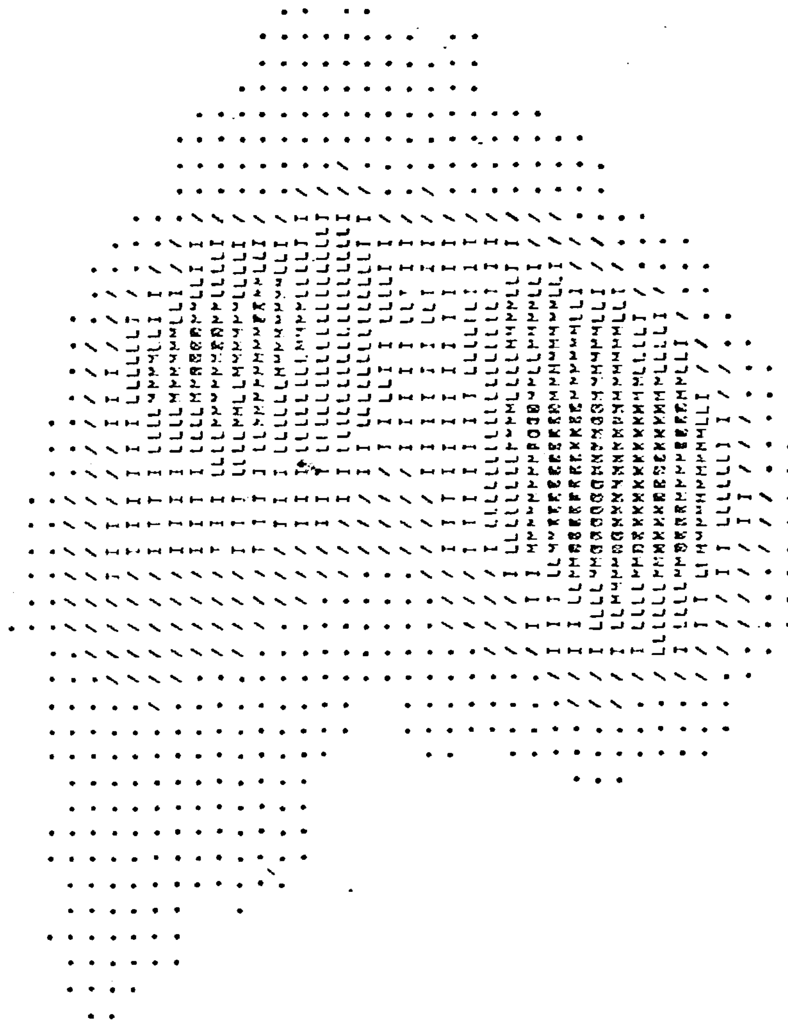

Figure 3. Computer printout of accumulation of ^{99}Tc (metastable) sulphur colloid used to localise the liver in the dog.

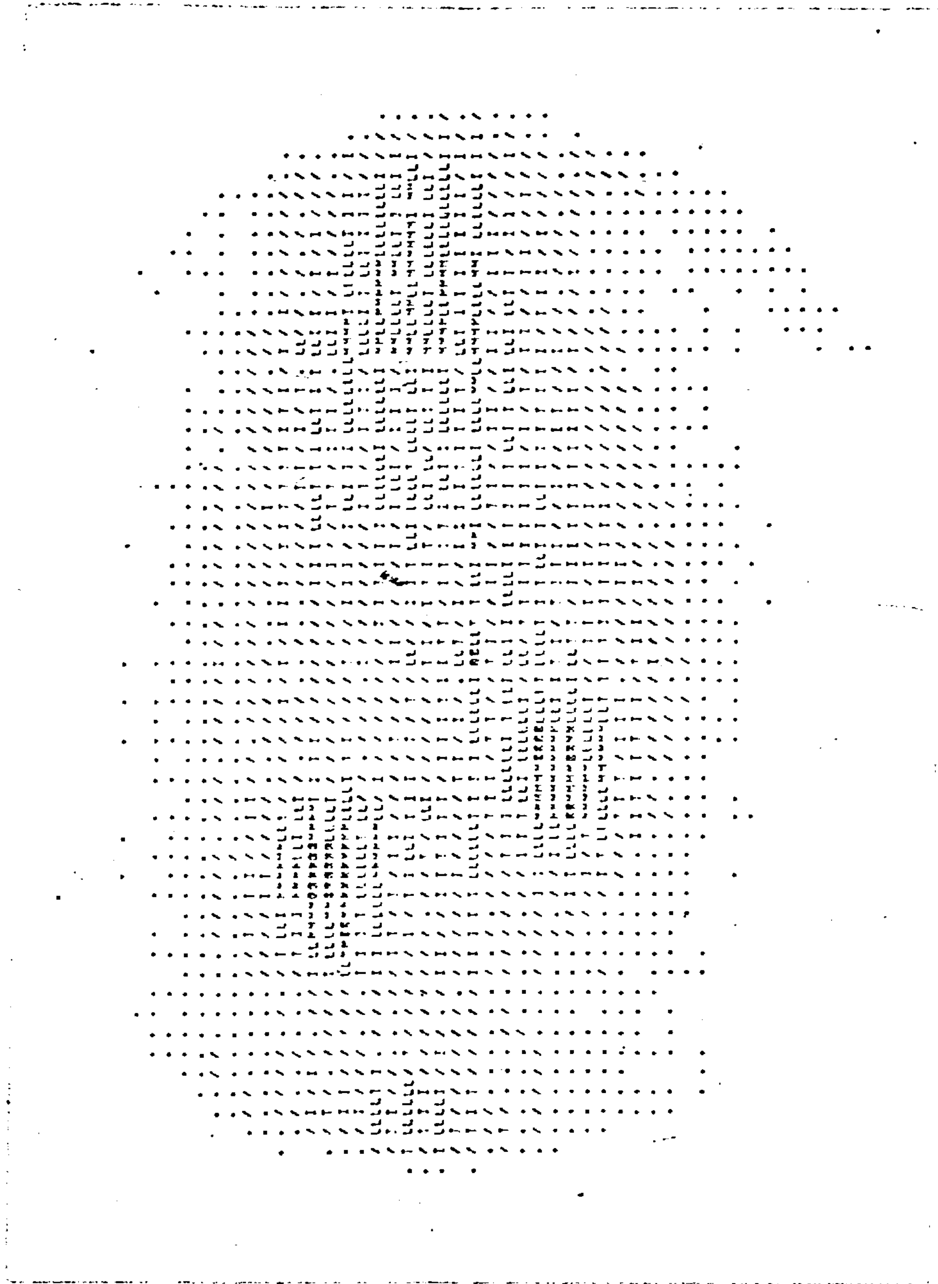

Figure 4. Computer printout of ^{131}I-labelled polyvinylpyrrolidone used to indicate distribution of radioactivity in the blood.

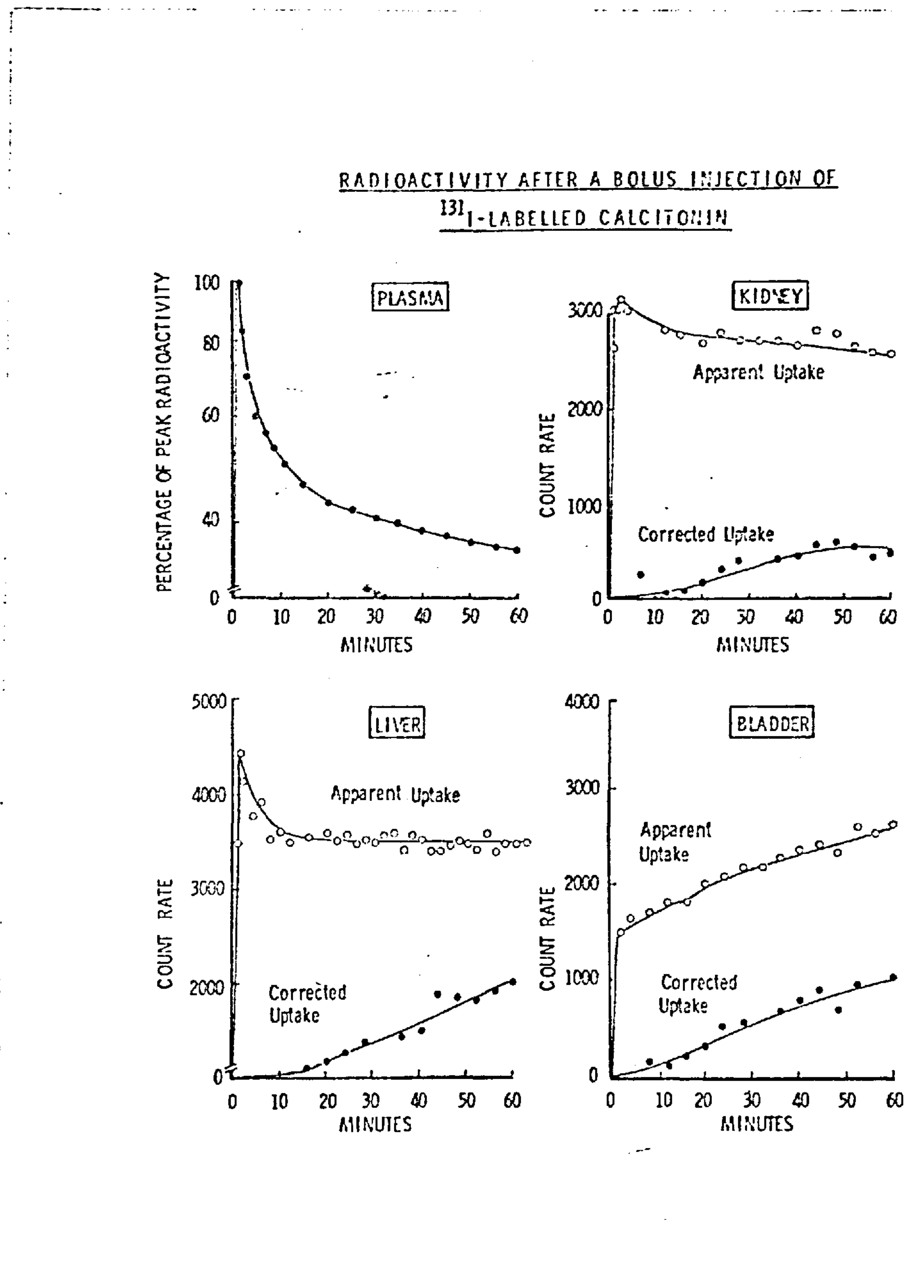

Figure 5. Diagrams showing the disappearance curve of radioactivity from the plasma and uptake of radioactivity in the kidney, bladder and liver of a dog following a single bolus injection of ^{131}I-labelled human calcitonin. Total uptake of radioactivity and net uptake after correction for radioactivity in blood and overlying tissue is indicated.

on the 16 lb dog in which 600 µCi of ^{131}I-calcitonin had been injected one hour previously, the kidneys were removed from the animal, and a transverse section was made and radioautographed. In the other, six rabbits from a single litter, each weighing 2 kg were starved overnight. On the following day they received 1 ml of Lugol's solution by mouth and were injected with a comparable dose of labelled hormone on an activity to weight basis. Immediately prior to administration of the labelled calcitonin, three were given 300 µg of unlabelled calcitonin. This amount was a thousand-fold greater than the amount of labelled hormone administered. One animal receiving labelled calcitonin and one animal receiving labelled calcitonin plus unlabelled calcitonin were killed 15, 30 or 60 minutes later. An additional control animal was given a similar amount of radioactive iodine alone. This animal was killed at 30 minutes. The kidneys of all animals were removed, and transverse sections were cut and radioautographed on Kodak Crystallex film.

Results.

