CALCITONIN - ORIGIN AND PURIFICATION

A thesis for the degree of
Doctor of Philosophy
University of London

Giraud Vernam Foster
B.S.(Hartford), M.D.(Baltimore)

Postgraduate Medical School
London, W. 12.

Abstract

An introduction to the dynamics of calcium metabolism with special reference to parathyroid hormone is presented in Part I of this thesis Part II, the corpus of original work, relates to calcitonin, the second major hormone involved in calcium homeostasis.

The existence of the plasma calcium-lowering hormone, calcitonin, has been proven by perfusion experiments in the dog and the goat. Evidence is presented to show that it is secreted by the thyroid and not by the parathyroid as previously thought. Histochemical studies on perfused thyroid tissue strongly suggest that it is produced by the "mitochondrion-rich" cells. Bioassays have been developed. The active principle has been extracted and purified a 120 fold by salt fractionation and gel filtration. The hormone is a polypeptide. It is relatively stable to heat, hydrogen peroxide oxidation and cysteine reduction, but is readily inactivated by trypsin or pepsin. Evidence is presented which suggests that the active moiety in crude extracts is associated with larger molecules.

Recent developments in calcitonin research and possible clinical applications of the hormone are discussed.

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PREFACE

The corpus of this thesis is presented chronologically in much the same order in which the work was originally done. Like many projects in unestablished fields the researching of a new hormone entails a unique set of problems which must be solved in sequence. The existence of the hormone must be proved, the origin determined, an assay developed and crude extracts prepared before a definitive attack can be made on its chemistry and mode of action. In generic terms three areas of approach are necessary in the following order: physiological, chemical and structural. These approaches reflect on a small scale the evolution of biochemical thinking which has developed over the past seventy years. It is without apology that much of the work reported in this thesis is of a physiological nature.

To present a more comprehensive picture of endocrine control of calcium homeostasis, a discussion of the general aspects of calcium metabolism with special reference to parathyroid hormone is included in Part I. This review serves as a foil to the work contained in the body of the thesis for three reasons. First, parathyroid hormone is the antagonist to calcitonin. Second, many of the investigators who have worked with this hormone have recently

turned their investigative efforts to calcitonin with the result that previous concepts of parathyroid control of calcium are undergoing revision. Third and finally, much of the research conducted on calcitonin closely parallels that of parathyroid hormone. The design of a few of the physiological experiments, some of the bioassay techniques and many of the purification procedures are common to both hormones. Much of the work done with parathyroid hormone over a sixty year period has been applied to calcitonin during the past two years.

That so much could be accomplished in so short a period of time is due largely to the genius of my supervisor, Dr. Iain MacIntyre. Under his aegis the project has never been without well defined direction. Experimentation peripheral to central problems has been scrupulously avoided. I am most grateful for his enthusiasm in times of despair, his caution in the face of success, and his friendship throughout my association with the project which has made my stay at the Postgraduate Medical School as pleasant as it has been rewarding.

My debts to others are too numerous to be completely recorded. I am particularly indebted to Professor Russell Fraser, who, remembering my wish to do thyroid perfusions which I had proposed five or six years ago, suggested that I work with Dr. MacIntyre.

I am most thankful for my association with Professor A.G.E. Pearse, who, with patience, has taught me much about histochemistry. I am grateful for the many kindnesses conferred on me by Professor E.C. Amoroso, F.R.S., who made possible much of the experimental work on larger animals.

To my immediate colleagues, I am indebted for their help and advice. The dog perfusion and bioassay studies were done in collaboration with Dr. M.A. Kumar and the studies of the effect of pH and salt concentration on crude extract with Dr. A. Baghdiantz. I am also indebted to Professor Pearse and his technicians for the preparation of the histochemically stained material.

Additionally, I would like to thank Miss Ella Slack, Miss Ann Edwards and Mr. Andre De Bats for their technical assistance, Mr. N.W. Please for his advice on the preparation of statistical material, Miss Dorothy Atkins for checking the bibliography, Miss Margaret Johnson for much of the photography, Miss Sonia Hills for drawing the illustrations and Miss Jill Francis for the preparation of the manuscript. Finally, I would like to acknowledge with gratitude the personal financial support given me by the Life Insurance Medical Research Fund and the American Heart Association.

PART I

CALCIUM HOMEOSTATIS AND THE ENDOCRINES

Calcium Homeostasis and the Endocrines

The first section of this review encompasses a brief discussion of the physiological and biochemical aspects of calcium absorption and excretion, internal calcium dynamics within the body, together with a descriptive commentary on the structure of bone. The second section surveys the field of parathyroid hormone research and includes a summary of pertinent historical aspects, assay systems, purification methods, studies of properties and techniques used in the elucidation of the modes and sites of action. Finally, brief mention is made of the understanding of control mechanism of calcium regulation prior to the present work.

This review is intended to serve as an introduction to the main body of this thesis, which describes the confirmation, elucidation of origin, purification and preliminary description of the properties of calcitonin, the second major hormone involved in calcium homeostasis.