In both dog and rabbit, radioactivity was strikingly increased within the cortex. In the rabbit this uptake was unaffected by prior administration of unlabelled hormone and was far greater than uptake of ^{131}Iodine alone. A representative autoradiograph is illustrated in Figure 6.

The finding that unlabelled hormone, in the dose given, did not appreciably prevent the uptake of labelled hormone is presumptive evidence that the binding sites within the kidney have an almost infinite capacity to bind hormone even at levels greatly in excess of what might be anticipated under physiological conditions.

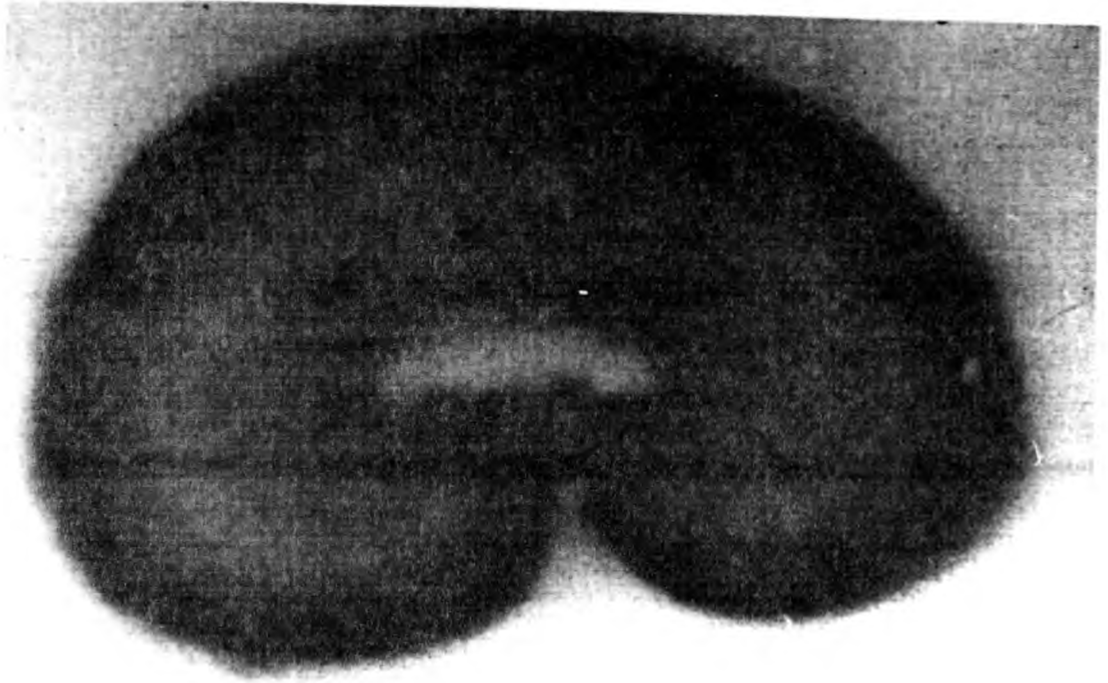

Figure 6. Autoradiograph of a transverse section of dog kidney obtained in an animal killed one hour after a bolus injection of ^{131}I-labelled human calcitonin. Radioactivity was concentrated within the cortex.

ARTERIOVENOUS DIFFERENCES ACROSS KIDNEY AND LIVER

The two previous studies indicated that the radioactivity following administration of labelled hormone was concentrated in both liver and kidney and excreted in the urine. To ascertain whether kidney and liver played a role in either the destruction or excretion of the hormone, arteriovenous differences were measured, across both organs, during infusion of unlabelled human calcitonin.

For this purpose, three greyhound dogs, weighing between 49 and 61 lbs, were studied. Each was anaesthetised with Nembutal and catheters were placed, under direct fluoroscopy, into a hepatic vein via the right external jugular vein and into a renal vein via a femoral vein (Figure 7). Unlabelled human calcitonin (1-2 mg) was infused into a forepaw vein for periods varying from 60 to 85 minutes. Simultaneous blood samples were collected, in chilled heparinised tubes, from the hepatic and renal veins and from the aorta at intervals during the infusions. In addition, in one animal, urine samples were obtained from a bladder catheter at timed intervals during and following infusion. In this dog the rate of destruction of calcitonin was also measured by collecting plasma for immunoassay from the femoral vein for 80 minutes after the infusion. Plasma was separated at 4° by centrifugation and all samples frozen until assayed.

Results.

In nineteen paired observations carried out on three dogs, the level of calcitonin in the hepatic and renal veins was significantly less than those in the aorta (Figure 8). The effects

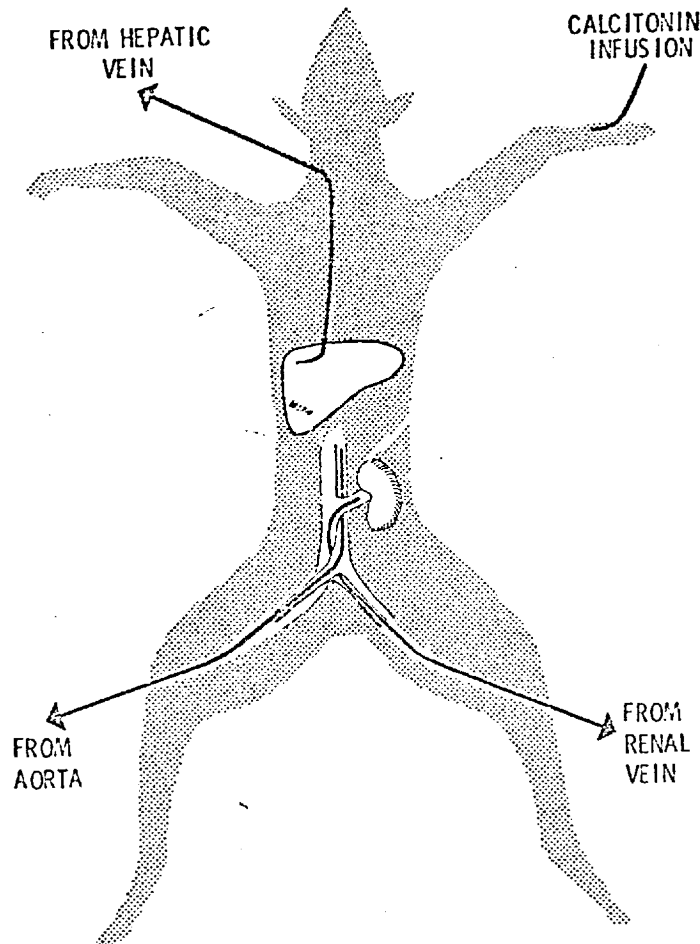

Figure 7. Schema indicating the positions of catheters for the administration of unlabelled hormone and for collection of blood samples from the renal and hepatic veins and aorta.

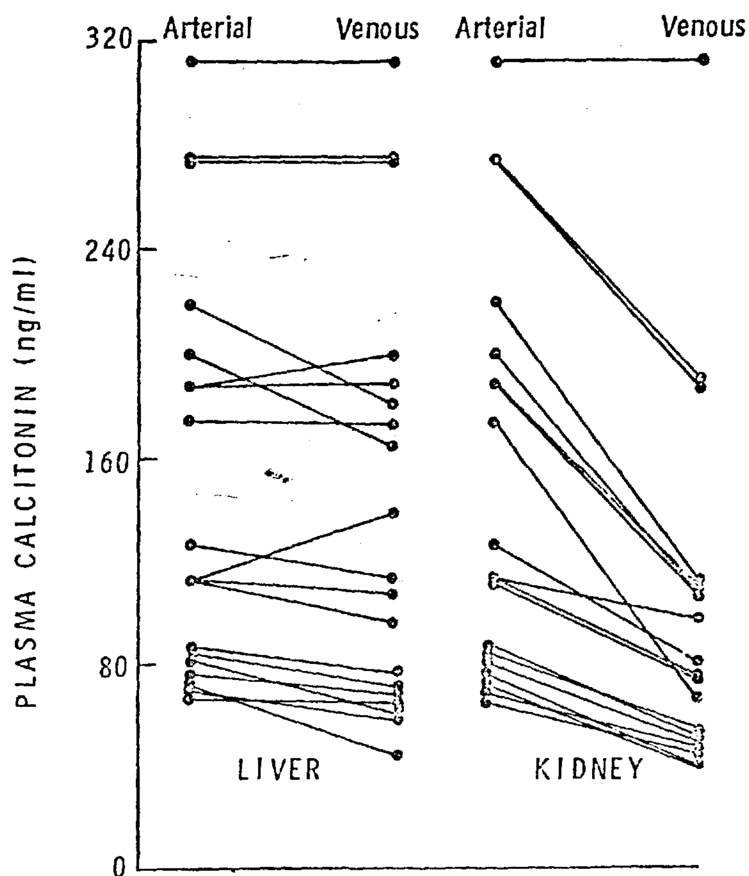

Figure 8. Arterio-venous differences of calcitonin observed across liver and kidney during infusion of unlabelled human calcitonin in the dog. Interconnecting lines indicate results in plasma samples obtained simultaneously. Only at arterial levels below 90 ng per ml was a significant difference observed across the liver. In contrast, renal venous blood contained only two-thirds as much hormone as arterial blood irrespective of the arterial level.

of both these organs on the circulating levels of the hormone will be separately discussed.

The arteriovenous differences across the liver were just significant ($p<0.04$) and appeared to be largely dependent upon the level of circulating hormone. At arterial levels between 90 and 320 ng/ml, there was no significant removal of the hormone. This may not, however, be true at lower levels. In one dog, in whom seven paired observations were made over a range of 70 to 90 ng/ml, there was consistently less hormone in the hepatic venous effluent ($p<0.03$). It was therefore tentatively concluded that if the liver did remove or degrade the hormone, its effect was only apparent at lower levels.

In contrast to the liver, the renal venous level of calcitonin was approximately 30% less than in arterial plasma, irrespective of the arterial level.

A crude approximation of the amount removed by the kidney was made from the assumption that the circulating blood volume in the dog is 93 ml/kg body weight (Sjorstrand, 1962) and that in the anaesthetised animal the blood flow through the kidneys is 23 ml/min/kg body weight (Selkurt, 1963). From this it follows that approximately 25% of the total blood volume passes through the kidneys every minute and, since one-third of the circulating hormone level was removed by these organs, 8% of the total calcitonin in plasma must be removed during this time.

No alteration in the immunoreactivity of the hormone in renal venous blood was apparent since the slope of its displacement curve produced was indistinguishable from that produced

by the synthetic hormone itself.

From these investigations it was concluded that the kidney and possibly the liver were responsible for the removal of hormone, and that, at the pharmacological levels studied, the kidney appeared to play the dominant role. It could not, however, be dismissed that at physiological levels the role of the liver might assume greater importance.

To assess whether the removal of hormone by kidney was due to degradation or excretion, urine samples from one animal were collected during and following a 90-minute infusion of unlabelled hormone. The levels of immunologically active hormone in the samples were measured by radioimmunoassay. The results are shown in Figure 9. Only 0.3% of hormone infused could be detected. From this it was concluded that the disappearance of calcitonin was principally due to renal uptake, and not to excretion, and that this uptake was most likely associated with degradation, since hormone was removed continuously even when large amounts were infused.

DISAPPEARANCE OF HORMONE IN INTACT AND NEPHRECTOMISED DOGS

The foregoing investigation indicated that the kidney and possibly the liver played a role in the removal of hormone from plasma. To confirm the role of the kidney and to assess its importance in the disappearance of the hormone, the following studies were carried out.

Methods.