GENERAL CONSIDERATIONS OF CALCIUM DYNAMICS

Calcium comprises 2 percent of the human body weight and is the most common cation. 99 percent is found in the bony skeleton where much of it is in dynamic equilibrium with the remaining 1 percent in soft tissue and blood (Rasmussen and Reifenstein, 1962). The equilibrium is one of an unsaturated solution of calcium ions in contact with a solid phase system regulated so that plasma levels are usually maintained within the range of 4.90 - 5.40 mEq/l. Control of blood levels is necessary for carrying out the many functions in which calcium participates.

In addition to its role in the formation of the endoskeleton and teeth, calcium is required for the proper maintenance of cell permeability, blood coagulation, cardiac rhythmicity, excitability of nerves, muscle contraction and many important enzyme reactions. The calcium blood level maintained to facilitate these functions is regulated with precision despite wide variations in dietary intake, utilization by bone growth and output during pregnancy and lactation. The secretions of the endocrine glands are in large measure responsible for the exacting process of homeostasis.

DYNAMICS OF CALCIUM METABOLISM

Absorption and Excretion

Figure 1 summarizes schematically the external dynamics of calcium metabolism in man (Rasmussen and Reifenstein, 1962). The amount of calcium in the diet is widely variable and exceeds many fold the amount absorbed by the gastro-intestinal tract. Despite variations in diet renal excretion of calcium remains relatively constant, suggesting that calcium absorption is well regulated. Absorption is increased by parathyroid hormone if sufficient Vitamin D is present to permit its action. Increased parathyroid hormone production is found with low calcium diet, periods of rapid skeletal growth or excessive loss of calcium during pregnancy or lactation. These conditions, by tending to lower plasma calcium, stimulate the parathyroids to secrete.

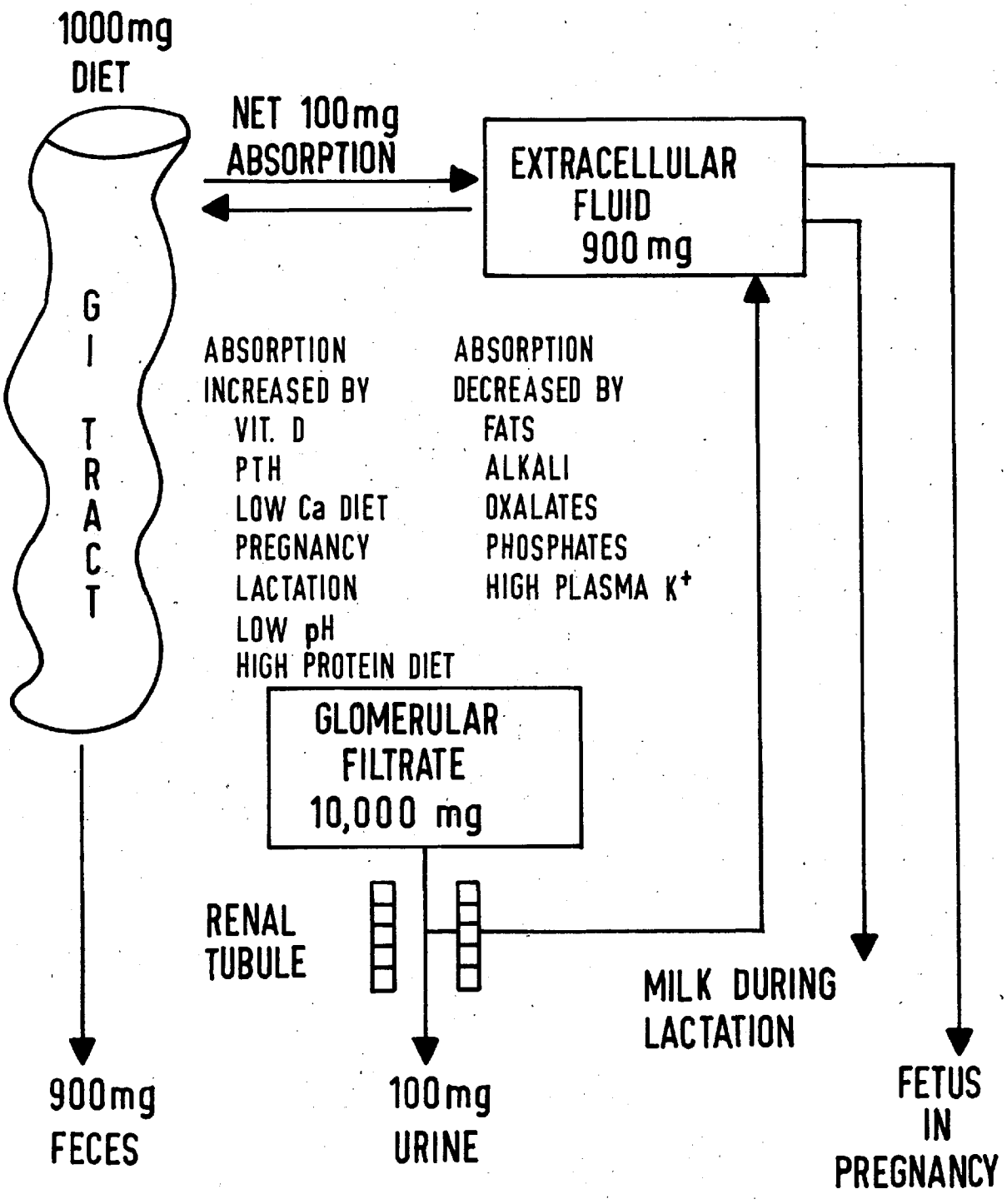


Figure 1. External calcium dynamics.
(Adapted from Rasmussen and Reifenstein, 1962).