Three conscious mongrel dogs, starved overnight and weighing

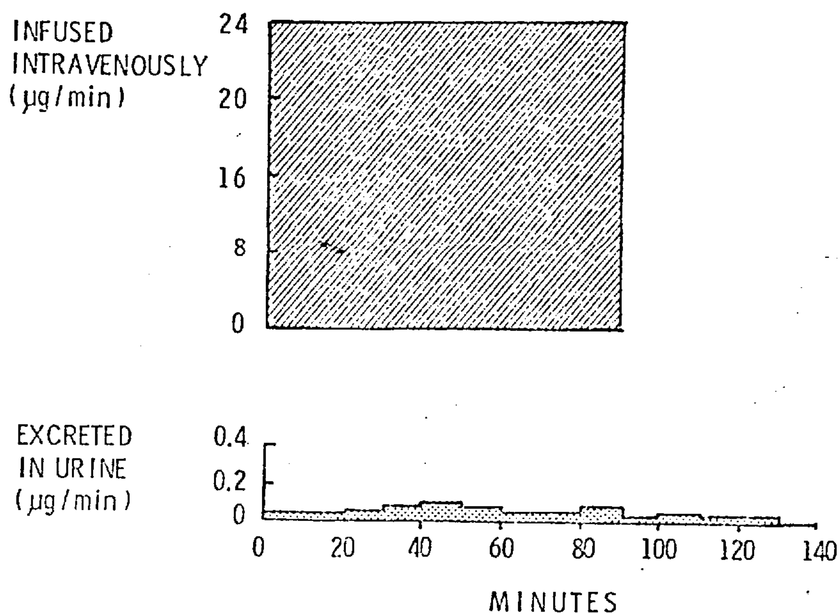

Figure 9. Immunoassayable hormone in urine during and following a 90-minute infusion of unlabelled human calcitonin. Note that the lower scale is expanded twenty-fold. It was calculated from these results that less than 0.4% of infused hormone was excreted in an immunologically detectable form.

between 30 and 45 lbs, were used. Each was restrained in an upright position. One to two milligrams of synthetic human calcitonin were infused over an 80-90 minute period via a catheter inserted into a vein in one forepaw, and blood samples were collected during and after the infusion from a catheter inserted in the vena cava via a vein in one hind paw. The volumes withdrawn were replaced by equal volumes of normal saline to obviate spurious effects due to reduction in blood volume. When blood was not being withdrawn, the catheter was filled with a dilute solution of sodium heparin to prevent clotting. At the conclusion of the infusion, blood samples were obtained at rapid intervals for 20 minutes in all animals, and in one animal at 5-minute intervals for a further 50 minutes.

Ninety minutes after the infusion, an acute injection of 60 µCi of ^{131}I-labelled human synthetic calcitonin (approximately 300 ng of hormone) in 1 ml of normal saline was administered intravenously to the dogs. To measure the disappearance of the labelled hormone, blood samples were again obtained at frequent intervals for 90 minutes.

Following these studies three of the animals were anaesthetised with sodium pentothal and their kidneys surgically excised. Post operative recovery was uneventful. The following day, the dogs were again infused with both labelled and unlabelled hormone as previously described. The dose of hormone administered to each animal was identical to that given the previous day.

Levels of calcitonin in blood samples obtained following infusion of unlabelled hormone were measured by radioimmunoassay. Blood samples collected following the infusion of labelled

hormone were treated as follows: each was centrifuged at 4°C, the plasma separated, and the radioactivity in 0.5 ml samples counted in a well counter. Subsequently, an equal volume of 10% trichloracetic acid was added to each aliquot. Following mixing for 10 minutes, each tube was centrifuged for 10 minutes at 3,000 RPM, the supernatant aspirated and the radioactivity in the precipitate counted.

Results.

Results of calcitonin levels obtained during and following unlabelled hormone infusion in one dog before and after nephrectomy are shown in Figure 10. Plateau levels after removal of the kidneys were higher than in the intact dog and the initial fast rate of disappearance was not seen.

The increased plateau level can be interpreted in one of two ways: either removal of the kidneys decreased the pool size available to the hormone or decreased the rate of degradation. Both explanations may account for the higher levels reached, since previous studies suggested that radioactivity from labelled hormone was accumulated by the kidneys and indicated that the kidneys were possibly responsible for degradation of the hormone.

The semi-logarithmic plot of the disappearance of unlabelled hormone from plasma following a continuous infusion before and after nephrectomy in one dog is shown in Figure 11. On analysis by a method which allowed the data points to be weighed according to their individual accuracy (Berman, Shan and Weiss, 1962), two exponential components were observed. The slow component, both

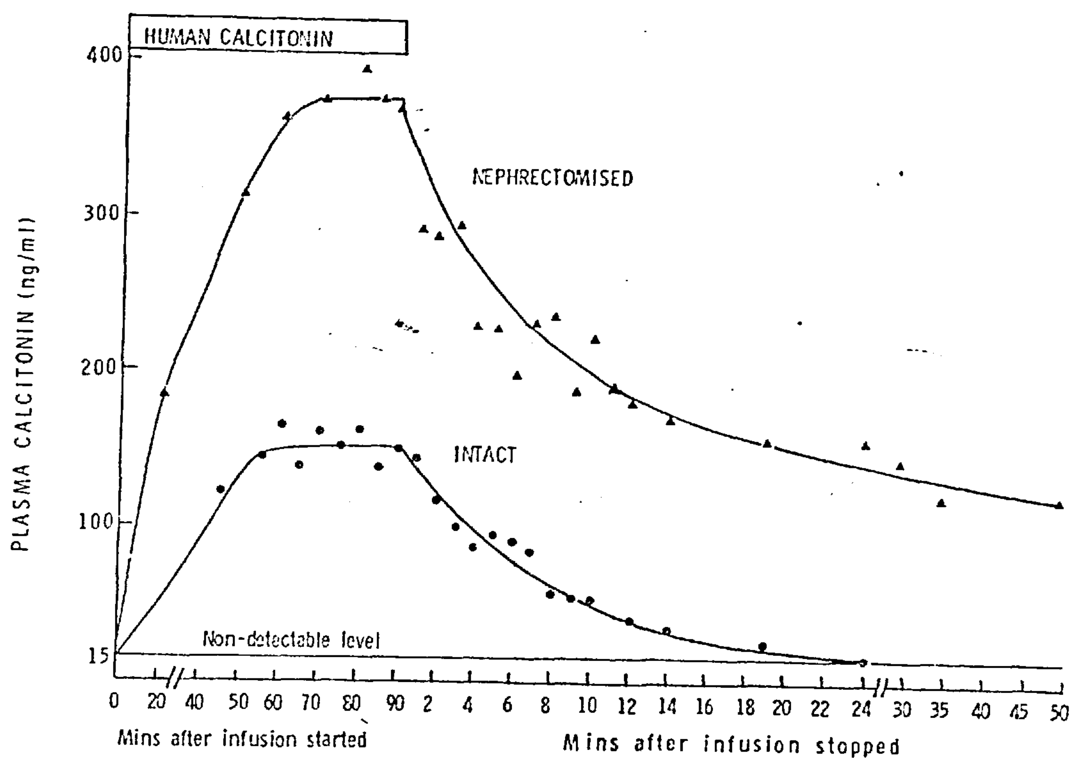

Figure 10. Results of a representative study indicating levels of calcitonin during and following a 90-minute infusion of unlabelled human hormone in a dog before and after nephrectomy. Following removal of the kidneys, calcitonin infusion produced higher levels of circulating hormone and the initial rapid rate of disappearance of hormone from plasma following infusion was altered.

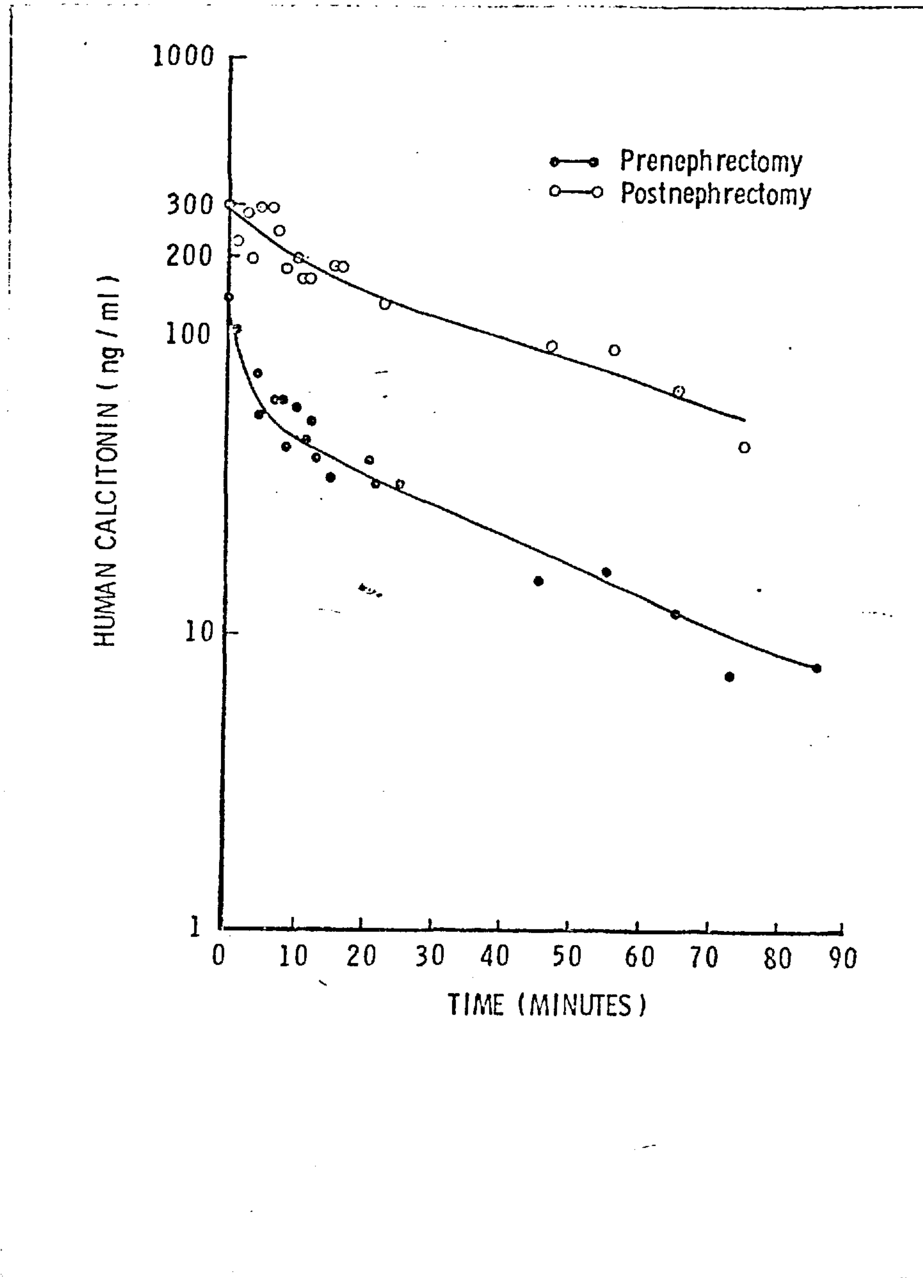

Figure 11. Log plot of the disappearance of unlabelled human calcitonin following a continuous infusion from the plasma of a dog before and after nephrectomy.

before and after nephrectomy, had a half-time of disappearance of approximately 40 minutes. In contrast, a component with a half-time of disappearance of approximately 3 minutes was found in the intact dog but was not measurable in the results from the nephrectomised dog. From these findings it was concluded that at these pharmacological levels the rapid rate of the disappearance of the hormone is due principally to renal destruction of the hormone. It cannot, however, be conclusively stated that no other factors contributed to the rapid disappearance of hormone, since the wide scatter in values obtained at high plasma levels of the hormone did not permit exclusion of a minor variation in the initial disappearance time of the hormone. The results obtained in the following study of the disappearance of calcitonin from plasma after a bolus injection of labelled hormone support these conclusions.