Absorption of calcium is also increased under acid conditions which permit greater solubility of calcium salts and with excessive lactose which may form a soluble calcium-lactose complex (Charley and Saltman, 1963). High protein diet further facilitates calcium absorption (Lengemann, Comar and Wasserman, 1957). Prevention of absorption is found under those circumstances such as diets high in fats or alkali, which promote the formation of insoluble soaps. Oxalates and phosphates in high concentrations are also responsible for the formation of insoluble complexes.

Calcium is lost from the body primarily in the feces as a result of non-absorption or active secretion into the intestinal lumen. Much smaller amounts are lost in the urine. Approximately 10 g of calcium are handled by the tubules a day of which 99 percent is reabsorbed under conditions of normocalcemia (Jahan and Pitts, 1948; Chen and Neuman, 1955; Poulos, 1957). Calcium in the urine diminishes to almost negligible quantities when plasma values fall below 3.8 mEq/l. Conversely, hypercalcuria occurs when excess calcium is added to the extracellular pool from bone (Copp, McPherson and McIntosh, 1960).

Internal Calcium Equilibrium

Factors involved in the internal dynamics of calcium metabolism are schematically represented in Figure 2 (Rasmussen and

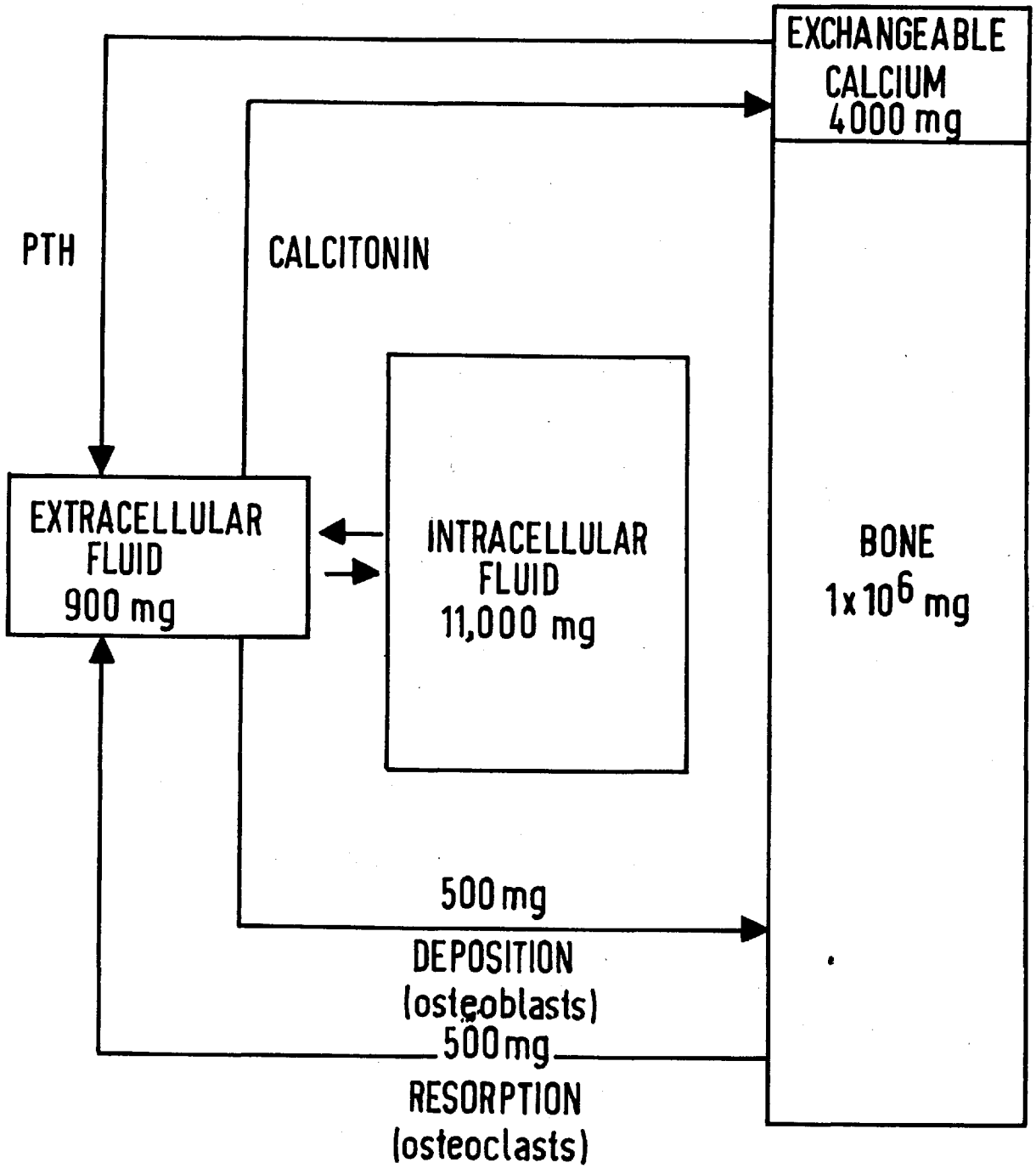


Figure 2. Internal calcium dynamics.