The combined findings of the three animals receiving ^{131}I-labelled hormone are shown in Figure 12. These data are presented as a plot of the percentage of the peak of radioactivity against time, where the highest amount of radioactivity recorded is expressed as 100%. This peak occurred 3 minutes after injection of the hormone.

The disappearance curves of the intact ^{131}I-labelled calcitonin exhibit two exponential components and the component associated with the slower rate of clearance does not appear to be affected by nephrectomy. This finding is in agreement with the results of previous experiments using unlabelled hormone.

Using a computer programme to fit two components to the data (Marquarat, 1963, 1964), the half-time of disappearance and

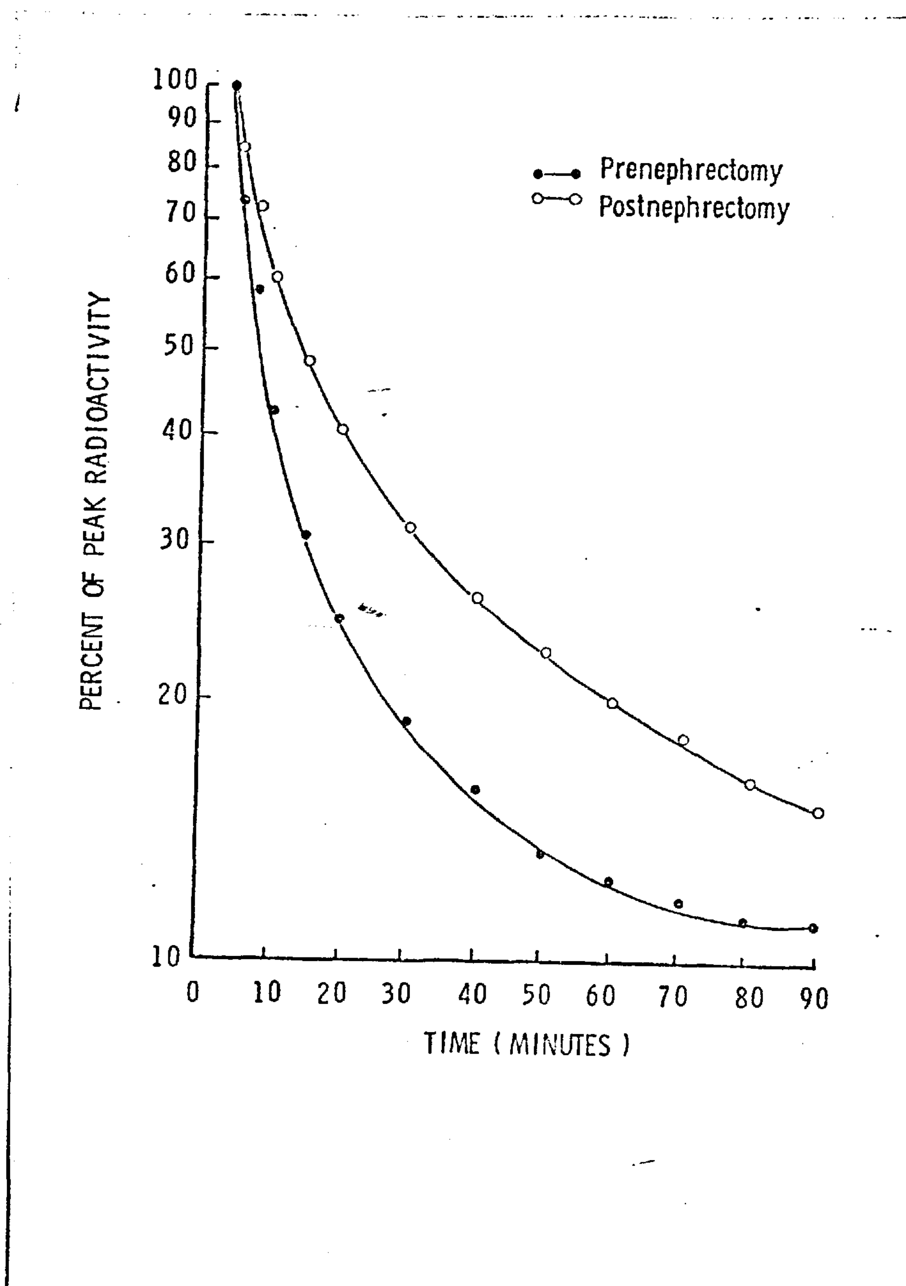

Figure 12. The mean disappearance of labelled human calcitonin (following single bolus injections) from the plasma of three dogs before and after nephrectomy. The early rate of disappearance was slower in the animals after nephrectomy than when intact. The later rate of disappearance was similar in both circumstances.

the relative pool sizes of the two compartments into which the hormone was distributed were then calculated.

In the intact animal, the relative sizes of the pools associated with the fast and slow exponents were calculated. It was assumed that, after nephrectomy, the size of the pool associated with the slow component remained constant as the half-time of disappearance remained constant. The relative size of the pool associated with the fast exponent, which had been altered by nephrectomy, was then compared with it (Figure 13). The change in the size of the fast pools before and after nephrectomy could then be compared (Table 1).

In the three dogs after nephrectomy the relative size of the pool associated with the fast component decreased in relation to the pool associated with the slow component. From these results it appears that the kidney accounts for the 55-70% of the initial removal of the hormone. These results suggest that the fast component could be further divided into two components, one of which was due to the kidney.

If the combined half-time of disappearance of both these components in the intact dog is 3.4 minutes, and the half-time of disappearance of the component remaining after nephrectomy is 4.8, it can be calculated that the half-time of disappearance due to the kidney is 10 minutes. This compares well with the disappearance time which can be calculated from the results of the A-V difference study where it was shown that 8% of circulating calcitonin was removed by the kidney per minute. The half-time of disappearance due to the kidney, calculated using this figure, would be 9.0 minutes.

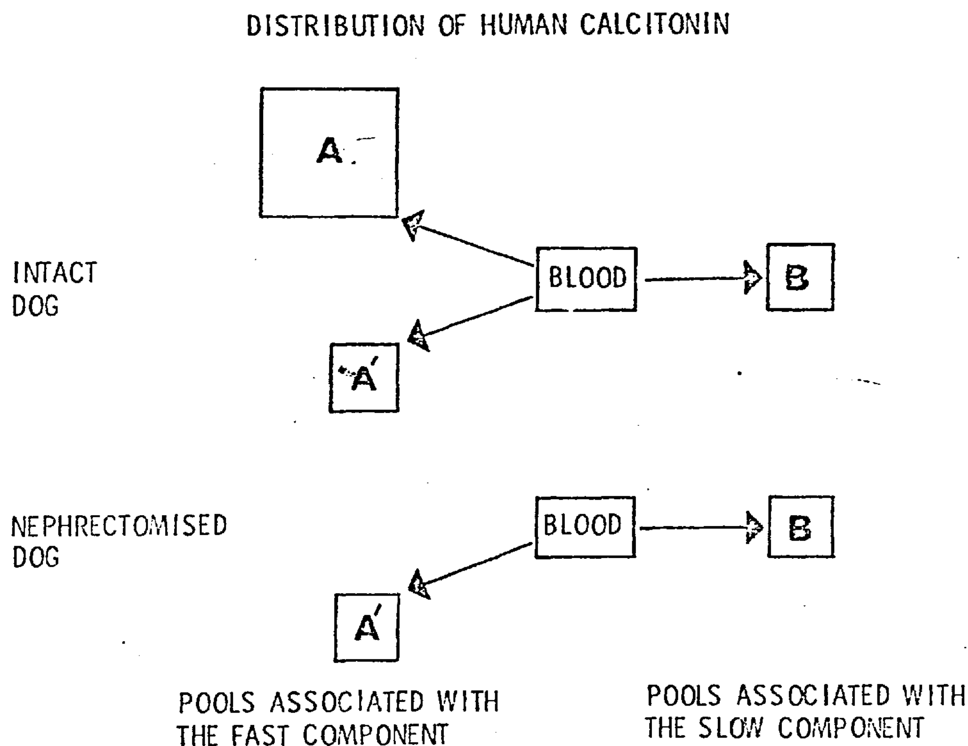

Figure 13. Diagrammatic representation of the pools into which ^{131}I-labelled calcitonin was distributed from the blood of a dog before and after nephrectomy. The pool B, associated with the slower rate of clearance was not altered by the removal of the kidney. The pool associated with the faster rate of clearance could be subdivided into pools A and A^1, of which the larger, A, was completely removed by nephrectomy.

Table 1. Half-times of disappearance of injected ^{131}I-labelled human calcitonin from pools related to the slow and fast exponents. The change in relative size of the fast pool is indicated following nephrectomy.

No.	Dog	Half-time of Disappearance (min)		Relative size of fast pool
		'Slow' pool	'Fast' pool	Intact:Nephrectomised
1	Intact	37.66	3.11	1.0:0.30
	Nephrectomised	39.38	3.71	
2	Intact	64.17	3.41	1.0:0.37
	Nephrectomised	48.46	5.25	
3	Intact	92.77	3.69	1.0:0.45
	Nephrectomised	82.11	5.25	

EVALUATION OF THE ROLE OF THE LIVER IN DEGRADATION

The following studies were carried out to assess whether or not the liver participates in the metabolism of the human hormone. This was done for three reasons. First, arteriovenous differences across the liver in one dog suggested that, at levels of hormone below 80 ng/ml, this organ might be responsible for the removal of some hormone. Second, it was not possible to exclude that there was a factor other than the kidneys that was responsible for the initial rapid disappearance of the hormone after infusion. Third, studies by other works (Milhaud and Hankiss, 1969; de Luise, Martin and Melick, 1970) have indicated that porcine hormone, in the rat, is removed principally by the liver.

Methods.

To assess whether or not the hormone might be excreted directly by the liver into bile, the contents of the gall bladder were collected from one dog, which had received ^{131}I-labelled calcitonin for gamma camera studies, and the total radioactivity was counted.

A second investigation was carried out to determine if the liver had any effect on the disappearance rate of the hormone. A 31 lb mongrel dog was starved overnight. The following day 1 ml of Lugol's solution was administered orally and the animal anaesthetised with Nembutal. As previously described, catheters were placed in the veins of both forepaws. Into one of these a single bolus injection of 20 µCi of synthetic human calcitonin, containing approximately 100 ng of hormone, was given. From the

other catheter venous blood samples were collected at one-minute intervals over a 50-minute period. The liver was then exposed by an upper abdominal midline incision and a functional hepatectomy was performed by ligation of the hepatic artery, portal vein, and bile duct. Within 5 minutes, the abdominal cavity was closed, and an identical bolus injection of ^{131}I-labelled human calcitonin was administered intravenously and plasma samples collected as before.

All blood samples were collected in chilled tubes. Following centrifugation and precipitation of intact hormone from plasma with trichloroacetic acid as previously described, the counts in the precipitate were counted in a well counter.

Results.

No appreciable radioactivity was found in the gall bladder contents collected following injection of the labelled hormone. This finding indicates that the hormone is not excreted by the liver.

Trichloroacetic acid precipitable labelled hormone in the blood was expressed as a percentage of the maximum peak value obtained after the bolus injection (Figure 14). As in other similar studies, this peak occurred within 3 minutes. The disappearance curves obtained, both before and after functional hepatectomy, were identical. The results of this study suggest, therefore, that the liver plays little or no role in the degradation of the hormone even when physiological levels are present. In these investigations administration of 100 ng of hormone could not have raised the initial circulating level above 50 pg/ml.