Reifenstein, 1962). Of the 900 mg of calcium in the extracellular fluid, about 250 mg are in the plasma where the concentration of calcium is approximately 40 percent greater. The increased concentration can be explained on the basis of binding to plasma proteins. Only 65 percent of the plasma calcium is freely diffusible: 52 percent is ionic calcium and 13 percent is calcium complexed with citrate, carbonate, phosphate and possibly sugars and some amino acids. Of the remaining 35 percent approximately 7 percent is bound to globulins and 27 percent to albumin. Aberrations in serum proteins, especially albumin, can readily cause changes in the total calcium value in plasma, while not affecting the concentration of ionic calcium. Ionic calcium concentration determines the regulation of the functions of calcium including the growth and maintenance of bone. The exchangeable calcium in bone and the calcium in the extracellular fluid pool are in continual interchange. The kinetics of this flux are under hormonal control by the parathyroids and the thyroid. Not only are these endocrine glands responsible for homeostasis of ionic calcium, but ionic calcium is responsible for the regulation of the secretion of their respective hormones.

Any discussion of ionic calcium must take into consideration the three common calcium complexing ions, $HPO_3^{=}$, citrate and HCO_3^{-}. Changes in concentration of these can affect the concentration

of calcium. Bone contains approximately 70 percent of all citrate in the body and 90 percent of all $HPO_4^{=}$. Factors which affect calcium movement in bone produce similar changes in these ions as well. Minor alterations of their respective solubility product coefficients caused by hormones or other agents may play an important part in shifts between the extracellular fluid compartment and bone.

Bone

Chemically the dry weight of bone consists of approximately 65-70 percent crystalline hydroxyapatite. The remaining 30-35 percent is organic matrix composed of 95 percent collagen and 5 percent proteins and carbohydrates and polymers of these with hexosamines and glucuronic acid. Studies on the ultrastructure using electron microscopy and low angle X-ray diffraction have shown collagen to be a rigid rod-shaped molecule approximately 2800A^{o} long and 14A^{o} wide. It is composed of three polypeptide chains (Gross, 1964). One of these chains most probably differs from the other two. Characteristically, collagen in all animal species contains a constant 33 ± 5 percent glycine and approximately 25 percent of the amino acids, proline and hydroxyproline (Gross, 1963). The probable presence of large numbers of γ-glutamyl peptide linkages may be an important determination in the secondary and tertiary structure. The three

polypeptide chains are left-handed helices oriented to one another in a right-handed superhelix.

Reconstitution studies have demonstrated that collagen in such a configuration is capable of initiating enucleation of crystals of hydroxyapatite. While the mechanics are not understood it has been speculated that the positively charged ϵ-amino groups of lysine may initiate crystallization by attracting negatively charged phosphate ions (McLean, 1958). Electromicrographs have shown these crystals are small rods or hexagonal prisms which are evenly distributed over the surface of the collagen frame. The configuration of the crystals is such that a high surface area is presented. Estimates up to a hundred acres for bone crystal surface have been made for man (Copp and Shim, 1963). All this surface is not in free equilibrium with the extracellular fluid. As crystals of hydroxyapatite form on the collagen, they displace the water molecules which make up the sphere of hydration surrounding individual fibrils. As the crystal population increases and the sphere of hydration decreases, the facility with which ions can enter is impaired and crystal formation stops. The structural characteristics of the relationship of hydroxyapatite to collagen account for why less than 1 percent of bone composes the exchangeable calcium pool. Presumably only those collagen fibrils which do not have their full complement of crystals are capable of presenting surfaces upon which exchange can take place. Conditions

which interfere with either collagen synthesis, the (Ca^{++}) (HPO_3^{-}) solubility coefficient or mucopolysaccharide formation will likewise affect hydroxyapatite deposition and hence bone formation.

The collagen molecules are longitudinally oriented within the basic microscopic unit of bone, the osteon or haversian system. The osteon is an irregular cylindrical structure about 150 microns in external diameter with branching surface (McLean, 1958). Within its centre is a narrow lumen, the haversian canal, which has its own microscopic blood supply. Branching from the haversian canal is a complex system of multiple interconnecting canalicules. These serve as the circulatory system connecting the central haversian canal with all the lacunae, the concentrically located housing units for the bone cells. The bone cells, called osteocytes, are of two varieties: osteoblasts and osteoclasts, both of which may have their origin from a common cell. Osteoblasts are involved in new bone formation especially in the process of laying down new collagen. Osteoclasts are involved in bone resorption. In non-growing bone the rates of new bone formation and resorption are equal. Hormonal factors which have an anabolic or catabolic effect on cells likewise affect the osteocytes and influence the kinetics of bone growth. Not to be forgotten are the changes in blood vessels, whose capillaries supply nutrition to the osteons. Changes in capillary permeability and aberrations in the constituents of blood itself also affect bone.

Microscopically bone can be divided into two types: (1) highly calcified, metabolically inactive cortical bone, which composes the diaphysis and cortex of long bone, and (2) the more metabolically active, less highly calcified spongiosa, made up of spicules called trabeculae, which composes the metaphysial portion. Both the external surface of the cortex of long bones and each trabecula is surrounded by an endosteal membrane. This membrane is highly cellular and is composed of osteoblasts which may play an important role under normal hormonal influence in regulating the (Ca^{++}) (HPO_3^{-}) solubility coefficient in bone.

As shown in Figure 2, two hormones effect the interchange of ionic calcium between the extracellular fluid and the exchangeable calcium pool in bone. The calcium mobilizing kinetics of parathyroid hormone are well on the way to being resolved. In the case of calcitonin, the plasma calcium-lowering hormone, the mechanisms involved have not been established but it is reasonable to assume that it promotes calcium deposition in bone.

CALCIUM MOBILIZING PARATHYROID HORMONE

To avoid confusion with other possible parathyroid factors, the accepted abbreviation, PTH, for parathyroid hormone, will hereafter be used for convenience to designate the calcium-mobilizing principle. Many excellent reviews have been written on this hormone

(Dragstedt, 1927; Albright, 1941; Greep, 1948; Bartter, 1954; Greep and Kenny, 1955; Irving, 1957; Neuman and Neuman, 1958; Kenny, 1960; Munson, 1960; Gerschwind, 1961; Rasmussen, 1961; Munson, 1961; Rasmussen and Reifenstein, 1962; Munson, Hirsch and Tashjian, 1963; Tashjian and Munson, 1963; Rasmussen and DeLuca, 1963; Fraser and King, 1964) to which the reader is referred for a more comprehensive appreciation of the subject.

Historical Aspects

The discovery of the parathyroids has been credited to Owen, who noted them during a post-mortem dissection of an Indian rhinoceros in 1862. They were subsequently rediscovered by Sandström in 1880, who described their occurrence in the dog, cat, rabbit, horse and ox and gave them their name. In Sandström's original description he noted only the internal parathyroids. Not until 1892 did Gley show that a second pair of external parathyroids existed. In the interim period a number of investigators had noted that tetany resulted in the dog when the entire thyro-parathyroid apparatus was removed. In these experiments it was thought that tetany was due to the removal of thyroid tissue. Gley, working with rabbits, showed that the thyroids together with their adjacent internal parathyroids could be removed without symptoms. However, if the external parathyroids, which are anatomically some distance from the

thyroid in the rabbit, were also removed, tetany followed. Vassale (1900; 1901) confirmed the observation in dogs and cats. In these species both internal and external parathyroids are found in close proximity to the thyroid. Previously, Welch (1898) had reported the first histological description of the parathyroid showing conclusively that they were anatomically distinct from thyroid tissue and Groschnuff (1896) had described their embryological development from the dorsal corners of the third and fourth pharyngeal pouches.

In a series of brilliant papers Erdheim (1903, 1904, 1906, 1911 and 1928) later demonstrated the relationship of the parathyroids to calcium metabolism and bone disease. He showed that following parathyroidectomy in the rat defects occurred in the skeleton and in the enamel of teeth. He also noted serum calcium aberrations in a patient with osteomalacia which preceded parathyroid enlargement. From these observations he deduced that (1) the parathyroids were responsible for the secretion of a hormone involved in the calcification of bone and (2) serum calcium levels affected parathyroid function. In 1904 Askanaszi described for the first time a patient at autopsy with a parathyroid adenoma and osteitis fibrosa, but failed to comment on the possible relationship.

Pashon and Urechie in 1907; MacCallum and Voegtlin in 1908; Frouin in 1909 and Luckhardt and Goldberg in 1923 all observed that

tetanic convulsions in parathyroidectomized animals could be prevented by the administration of calcium salts. A most excellent review of early physiological experiments is given by MacCallum (1912) in which the following classical description of a dog in tetany appears.

> If we parathyroidectomize an animal, symptoms appear only after a lapse of a day or two....Its effects become evident in the form of muscular twitchings over the whole body, a curious fibrillory quavering of the muscles of the tongue, tonic contractions of the facial muscles, which distort the face, or the laryngeal muscles which produce a stridorous breathing and of all the body which stiffen the limbs and the trunk and render the animal awkward and helpless. With this rigidity come more and more violent clonic convulsions of all the muscles - the jaws snap, biting the tongue and the whole body is thrown into the most intense spasms....These are the most striking symptoms but there are also sensory disturbances, parasthesias and painful disturbances of the alimentary tract, of the vasomotors etc., as well as underlying cachexia which may lead to the death of the animal. In watching these symptoms one is impressed that they arise from some disturbance of the nervous system.