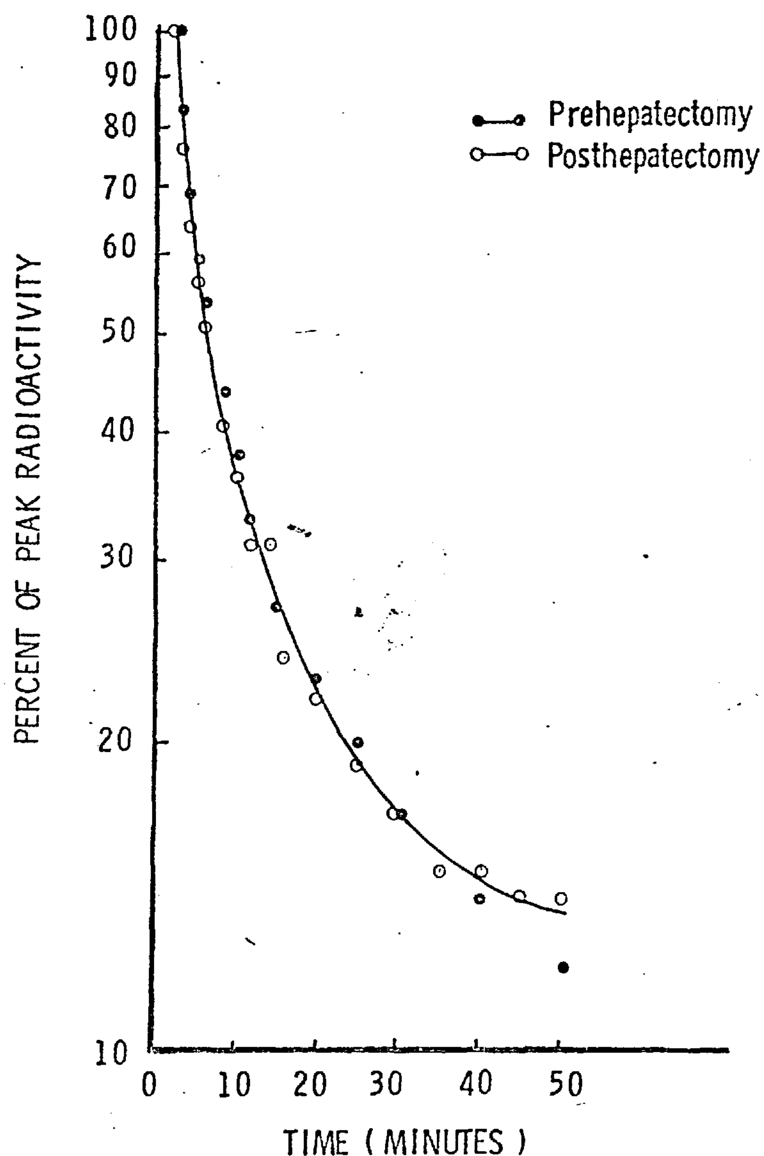

Figure 14. The disappearance of trichloroacetic acid precipitable human calcitonin activity from the plasma of a dog before and after functional hepatectomy. This procedure had no effect on the rate of disappearance of the hormone.

It is important to point out that hepatectomy results in death within 1 to 4 hours. For this reason metabolic abnormalities may have altered not only the rate of metabolism of the hormone but also the pool space available for its distribution.

From the results of both experiments, it would appear that the liver plays little or no role in the removal of the human hormone.

GENERAL DISCUSSION

From these studies in the dog, it was concluded that radioactivity following injection of labelled human calcitonin is taken up by both kidney and liver, that the kidney plays a prominent role in the removal of the hormone, that removal of the hormones by the kidney is due to degradation rather than excretion, that radioactivity following administration of labelled hormone is concentrated by the cortex, that the blood clearance curve for the hormone has two exponentials, one of approximately 3 minutes and another in the order of 40 minutes, and that the rapid phase of disappearance is due principally to renal destruction of the hormone.

The half-times of disappearance for human calcitonin, reported in this chapter, are in good agreement with those obtained with porcine hormone. Potts (1969) found a half-time of between 5 and 15 minutes in rabbit, West, O'Riordan and Care (1969) observed two half-times of 5 and 45 minutes in pig, and Neer, Parsons, Krane, Deftos, Shields and Potts (1970) a half-time of 15 minutes in man. The only estimate of the half-life of the human hormone in man has suggested that it is less than

15 minutes. This conclusion was drawn from the disappearance of biological activity in blood following the removal of a medullary carcinoma tumour in one patient (Cunliffe, Black, Hall, Johnson, Hudgson, Shuster, Gudmundsson, Joplin, Williams, Woodhouse, Galante and MacIntyre, 1968).

The view that the kidney is responsible for the degradation of the hormone is supported by the observation of Ardaillou and his colleagues (Ardaillou, Sizonenko, Meyrier, Vallée and Beaugas, 1970) who observed that ^{131}I-labelled human calcitonin is removed by the kidney in man and that only 4% is excreted intact in the urine. From their data it was deduced that the renal uptake is a consequence of tubular reabsorption and destruction of the filtered hormone. The fate of calcitonin may therefore be analogous to that of insulin. Chamberlain and Stimmler (1967) demonstrated that this latter peptide was filtered by the glomeruli and reabsorbed and destroyed in the proximal tubules.

The conclusion that the removal of human calcitonin is principally by the kidney, is in direct contradiction to the findings of others using the porcine hormone. De Luise, Martin and Melick (1970) investigated the distribution of ^{131}I-labelled porcine calcitonin in rats. Of the tissues studied, only liver and kidney showed concentrations greater than that found in plasma. Of these two organs, the greater concentration was found in liver. This accumulation by liver was decreased by simultaneous administration of unlabelled hormone. These findings, as well as those of Milhaud and Hankiss (1969) who found that liver homogenates inactivated biological activity, indicate that the liver was responsible for the early clearance of porcine hormone from plasma.

Porcine calcitonin differs from human calcitonin in eighteen of its thirty-two amino acids. The observation that the rapid disappearance of the human hormone is due principally to action of the kidney, as reported in this chapter and now confirmed by de Luise, Martin, Greenberg and Michaelangeli (personal communication), and not the liver can best be explained by the difference in its chemical structure. It is therefore tentatively proposed that the choice of organ by which the hormone is removed is programmed by its amino acid sequence. It therefore follows that it should be possible to prepare synthetic analogues with prolonged biological activity.

BIBLIOGRAPHY

Abraham, G.E. (1969): Solid phase radioimmunoassay of estradiol-17β. J. clin. Endocr. 29, 866-70.

Addison, G.M. and Hales, C.N. (1971): Methods involving labelled antibody (discussion). In: Radioimmunoassay Methods, 481-7. Ed: Kirkham, K.E. and Hunter, W.M., Churchill Livingstone, Edinburgh and London.

Aliapoulios, M.A., Goldhaber, P. and Munson, P.L. (1966): Thyrocalcitonin inhibition of bone resorption induced by parathyroid hormone in tissue culture. Science 151, 330-1.

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

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

Ardaillou, R., Sizonenko, P., Meyrier, A., Vallée, G. and Beaugas, C. (1970): Metabolic clearance rate of radioiodinated human calcitonin in man. J. clin. Invest. 49, 2345-52.

Ardaillou, R., Vuagnat, P., Milhaud, G. and Richet, G. (1967): Effets de la thyrocalcitonine sur l'excrétion rénale des phosphates, du calcium et des ions H^+ chez l'homme. Nephron 4, 298-314.

Arnaud, C.D., Littledike, T. and Tsao, H.S. (1970): Calcium homeostasis and the simultaneous measurement of calcitonin and parathyroid hormone in the pig. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 95-101. Ed: Taylor, S. and Foster, G.V. Heinemann Medical Books Ltd., London.

Arnaud, C.D., Littledike, T., Tsao, H.S. and Kaplan, E.L. (1968): Radioimmunoassay for calcitonin: a preliminary report. Proc. Mayo Clin. 43, 496-502.

Arnon, R. and Sela, M. (1969): Antibodies to a unique region in lysozyme provoked by a synthetic antigen conjugate. Proc. nat. Acad. Sci. 62, 163-70.

Beardwell, C.G. and Wright, A.D. (1968): Radioimmunoassay of arginine vasopressin. In: Third International Congress of Endocrinology (Abstract 119). Ed: Gual, C. Excerpta Medica International Congress Series No. 157, Amsterdam.

Bell, P.H., Barg, W.F. Jr., Colucci, D.F., Davies, M.C., Dziobkowski, C., Englert, M.E., Heyder, E., Paul, R. and Snedeker, E.H. (1968): Purification and structure of porcine calcitonin - 1. J. Amer. chem. Soc. 90, 2704-6.

Berman, M., Shahn, E. and Weiss, M.F. (1962): The routine fitting of kinetic data to models: a mathematical formulation for digital computers. Biophys. J. 2, 275-88.

Berson, S.A. and Yalow, R.S. (1959): Quantitative aspects of the reaction between insulin and insulin-binding antibody. J. clin. Invest. 38, 1996-2016.

Berson, S.A. and Yalow, R.S. (1964): Immunoassay of protein hormones. In: The Hormones 4, 557-630.

Berson, S.A. and Yalow, R.S. (1968): Immunochemical heterogeneity of parathyroid hormone in plasma. J. clin. Endocr. 28, 1037-47.

Berson, S.A., Yalow, R.S., Bauman, A., Rothschild, M.A. and Newerly, K. (1956): Insulin I^{131} metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin treated subjects. J. clin. Invest. 35, 170-90.

Berson, S.A., Yalow, R.S., Glick, S.M. and Roth, J. (1964): Immunoassay of protein and peptide hormones. Metabolism 13, 1135-53.

Birge, S.J. and Avioli, L.V. (1969): Glucagon induced hypocalcaemia in man. J. clin. Endocr. 29, 213-8.

Borle, A.B. (1969): Effects of thyrocalcitonin on calcium transport in kidney cells. Endocrinology 85, 194-9.

Borth, R. (1970): Discussion on theoretical aspects of saturation analysis. Acta endocr. (Kbh.) Suppl. No. 147, 32-6.

Boyd, G.W. and Peart, W.S. (1968): The production of high-titre antibody against free angiotensin II. The Lancet ii, 129-33.

Brewer, H.B. (1970): Bovine thyrocalcitonin. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 14-27. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Brewer, H.B. and Ronan, R. (1969): The amino acid sequence of bovine thyrocalcitonin. Proc. nat. Acad. Sci. 63, 940-7.

Brown, R.K. (1963): Immunologic studies of bovine ribonuclease derivatives. Ann. N.Y. Acad. Sci. 103, 754-64.

Bussolati, G., Carvalheira, A.F. and Pearse, A.G.E. (1968): Immunofluorescence studies on the source of calcitonin and the effect of immunisation with calcitonin on the thyroid gland of the guinea pig. In: Proceedings of the Symposium on Thyrocalcitonin and the 'C' Cells, 133-42. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Butt, W.R. (1969): Chemistry of gonadotrophins in relation to their antigenic properties. Acta Endocr. (Kbh) 63 (Suppl. 142), 13-30.

Byfield, P.G.H. (Personal communication): Presence of a hypocalcaemic factor in dogfish ultimobranchial glands.

Byfield, P.G.H. (1970): Human calcitonin: purification and immunochemistry. Ph.D. Thesis. University of London.

Catt, K.J. (1969): Discussion on labelling and separation. In: Protein and Polypeptide Hormones, 610. Ed: Margoulies, M. Excerpta Medica International Congress Series No. 161, Amsterdam.

Catt, K.J., Niall, H.D. and Tregear, G.W. (1967): A solid phase disc radioimmunoassay for human growth hormone. J. Lab. clin. Med. 70, 820-30.