To substantiate this idea MacCallum subsequently (1911-1912) extended his earlier work in dogs and demonstrated that blood from a dog in tetany transfused into a normal healthy dog could not produce tetany in the recipient animal. This was an important advance which rectified the misconception that tetany was caused by a toxin circulating in the blood. He showed tetany was due to hyperexcitability of peripheral nerves which were dependent upon a change in the character of the blood. This was demonstrated by two classical

experiments. In one he proved that tetany did not appear in the leg of a dog if the nerves had been previously cut. In the second experiment he isolated the leg of a normal dog with the exception of the nerve supply, which was left intact. The vessels of the isolated limb were then perfused with the blood of a dog in tetany and the characteristic twitching of tetany was observed in the isolated preparation.

At the same time that calcium changes were being studied an appreciation of the effect of parathyroid control of phosphate grew out of the work of Greenwald (1911). Greenwald showed that blood phosphate increased and urinary phosphate decreased in parathyroidectomized dogs.

Low calcium as a stimulus for parathyroid hyperfunction was suggested by the experiments of Marine in 1914. He observed parathyroid hypertrophy and hyperplasia in birds maintained on prolonged calcium deficient diets. A few years later Schlagenhaufer (1916) and Maresch (1916) suggested that enlargements of the parathyroids might not only be caused by aberrations of calcium but might also be their cause. Hoffheimz (1925) gave further credence to this concept by describing the first post-mortem case of primary hyperplasia. Application of this idea in 1925 by Mandl in Germany resulted in the first clinically diagnosed case of a parathyroid adenoma

successfully treated surgically. With the advent in the 1920's of improved procedures for the determination of blood calcium, the number of reported cases grew rapidly so that by 1937 Albright was able to report on thirty-five operated patients. The relationship of tetany to ionized and protein bound calcium was established by McLean and Hastings (1935). In 1937 Petersma reported on the first patient diagnosed as having a hyperfunctioning parathyroid carcinoma. Numerous other clinical observations followed, including the observation of the frequent occurrence of peptic ulcer with primary hyperparathyroidism by Rogers in 1946.

Concurrent with the development of improved analytical techniques for the measurement of blood calcium began a wave of interest in the physiological effects. Collip and his collaborators are generally credited as being the first to prepare parathyroid extracts with calcium-mobilizing activity. Certainly their work stands out as the most exacting of the earlier investigators. A number of workers in the few preceding years, however, had reported calcium-raising activity in a variety of preparations. As early as 1913 MacCallum and Vogel had prepared an extract which alleviated tetany in both patients and dogs. These extracts were exceedingly crude having been prepared by grinding bovine or dog parathyroid tissue in a mortar with sand and Ringer's solution.

Interestingly, these extracts were effective when given intravenously or intramuscularly, but not when taken orally. These observations in retrospect show the polypeptide nature of the hormone and its inactivation by peptidases in the stomach. Grove and Vines (1922) used a preparation made for them by Parke Davis and Company for the treatment of calcium deficiency, which they believed was the cause of chronic infections. Unfortunately no description of the method of preparation was given. The following year Berman (1923-1924) prepared a crystalline material from an acid alcohol extract of bovine parathyroid glands which had hypercalcemic activity. Most likely the crystals observed were those of sodium chloride. Acid alcohol normally inactivates parathyroid hormone (Tweedy and Torigoe, 1932) but might not totally inactivate crude preparations. Hanson (1924 a, b, c) without doubt did prepare active extracts before Collip. He extracted minced glands with boiling hydrochloric acid and recovered activity after filtration by either evaporation or precipitation with phosphotungstic acid. The preparation called Hydrochloric X Sicca, when given intramuscularly or intravenously, successfully prevented tetany in thyro-parathyroidectomized dogs. Much of Hanson's work is tarnished by his claim to have discovered a general panacea to cure everything from ulcers to pruritis ani.

Collip published his classical papers on extracts in 1925. Like Hanson he prepared a hot hydrochloric acid digest of bovine parathyroid tissue (Collip, 1925). After filtration the crude extract was cooled, made slightly alkaline and subjected to two successive acid precipitation steps. A single injection of this extract was found to raise the serum calcium in a normal dog to a maximum in 5 - 9 hr. (Collip, Clark and Scott, 1925). A dose response curve was derived upon which an assay was developed using dogs (Collip and Clark, 1925). This assay is still the official assay in the U.S. Pharmacopia (1962).

It remained for Tweedy and his collaborators to clarify and enlarge upon the findings of Collip. These workers (Tweedy and Chandler, 1929) corrected the erroneous impression (Collip, Clark and Scott, 1925) that parathyroid extracts were equally active in intact and parathyroidectomized animals. They showed that parathyroidectomized rats were more sensitive to extracts within the first 25 days following operation. Use of this observation was made by later investigators to develop further assay systems. Tweedy also contributed to purification of parathyroid hormone. He showed that hydrochloric acid digests retained more activity if they were heated but not boiled (Tweedy, Smullen and Bell, 1936), that much of the inactive protein in acid digests could be precipitated with acetone (Tweedy, 1930) and that the activity remaining could be recovered by a trichloracetic acid precipitation.

In addition he noted that partially purified hormone was readily soluble in 90-100 percent phenol (Tweedy, 1930). Phenol has subsequently been almost universally adopted in preliminary extraction procedures since Aurbach first began using it in 1953.

Biological Assays

The great strides in PTH research during the past decade have been made possible by the development of feasible assay systems. The original assay procedure developed by Collip and Clark (1925) was exceedingly cumbersome and required the use of a minimum of ten dogs. The definition of a unit as one-hundredth of that amount of PTH which raises the blood calcium 1 mg/100 ml plasma in 16-18 hr. in ten consecutive dogs has been officially adopted by the U.S. Pharmacopia (1962) and is internationally accepted. Since most investigators using this method have found it necessary to wait a week between assay trials, a sizeable kennel must be maintained to carry out even a few assays a week. The adoption of the rat as an assay animal (Davies and Gordon, 1954; Tepperman, L'Heureux and Wilhelmi, 1947) was a major improvement.

Most systems used today in assaying biological activity are based on the rigorously controlled studies of Munson and his colleagues (Munson, Kenny and Iseri, 1953; Munson, 1955). These investigators used 50-60 day old male rats, maintained on a low calcium diet for four

days prior to assay trial. U.S.P. standardized PTH in varying concentrations from 5-20 units and unknown PTH were administered subcutaneously to different groups of rats immediately after parathyroidectomy by hot wire cauterization. After 6 hr. blood was drawn by cardiac puncture and plasma calcium measured. A modification of this procedure (Kenny, Vine and Munson, 1954; Kenny and Munson, 1959; Munson, 1961) made it possible to assay the phosphaturic effect of PTH instead of its calcium-mobilizing property. Groups of parathyroidectomized rats were kept in metabolic cages after subcutaneous injection of PTH and urine was collected over a 6 hr. period. The pooled collections from each group were assayed for inorganic phosphate. In comparative studies between intact and parathyroidectomized animals, greater sensitivity was observed in the operated animals. In contrast to the assay based on the calcium-mobilizing property of PTH, animals maintained on a normal stock diet prior to assay exhibited a far more sensitive phosphaturic response to PTH than animals on a low calcium diet (Kenny and Munson, 1955).

A modification of the Munson technique by MacIntyre (MacIntyre, Boss and Troughton, 1963) led to a still greater improvement. Parathyroid extracts were slowly infused over a 1 hr. period into the caudal veins of fasted parathyroidectomized rats. Urine from

each rat was collected and assayed for inorganic phosphate. Using this method concentrations of PTH as low as 0.5 units/ml have been detected. Almost as great sensitivity has been reported recently (Lemon, 1962) using a technique measuring urinary P/Ca ratios in intact fasted Swiss mice injected subcutaneously. Animals were first hydrated by subcutaneous injections of saline. Both the latter assays appear to achieve somewhat better results because of forced hydration of the test animals to insure adequate urinary outputs.

A number of other biological assays have been described in the recent literature (Thieblot, Simonnet and Bataille, 1954; Iwanowska, Deptuła and Symk, 1956; Polin, Sturkie and Hunsaker, 1957; Clark, Bowers and Geoffroy, 1960; Dulce, Bauditz and Siegmund, 1961; Mouzas and Weiss, 1961) using similar techniques, but none has been universally accepted. All biological assays have proved laborious because of the need to stock large colonies of animals and perform parathyroidectomies to obtain maximum sensitivity to PTH. Inherent in all these methods are two further problems which need consideration. First, in assays using routes of administration other than intravenous, the suspending vehicle exerts a profound effect (Rasmussen 1959; Tashjian and Munson, 1963). Rasmussen (1959) found extracts exhibited maximum action

relatively rapidly if given in saline. If the same extract was suspended in either sesame oil, heparin or gelatine, the effect was not only prolonged but the hypercalcemic response was noticeably greater. Secondly, the rate with which PTH is absorbed from an injected site is related to its degree of purity. Early work on the purification of PTH, using dog assays (Rasmussen and Westall, 1956), demonstrated that the relatively crude extracts showed maximum activity after 16-18 hr., partially purified material had maximum activity after only 6 hr. and no activity at 18 hr. The most probable explanation for this discrepancy is the elimination of proteins associated with PTH, which delay absorption (Reichert and L'Heureux, 1961). In summary, at least five factors influence the responses observed in bioassays: species and strain variations, degree of hydration of assay animals, route of administration, suspending vehicle and purity of extract.

Currently several new approaches to the problem of PTH assay are being explored. Berson and his colleagues (Berson, Yallow, Aurbach and Potts, 1963; Berson, Yalow, Glick and Roth, 1964; Tashjian, Levine and Munson, 1962; Tashjian and Munson, 1963) have been investigating methods of immunoassay. The latter investigators claim that their method is capable of assaying as little as 24 mμg (84 mU/ml) with an index of precision of 0.11 compared

with earlier techniques (Munson, Kenny and Iseri, 1953; Munson, 1955) of 0.23 $\pm$ 0.5 in which calcium was measured after parathyroid extracts were administered to parathyroidectomized rats. A second approach being investigated by Aurbach, Houston and Potts (1964) appears more immediately promising. These investigators, using in vitro systems, have shown that PTH stimulates the degradation of succinate to carbon dioxide in rat liver mitochondria. The effect was found to be highly specific for PTH, not dependent on ATP in the presence of oligomycin, and to be brought about by the oxidation of reduced pyridine nucleotide. By measuring carbon dioxide produced from succinate in the presence of PTH, they have developed an assay which is capable of detecting amounts of PTH as low as 0.2 units/ml.