Catt, K.J. and Tregear, G.W. (1967): Solid phase radioimmunoassay in antibody coated tubes. Science 158, 1570-1.

Catt, K.J., Tregear, G.W., Burgher, H.G. and Skermer, C. (1970): Antibody-coated tube method for radioimmunoassay of human growth hormone. Clin. chim. Acta 27, 267-79.

Care, A.D., Bates, R.F.L. and Gitelman, H.J. (1969): The role of glucagon in the release of thyrocalcitonin. J. Endocr. 43, p. IV.

Care, A.D. and Bruce, J.B. (1971): Calcitonin-releasing substances: structure-activity relationship. J. Endocr. 49, p. viii-ix.

Care, A.D., Cooper, C., Duncan, T. and Orimo, H. (1968): The direct measurement of thyrocalcitonin secretion rate in vivo. In: Parathyroid Hormone and Thyrocalcitonin (Calcitonin), 417-27. Ed: Talmage, R.V. and Bélanger, L.F. Excerpta Medica, Amsterdam.

Carvalheira, A.F. and Pearse, A.G.E. (1968): Cytochemical evidence for the ultimobranchial origin of the 'C' cells in rodent thyroid. In: Proceedings of the Symposium on Thyrocalcitonin and the 'C' Cells, 122-6. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Chamberlain, M.J. and Stimmler, L. (1967): Renal handling of insulin. J. clin. Invest. 46, 911-19.

Chard, T. (1971): Observations on the uses of a mathematical model in radioimmunoassay. In: Radioimmunoassay Methods, 595-8. Ed: Kirkham, K.E. and Hunter, W.M., Churchill Livingstone, Edinburgh and London.

Chard, T., Kitau, M.J. and Landon, J. (1970): The development of a radioimmunoassay for oxytocin: radioiodination, antibody production and separation techniques. J. Endocr. 46, 269-78.

Chase, L.R., Fedak, S.A. and Aurbach, G.D. (1969): Activation of skeletal adenyl cyclase by parathyroid hormone in vitro. Endocr. 84, 761-8.

Clark, M.B., Boyd, G.W., Byfield, P.G.H. and Foster, G.V. (1969): A radioimmunoassay for human calcitonin M. The Lancet ii, 74-7.

Cooper, C.W., Deftos, L.J. and Potts, J.T. Jr. (1971): Endocrinology, in press.

Copp, D.H., Brooks, C.E., Low, B.S., Newsome, F., O'Dor, R.K., Parkes, C.O., Walker, V. and Watts, E.G. (1970): Calcitonin and ultimobranchial function in lower vertebrates. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 281-94. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Copp, D.H., Cameron, E.C., Cheney, B.A., Davidson, A.G.F. and Henze, K.G. (1962): Evidence for calcitonin - a new hormone from the parathyroid that lowers blood calcium. Endocr. 70, 638-49.

Copp, D.H., Cockcroft, D.W., Kueh, Y. and Melville, M. (1968): Calcitonin - ultimobranchial hormone. In: Calcitonin - Proceedings of the Symposium on Thyrocalcitonin and the 'C' Cells, 306-21. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Crumpton, M.J. (1968): Conformation of the growing polypeptide chain. Biochem. J. 108, p. 18-19.

Crumpton, M.J., Law, H.D. and Strong, R.C. (1970): The C-terminal antigenic site of sperm-whale myoglobin: the immunological activities of synthetic peptides related to the C-terminus of myoglobin. Biochem. J. 116, 923-5.

Cunliffe, W.J., Black, M.M., Hall, R., Johnson, I.D.A., Hudgson, R., 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, 63-6.

Deftos, L.J., Bury, A.E., Mayer, G.P., Habener, J.F., Singer, F.R., Powell, D. and Potts, J.T. (in press): Radioimmunoassays for calcitonins: chemical and experimental studies. In: Endocrinology 1971 - Proceedings of the Third International Symposium. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

de Luise, M., Martin, T.J., Greenberg, P. and Michaelangeli, V. (in preparation): Metabolism of human, pig and salmon calcitonins in the rat.

de Luise, M., Martin, T.J. and Melick, R.A. (1970): Inactivation and degradation of porcine calcitonin by rat liver, and relative stability of salmon calcitonin. J. Endocr. 48, 181-8.

Dietrich, F.M. and Rittel, W. (1970): Antigenic site(s) of synthetic human calcitonin M. Nature (London) 225, 75-6.

Doty, S.B., Schofield, B.H. and Robinson, R.A. (1968): The electron microscope identification of acid phosphatase and adenosine triphosphatase in bone cells following parathyroid extract and thyrocalcitonin administration. In: Parathyroid Hormone and Thyrocalcitonin (Calcitonin), 169-181. Ed: Talmage, R.V. and Bélanger, L.F., Excerpta Medica, Amsterdam.

Dube, W.J., Bell, G.O. and Aliapoulios, M.A. (1969): Thyrocalcitonin activity in metastatic medullary thyroid carcinoma. Further evidence for its parafollicular cell origin. Arch. intern. Med. 123, 423-7.

Dube, W.J., Goldsmith, R.S., Arnaud, S.B. and Arnaud, C.D. (1971): Hyperparathyroidism secondary to long-term therapy of Paget's disease of bone with calcitonin. Clin. Res. 19, 371.

Edwards, C.R.W., Chard, T., Kitau, M.J. and Forsling, M.L. (1970): The development of a radioimmunoassay and plasma extraction method for vasopressin. J. Endocr. 48, p. xi-xii.

Eisentraut, A.M., Whissen, N. and Unger, R.H. (1968): Incubation damage in the radioimmunoassay for human plasma glucagon and its prevention with "Trasylol". Am. J. med. Sci. 255, 137-42.

Ekins, R.P. (1969): Specificity of radioimmunoassay. In: Protein and Polypeptide Hormones, 575-82. Ed: Margoulies, M., Excerpta Medica International Congress Series, No. 161, Amsterdam.

Ekins, R.P., Newman, G.B. and O'Riordan, J.L.H. (1968): Theoretical aspects of "saturation" and radioimmunoassay. In: Radioisotopes in Medicine: In Vitro Studies, Oak Ridge Symposium, 1967, 59-100. Ed: Hayes, R.L., Goswitz, F.A. and Murphy, B.E.P., U.S. Atomic Energy Commission, Dept. of Tech. Information.

Evered, D.C., Chapman, C. and Hayter, C.J. (1970): Measurement of plasma digoxin concentration by radioimmunoassay. Brit. med. J. 3, 427-8.

Faiman, C. and Ryan, R.J. (1967): Radioimmunoassay for human follicle stimulating hormone. J. clin. Endocr. 27, 444-7.

Farr, R.S. (1958): A quantitative immunochemical measure of the primary interaction of I*BSA and antibody. J. infect. Dis. 103, 239-62.

Foster, G.V., Baghdiantz, A., Kumar, M.A., Slack, E., Soliman, H.A. and MacIntyre, I. (1964): Thyroid origin of calcitonin. Nature (London) 202, 1303-5.

Foster, G.V., Doyle, F.H., Bordier, P. and Matrajt, H. (1966): Effect of thyrocalcitonin on bone. Lancet ii, 1428-31.

Foster, G.V., Doyle, F.H., Bordier, P., Matrajt, H. and Tun-Chot, S. (1967): Roentgenologic and histologic changes in bone produced by thyrocalcitonin. Amer. J. Med. 43, 691-5.

Foster, G.V., Joplin, G.F., MacIntyre, I., Melvin, K.E.W. and Slack, E. (1966): Effect of thyrocalcitonin in man. Lancet i, 107-9.

Foster, G.V., MacIntyre, I. and Pearse, A.G.E. (1964): Calcitonin production and the mitochondrian-rich cells of the dog thyroid. Nature (Lond.) 203, 1029-30.

Franchimont, P. (1969): Radioimmunoassay of gonadotrophic hormones. In: Protein and Polypeptide Hormones, 99-116. Ed: Margoulies, M., Excerpta Medica International Congress Series No. 161., Amsterdam.

Freund, J. (1951): The effect of paraffin oil and mycobacteria on antibody formation and sensitisation. Am. J. clin. Path. 21, 645-56.

Friedman, J. and Raisz, L.G. (1965): Thyrocalcitonin: inhibitor of bone resorption in tissue culture. Science 150, 1465-6.

Gaillard, P.J. (1967): Bone culture studies with thyrocalcitonin. Proc. Kon. Nederl. Akad. Wet. (Biol. Med.) 70, 309-20.

Galante, L., Gudmundsson, T.V., Horton, R., Woodhouse, N.J.Y., MacIntyre, I., Burrows, P. and Robinson, C.J. (1970): Studies in calcitonin M and D. Calcified Tissue Research 4, Suppl. 84-5.

Galante, L., Gudmundsson, T.V., Matthews, E.W., Tse, A., Williams, E.D., Woodhouse, N.J.Y. and MacIntyre, I. (1968): Thymic and parathyroid origin of calcitonin in man. Lancet ii, 537-8.

Galante, L., Horton, R., Wiggins, R.C., Gudmundsson, T.V. and MacIntyre, I. (1970): The calcitonin-like action of streptomycin. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 427-33. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Gittes, R.F. and Irving, G.L. (1965): Thyroid and parathyroid roles in hypercalcaemia: evidence for a thyrocalcitonin-releasing factor. Science 148, 1737-9.

Goldhaber, P., Stern, B.D., Glimcher, M.J. and Chao, J. (1968): The effects of parathyroid extract and thyrocalcitonin on bone remodelling in tissue culture. In: Parathyroid Hormone and Thyrocalcitonin (Calcitonin), 182-95. Ed: Talmage, R.V. and Bélanger, L.F., Excerpta Medica, Amsterdam.

Goodfriend, T.L., Levine, L. and Fasman, G.D. (1964): Antibodies to bradykinin and angiotensin: a use of carbodiimides in immunology. Science 144, 1344-6.

Goodman, J.W. (1969): Immunochemical specificity: recent concepted advances. Immunochemistry 6, 139-49.

Goshi, N., Scott, B.L. and Foster, G.V. (in press): Functional variations and acid phosphatase reactivity in osteoclasts. J. Cell Biol.

Graham-Smith, D. (1968): The carcinoid syndrome. Hospital Medicine, February, 558-66.

Greenwood, F.C. (1969): Discussion on specificity of radio-immunoassay. In: Protein and Polypeptide Hormones, 565. Ed: Margoulies, M., Excerpta Medica International Congress Series No. 161, Amsterdam.

Gudmundsson, T.V., Galante, L., Horton, R., Matthews, E.W., Woodhouse, N.J.Y., MacIntyre, I. and Nagant de Deuxchaisnes (1970): Human plasma calcitonin. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 102-9. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

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, 460-77.

Guttman, St., Pless, J., Sandrin, E., Jaquenoud, P-A., Bossert, H. and Willems, H. (1968): Synthese des Thyreocalcitonins. Helv. chim. Acta 51, 1155-8.

Guttman, St., Pless, J., Huguenin, R.L., Sandrin, E., Bossert, H. and Zehnder, K. (1969): Synthese von Salm - calcitonin, einem hochaktiven hypocalcämischen Hormon. Helv. chim. Acta 52, 1789-95.

Hall, R., Amos, J., Garry, R. and Buxton, R.L. (1970): Thyroid stimulating hormone response to synthetic thyrotropin releasing hormone in man. Brit. med. J. 2, 274-7.

Hamilton, J. (personal communication).

Hanna, S., Harrison, M., MacIntyre, I. and Fraser, R. (1960): The syndrome of magnesium deficiency in man. The Lancet ii, 172-5.

Harris, B.F., Orr, A.H. and Bagshawe, K.D. (1967): Automated radioimmunoassay for HCG and LH. Technicon European Symposium 1967, p. 18. Automation in Analytical Chemistry.

Herbert, V., Lau, K.S., Gottlieb, C.W. and Bleicher, S.J. (1965): Coated charcoal immunoassay of insulin. J. clin. Endocr. Metab. 25, 1375-84.

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, 244-52.

Hirsch, P.F., Voelkel, E.F. and Munson, P.L. (1964): Thyrocalcitonin: hypocalcemic, hypophosphatemic principle of the thyroid gland. Science 146, 412-3.

Hunter, W.M. (1971): The preparation and assessment of iodinated antigens. In: Radioimmunoassay Methods, 3-23. Ed: Kirkham, K.E. and Hunter, W.M., Churchill Livingstone, Edinburgh and London.

Hunter, W.M. and Greenwood, F.C. (1962): Preparation of iodine-131--labelled growth hormone of high specific activity. Nature (Lond.) 194, 495-6.

Hurn, B.A.L. and Landon, J. (1971): Antisera for radioimmunoassay. In: Radioimmunoassay Methods, 121-42. Ed: Korkham, K.E. and Hunter, W.M., Churchill Livingstone, Edinburgh and London.

Jeffcoate, S.L. (1971): Hapten-specificity of antisera to steroid-protein conjugates. In: Radioimmunoassay Methods, 151-5. Ed: Kirkham, K.E. and Hunter, W.M., Churchill Livingstone, Edinburgh and London.

Johnson, C.C. Jr. and Deiss, W.P. Jr. (1966): An inhibitory effect of thyrocalcitonin on calcium release *in vivo* and on bone metabolism *in vitro*. Endocrinology 78, 1139-43.

Jowsey, J. and Simons, G.W. (1968): Normocalcaemia in relation to cortisone secretion. Nature (Lond.) 217, 1277-9.

Kahnt, F.W., Riniker, B., MacIntyre, I. and Neher, R. (1968): Thyrocalcitonin I. Isolierung und Charakterisierung wirksamer Peptide aus Schweineschilddrüsen. Helv. chim. Acta 51, 214-7.

Kalina, M., Foster, G.V., Clark, M.B. and Pearse, A.G.E. (1970): C cells in man. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 268-73. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Kalu, D.N. and Foster, G.V. (1971): Effect of calcitonin on ^{3}H proline incorporation into bone hydroxyproline in the rat. J. Endocr. 49, 233-41.

Kaplan, E.L., Peskin, G.W. and Arnaud, C.D. (1969): Non-steroid, calcitonin-like factor from the adrenal gland. Surgery 66, 167-74.

Keutmann, H.T., Parsons, J.A., Potts, J.T. Jr. and Schleuter, R.J. (1970): Isolation and chemical properties of two calcitonins from salmon ultimobranchial glands. J. biol. Chem. 245, 1491-6.

Knight, R.A., Caldwell, C. and Audhya, T.A. (1971): Automation of a radioimmunoassay for human growth hormone, using the 'Analmatic'. In: Radioimmunoassay Methods, 598-606. Ed: Kirkham, K.E. and Hunter, W.M., Churchill Livingstone, Edinburgh and London.

Korenman, S.G., Perrin, L.E. and McCallum, T.P. (1969): A radioligand binding assay system for estradiol measurement in human plasma. J. clin. Endocr. 29, 879-83.

Kumar, M.A., Foster, G.V. and MacIntyre, I. (1963): Further evidence for calcitonin, a rapid-acting hormone which lowers plasma-calcium. Lancet ii, 480-2.

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, 469-75.

Landon, J., Girard, J. and Greenwood, F.C. (1969): The specificity of a radioimmunoassay for human plasma ACTH. In: Protein and Polypeptide Hormones, 29-31. Ed: Margoulies, M., Excerpta Medica International Congress Series No. 161, Amsterdam.

Leaf, A. (1967): Membrane effects of antidiuretic hormone. Amer. J. Med. 42, 745-56.

Le Douarin, N. and Le Lièvre, C. (1970): Demonstration de l' origine neural des cellules à calcitonin due corps ultimobranchial chez l'embryon de Poulet. C.R. Acad. Sci. D., Paris 270, 2857-60.

Leeksma, C.H.W., De Graaff, J. and DeCock, J. (1957): Hypercalcemia in adrenal insufficiency. Acta med. scand. 156, 455-8.

Lequin, R.M., Hackeng, W.H.L. and Schopman, W. (1969): A radioimmunoassay for porcine calcitonin. J. Endocr. 44, 283-284.

Lefkowitz, R.J., Roth, J. and Pastan, I. (1970): 'Radio Receptor Assay' of ACTH: a new assay for polypeptide hormones. J. clin. Invest. 49, p. 56a.

MacIntyre, I. (1961): Flame photometry. Advances in Clinical Chemistry 4, 1-28.

MacIntyre, I. (1970): Human calcitonin: practical and theoretical consequences. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 1-13. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

MacIntyre, I. (in press): Human calcitonin: physiological and therapeutic use. In: Proceedings of the Fourth Parathyroid Conference, Chapel Hill, 1971. Excerpta Medica, Amsterdam.

MacIntyre, I. (in press): Studies on the effects of human synthetic calcitonin in experimental animals and in man. In: Endocrinology 1971 - Proceedings of the Third International Symposium, Ed: Taylor, S., Heinemann Medical Books Ltd., London.

MacIntyre, I., Parsons, J.A. and Robinson, C.J. (1967): The effect of thyrocalcitonin on blood-bone calcium equilibrium in the perfused tibia of the cat. J. Physiol. (Lond.) 191, 393-405.

McLean, F.C. and Urist, M.R. (1961): Bone: an introduction to the physiology of skeletal tissue. 2nd Edn. The University of Chicago Press, Chicago.

MacManus, J.P. and Whitfield, J.F. (1970): Inhibition by thyrocalcitonin of the mitogenic actions of parathyroid hormone and cyclic adenosine-3'5'-monophosphate on rat thymocytes. Endocrinology 86, 934-9.

Marcus, R., Heersche, J.N.M. and Aurbach, G.D. (1971): Effects of calcitonin on formation of 3'5'-AMP in bone and kidney. Presented at the 53rd Annual Meeting of the U.S. Endocrine Society.

Marquardt, D.W. (1963): An algorithm for least squares estimation of non-linear parameters. J. Soc. Indust. App. Math. 11, 431.

Marquardt, D.W. (1964): I.B.M. Share Programme No. 3094.

Melvin, K.E.W. and Tashjian, J.R. Jr. (1968): The syndrome of excessive thyrocalcitonin produced by medullary carcinoma of the thyroid. Proc. nat. Acad. Sci. (Wash.) 59, 1216-22.

Melvin, K.E.W., Voelkel, E.F. and Tashjian, A.H. Jr. (1970): Medullary carcinoma of the thyroid: stimulation by calcium and glucagon of calcitonin secretion. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 487-96. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Meyer, J.S. and Abdel-Bari, W. (1968): Granules and thyrocalcitonin-like activity in medullary carcinoma of the thyroid gland. New Engl. J. Med. 278, 523-9.

Midgley, A.R. Jr. (1966): Radioimmunoassay: a method for human chorionic gonadotrophin and human luteinising hormone. Endocrinology 79, 10-18.

Midgley, A.R. Jr. and Jaffe, R.B. (1968): Regulation of human gonadotrophins: IV. Correlation of serum concentration of follicle stimulating and luteinizing hormones during the menstrual cycle. J. clin. Endocr. Metab. 28, 1699-1703.

Midgley, A.R. Jr., Niswender, G.B. and Ram, S.R. (1969): Hapten - radioimmunoassay: a general procedure for the estimation of steroidal and other haptenic substances. Steroids 13, 731-7.

Mikhail, G., Wu, C.H., Ferin, M. and Vande Wiele, R.L. (1970): Radioimmunoassay of estrone and estradiol. Acta endocr. (Kbh), Suppl. No. 147, p. 347-56.

Miles, L.E.M. and Hales, C.N. (1968): Labelled antibodies and immunological assay systems. Nature (Lond.) 219, 186-9.

Milhaud, G., Calmettes, C., Raymond, J.P., Bignon, J. and Moukhtar, M.S. (1970): Carcinoide sécrétant de la thyrocalcitonine. C.R. Acad. Sc.(D),(Paris) 270, 2195-8.

Milhaud, G. and Hankiss, J. (1969): Inactivation de la thyrocalcitonine par divers organes. C.R. Acad. Sci. (D),(Paris) 268,

Milhaud, G. and Job, J.C. (1966): Thyrocalcitonin: effect on idiopathic hypercalcaemia. Science 154, 794-5.

Milhaud, G., Pérault, A.M. and Moukhtar, M.S. (1965): Étude du mechanisme de l'action hypocalcemiante de la thyrocalcitonine. C.R. Acad. Sci. (Paris) 261, 813-6.

Milhaud, G., Tubiana, M., Parmentier, C. and Coutris, G. (1968): Epithélioma de la thyröide sécrétant de la thyrocalcitonine. C.R. Acad. Sci. (D), (Paris) 266, 608-10.

Mills, B.G., Haroutinian, M., Bordier, P.J. and Tun-Chot, S. (in press): Effects of porcine calcitonin on the fine structure, morphology and number of bone cells in the rabbit. In: Endocrinology 1971 - Proceedings of the Third International Symposium. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Morain, W.D. and Aliapoulios, M.A. (1968): Comparative hypocalcemic effect of thyrocalcitonin and glucagon. In: Third International Congress of Endocrinology (Abstract 15). Ed: Gual, C., Excerpta Medica International Congress Series No. 157, Amsterdam.

Morgan, C.R. and Lazarow, A. (1962): Immunoassay of insulin using a two-antibody system. Proc. Soc. exp. Biol. (N.Y.) 110, 29-32.

Moseley, J.M., Matthews, E.W., Breed, R.H., Galante, L., Tse, A. and MacIntyre, I. (1968): The ultimobranchial origin of calcitonin. Lancet i, 100-10.

Murphy, B.E.P. (1967): Some studies of the protein-binding of steroids and their application to the routine micro and ultramicro measurement of various steroids in body fluids by competitive protein-binding radio assay. J. clin. Endocr. Metab. 27, 973-90.

Neer, R.M., Parsons, J.A., Krane, S.M., Deftos, L.J., Shields, C.L. and Potts, J.T. Jr. (1970): Pharmacology of calcitonin: human studies. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 547-54. Ed: Taylor, S. and Foster, G.V., London.

Neher, R., Riniker, B., Maier, R., Byfield, P.G.H., Gudmundsson, T.V. and MacIntyre, I. (1968): Human calcitonin. Nature (Lond.) 220, 984-6.

Neher, R., Riniker, B., Zuber, H., Rittel, W. and Kahnt, F.W. (1968): Thyrocalcitonin II Struktur von alpha,Thyrocalcitonin. Helv. chim. Acta 51, 917-24.

Niall, H.D., Keutmann, H.T., Copp, D.H. and Potts, J.T. Jr. (1969): Aminoacid sequence of salmon ultimobranchial calcitonin. Proc. nat. Acad. Sci. (Wash.) 64, 771-8.

Nisbet, J. and Nordin, B.E.C. (1968): Thyrocalcitonin inhibition of bone resorption in tissue culture. In: Proceedings of the Symposium on Thyrocalcitonin and the 'C' Cells, 230-7. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Nordin, B.E.C. and Peacock, M. (1970): The role of the kidney in serum calcitonin homeostasis. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 472-82. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Oates, J.A., Pettinger, W.A. and Doctor, R.D. (1966): Evidence for the release of bradykinin in carcinoid syndrome. J. clin. Invest. 45, 173-8.

Odell, W.D., Reichert, L.E. and Bates, R.W. (1969): Pitfalls in the radioimmunoassay of carbohydrate containing polypeptide hormones. In: Protein and Polypeptide Hormones, Proceedings of the International Symposium, 124-8. Ed: Margoulies, M., Excerpta Medica International Congress Series No. 161, Amsterdam.

Orimo, H., Fujita, T., Ohata, M., Okano, K. and Yoshikawa, M. (in press): Ultimobranchial calcitonin of anguilla japonica: its chemical properties and physiological significance. In: Endocrinology 1971 - Proceedings of the Third International Symposium. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Paloyan, E. (1967): Recent developments in the early diagnosis of hyperparathyroidism. Surg. Clin. N. Amer. 47, 61-9.

Paloyan, E., Paloyan, D. and Harper, P.V. (1967): Glucagon-induced hypocalcaemia. Metabolism 16, 35-9.

Parkinson, D.K. and Radde, I.C. (1970): Calcitonin action on membrane ATPase - a hypothesis. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 466-71. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Parsons, J.A. (1968): Effect of added protein on the apparent potency of thyrocalcitonin. In: Calcitonin - Proceedings of the Symposium on Thyrocalcitonin and the 'C' Cells, 36-41. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Pearse, A.G.E. (1966): The cytochemistry of the thyroid C cells and their relationship to calcitonin. Proc. roy. Soc. B. 164, 478-87.

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. 170, 71-80.

Pearse, A.G.E. (1970): The characteristics of the C cell and their significance in relation to those of other endocrine polypeptide cells and to the synthesis, storage and secretion of calcitonin. In; Calcitonin 1969 - Proceedings of the Second International Symposium, 125-40. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Pearse, A.G.E. and Polak, J.M. (1971, in press): Neural crest origin of the C cells and their comparative cytochemistry and ultrastructure in the ultimobranchial gland. Proceedings of the 4th Parathyroid Conference, Chapel Hill. Excerpta Medica, Amsterdam.

Pors Nielsen, S., Buchanan-Lee, B., Matthews, E.W., Moseley, J.M. and Williams, C.C. (1970): The effect of synthetic porcine alpha-calcitonin on the renal elimination of magnesium, sodium and inorganic phosphate in normal and parathyroidectomised rats. J. Endocr. 48, p. vii.

Potts, J.T. Jr. (personal communication).

Potts, J.T. Jr. (1969): Comparative study of methods of separation. In: Protein and Polypeptide Hormones, 646-9. Excerpta Medica International Congress Series No. 161, Amsterdam.

Potts, J.T. Jr. (1970): Recent advances in thyrocalcitonin research. Fed. Proc. 29, 1200-5.

Potts, J.T. Jr., Niall, H.D. and Deftos, L.J. (in press): Calcitonin. Current Topics in Experimental Endocrinology. Ed: Martini, C. and James, V.H.T., Academic Press, N.Y.

Potts, J.T. Jr., Niall, H.D., Keutmann, H.T., Brewer, H.B. Jr. and Deftos, L.J. (1968): The amino acid sequence of porcine thyrocalcitonin. Proc. nat. Acad. Sci. (Wash.) 59, 1321-8.

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

Potts, J.T. Jr., Sherwood, L.M., O'Riordan, J.L.H. and Aurbach, G.D. (1967): Radioimmunoassay of polypeptide hormones. Advanc. intern. Med. 13, 183-240.

Rasmussen, H. (1961): Parathyroid hormone - nature and mechamism of action. Amer. J. Med. 30, 112-28.

Rasmussen, H., Feinblatt, J., Nagata, N. and Pechet, M. (1970): Effect of ions upon bone cell function. Fed. Proc. 29, 1190-7.

Rasmussen, H. and Pechet, M.M. (1970): Calcitonin. Sci. Amer. 223, 42-50.

Raud, H.R. and Odell, W.D. (1969): The radioimmunoassay of human thyrotrophin. Br. J. Hosp. Med. 2, 1366-76.

Reiss, E. and Canterbury, J.M. (1968): A radioimmunoassay for parathyroid hormone in man. Proc. Soc. expl. Biol. (N.Y.) 128, 501-4.

Reynolds, J.J., Dingle, J.T., Gudmundsson, T.V. and MacIntyre, I. (1968): Bone resorption in vitro and its inhibition by calcitonin. In: Proceedings of the Symposium on Thyrocalcitonin and the 'C' Cells, 223-9. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

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 Charakterisierung. Helv. chim. Acta 51, 1738-42.

Rittel, W., Brugger, M., Kamber, B., Riniker, B. and Sieber, P. (1968): Thyrocalcitonin III. Die synthese des alpha-thyrocalcitonins. Helv. chim. Acta 51, 924-8.

Robertson, D.R. (1970): The role of the amphibian ultimobranchial gland on sites of calcium mobilisation. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 311-9. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Robinson, C.J., Martin, T.J., Matthews, E.W. and MacIntyre, I. (1967): Mode of action of thyrocalcitonin. J. Endocr. 39, 71-9.

Robinson, C.J., Matthews, E.W. and MacIntyre, I. (1968): The effect of parathyroid hormone and thyrocalcitonin on the intestinal absorption of calcium and magnesium. In: Les Tissus Calcifiés: Vth European Symposium, 279-82. Ed: Milhaud, G., Owen, M. and Blackwood, H.J.J., Société D'Edition D'Enseignement Supérieur, 5, Place de la Sorbonne, Paris 5e.

Rosselin, G., Assam, 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, 355-8.

Samols, E.S. and Bilkus, D. (1964): A comparison of insulin immunoassays. Proc. Soc. exp. Biol. (N.Y.) 115, 79-84.

Schwyzer, R. (1969): Relationship of structure to activity of polypeptide hormones. In: Protein and Polypeptide Hormones, 201-7. Ed: Margoulies, M., Excerpta Medica International Congress Series No. 161, Amsterdam.

Selkurt, E.E. (1963): The renal circulation. In: Handbook of Physiology, section 2. Circulation, Vol. II, 1457-1516. American Physiological Society, Washington.

Sieber, P., Brugger, M., Kamber, B., Riniker, B. and Rittel, W. (1968): Die Synthese von Calcitonin M. Menschliches Calcitonin IV. Helv. chim. Acta 51, 2057-61.

Smith, T.W., Bulter, V.P. and Haber, E. (1969): Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay. New Engl. J. Med. 281, 1212-6.

Sjorstrand, T. (1962): Blood volume. In: Handbook of Physiology, Section 2. Circulation, Vol. I., 51-62. American Physiological Society, Washington.

Sturtridge, W.C. and Kumar, M.A. (1968): An improved bioassay for calcitonin. J. Endocr. 42, 501-3.

Talmage, R.V. and Klein, D.C. (1968): Relation of endogenous thyrocalcitonin secretion to plasma calcium levels. In: Calcitonin - Proceedings of the Symposium on Thyrocalcitonin and the 'C' Cells, 261-7. Ed: Taylor, S., Heinemann Medical Books Ltd., London.

Tashjian, A.H. Jr. (1965): Homeostasis of plasma calcium: effects of actinomycin D, parathyroidectomy and thyrocalcitonin. Endocr. 77, 375-81.

Tashjian, A.H. Jr., Bell, P.H. and Levine, L. (1970): Immunochemistry of thyrocalcitonins: species differences and studies of the relationship between structure and immunological activity. In: Calcitonin 1969 - Proceedings of the Second International Symposium, 359-75. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Tashjian, A.H. Jr., Frantz, A.G. and Lee, J.B. (1966): Pseudohypoparathyroidism: assays of parathyroid hormone and calcitonin. Proc. nat. Acad. Sci (Wash.) 56, 1138-42.

Tashjian, A.H. Jr., Howland, B.G., Melvin, K.E.W. and Hill, C.S. (1970): Immunoassay of human calcitonin. New Eng. J. Med. 283, 890-5.

Tashjian, A.H. Jr. and Melvin, K.E.W. (1968): Medullary carcinoma of the thyroid gland: studies of thyrocalcitonin in plasma and tumour extracts. New Eng. J. Med. 279, 279-83.

Tenenhouse, A. and Rasmussen, H. (1968): Parathyroid hormone, thyrocalcitonin and ascites tumour cells. In: Parathyroid Hormone and Thyrocalcitonin (Calcitonin), 392-400. Ed: Talmage, R.V. and Bélanger, L.F., Excerpta Medica, Amsterdam.

Tenenhouse, A., Rasmussen, H. and Nagata, N. (1969): Parathyroid hormone, 3'5'-AMP and Ca^{++}. In: *Calcitonin 1969 - Proceedings of the Second International Symposium*, 418-26. Ed: Taylor, S. and Foster, G.V., Heinemann Medical Books Ltd., London.

Thomas, K., Nash, D. and Ferin, J. (1969): Chemical precipitation techniques for the radioimmunologic quantitation of gonadotrophins with special reference to the organic solvent dioxane. Acta endocr. (Kbh.), Suppl. 142., 279-99.

Toto, P.D. and Magon, J.J. (1966): Histogenesis of osteoclasts. J. dent. Res. 45, 225-8.

Tubiana, M., Milhaud, G., Coutris, G., Lacour, J., Parmentier, C. and Bok, B. (1968): Medullary carcinoma and thyrocalcitonin. Brit. Med. J. 4, 87-9.

Vernon, P. and Glass, H.I. (1971): An off-line digital system for use with a gamma camera. Phys. in Med. Biol. 16, 405-15.

Wells, H. and Lloyd, W. (1968): Possible involvement of cyclic AMP in the actions of thyrocalcitonin and parathyroid hormone. In: *Parathyroid Hormone and Thyrocalcitonin (Calcitonin)*, 332-3. Ed: Talmage, R.V. and Bélanger, L.F., Excerpta Medica, Amsterdam.

Welsch, V. and Perase, A.G.E. (1969): Electron cytochemistry of BuChE and AChE in thyroid and parathyroid C cells under normal and experimental conditions. Histochemie 17, 1-10.

West, T.E.T., O'Riordan, J.L.H. and Care, A.D. (1969): The clearance of homologous calcitonin. J. Endocr. 45, 495-504.

Wide, L. (1969): Radioimmunoassay employing immunoadsorbents. Acta endocr. (Kbh.), Suppl. 142, 207-21.

Wide, L. (1971): Solid phase antigen-antibody systems. In: *Radioimmunoassay Methods*, 405-12. Ed: Kirkham, K.E. and Hunter, W.M., Churchill Livingstone, Edinburgh and London.

Wide, L., Roos, P. and Gemzell, C. (1961): Immunological determination of human pituitary luteinising hormone. Acta endocr. (Kbh.) 37, 445-9.

Williams, C.C., Matthews, E.W., Moseley, J.M. and MacIntyre, I. (in press): The effects of synthetic human and salmon calcitonins on electrolyte excretion in the rat.

Woodhouse, N.J.Y., Reiner, M., Bordier, Ph., Kalu, D.N., Fisher, M., Foster, G.V., Joplin, G.F. and MacIntyre, I. (1971): Human calcitonin in the treatment of Paget's bone disease. Lancet i, 1139-43.

Yalow, R.S. (1969): Incubation damage to labelled porcine calcitonin. In: Proteins and Polypeptide Hormones, 624-6. Ed: Margoulies, M., Excerpta Medica International Congress Series No. 161, Amsterdam.

Yen, S.S.C., Llerna, O., Little, B. and Pearson, O.H. (1968): Disappearance rates of endogenous luteinizing hormone and chorionic gonadotrophin in man. J. clin. Endocr. 28, 1763-7.

Zileli, M.S., Kanra, G., Ürünay, G., Güner, T. and Caglar, S. (1968): Evidences for a hypocalcemic factor from pituitary gland. Experientia 24, 960-1.