Further development of these latter two techniques may make current biological assays obsolete. The development of an assay for PTH has closely paralleled the development in the purification of the hormone itself. Each step forward in one area has made possible the next step in the other.

Purification and Properties

The first major advance in the purification of PTH since the initial extraction by Collip and Clark (1925) was that of Ross and Wood in 1942. These investigators prepared extracts with 2-3 times

the potency of the original extracts by fractionation of a hydrochloric acid digest of beef parathyroid glands first with alcohol, then with ammonium sulphate and, finally, by absorption of the active principle on benzoic acid. This method was followed by notable improvements by L'Heureux and his collaborators (L'Heureux, Tepperman and Wilhelmi, 1947) who found that more potent preparations could be made using acetone fractionation if parathyroid tissue was first adequately defatted. Subsequently, different extracting agents were tried on acetone dried parathyroid tissue. Aurbach (1953) achieved marked success using 90 percent W/V phenol coupled with a trichloroacetic acid precipitation step, Davies and Gordon (1955) using 80 percent V/V acetic acid and Kenny and his collaborators (Kenny, Rosenberg and Beutner, 1956) with cold 0.1-N hydrochloric acid.

In the years that followed a succession of separation methods were tried which made use of differences in charge and size of molecular species. A 4-5 fold increase in activity over the crude extract was achieved by Davies and Gordon (1955), using ultrafiltration through a Visking membrane. Rasmussen (1957) tried zonal electrophoresis on polyvinyl chloride and obtained enough material to estimate the molecular weight of PTH as 10,000 by equilibrium centrifugation. The Harvard group obtained partial

purification by using a combination of an ammonium sulphate fractionation procedure coupled with gradient elution with sodium chloride of the active principle from a carboxymethyl cellulose column (Friedman and Munson, 1958a; Friedman and Munson, 1958b; Munson, 1959). A notable advance was made in 1959 when Rasmussen and Munson independently reported that partially purified PTH lost activity through oxidation and that this activity could be partially restored by reduction with cysteine. Phenol extractions followed by trichloroacetic acid precipitation and countercurrent distribution fractionation yielded sufficient material to re-estimate the molecular weight at 9,500 on the basis of both amino acid composition and sedimentation rate. PTH was found to be a single chain molecule, since it contained no cystine and had a single N-terminal amino acid, alanine (Rasmussen and Craig, 1961a). The following year an alternate method of purification was worked out using Sephadex G-50 with ammonium acetate pH 4.72 as eluant (Rasmussen and Craig, 1962b).

At the present writing excellent purification results are being obtained by Rasmussen, Sze and Young (1964). These investigators are extracting acetone dried glands with 8$\underline{\underline{M}}$-urea - 0.2$\underline{\underline{N}}$-hydro - chloric acid - 0.1$\underline{\underline{M}}$-cysteine. Inactive proteins are precipitated with an acetone-acetic acid-sodium chloride mixture and the activity recovered by ether precipitation. Further inactive material is

removed by a second precipitation with sodium chloride. The supernatant is adjusted with a 45 percent solution with trichloroacetic acid. The resulting precipitate is washed with ether, passed through an Amberlite IRA 400 column, lyophilized and applied to a Sephadex G-100 column using ammonium acetate, pH 4.7, as eluant. Five peaks are obtained of which the fourth contains PTH activity. Two points of evidence suggest that the final product is still not homogeneous. First, acid hydrolysis and amino acid analysis have shown the presence of one mole of cysteine per mole of PTH. Second, immunodiffusion techniques, using antisera prepared from the trichloroacetic acid powder, have shown multiple precipitation lines (Aurbach and Potts, 1964).

More recently Potts and Aurbach (1965) have further improved on the Rasmussen technique. They have applied the unlyophilized fourth peak obtained from Sephadex G-100 (Aurbach and Potts, 1964) to a carboxymethyl cellulose column. The gradient was developed with ammonium acetate 0.01M, pH 5.3, to 0.33M, pH 7.0. Four peaks were obtained of which the third, or main peak, contained all the activity. From electrophoretic and immunodiffusion techniques they have estimated that their final product is 95 percent pure.

Further studies on their purified PTH (Aurbach and Potts, 1965; Potts and Aurbach, 1965; Potts, Aurbach and Sherwood, in press)

have shown that it is a single chain polypeptide with a molecular weight of approximately 9,000 by sedimentation rate determinations, composed of 75 residues containing seventeen common amino acids and no cysteine (See Figure 3). Digestion with carboxypeptidase removed at least fourteen amino acids from the COOH-terminal portion of the molecule and leucine aminopeptidase at least an additional thirteen more from the NH_2-terminal end. The remaining central core was found to be still biologically active. Cleavage with cyanogen bromide to convert methionine to homoserine did not result in loss of activity, demonstrating that methionine (not found in the central core) was not necessary for PTH's biological effects. This latter finding was not in agreement with previous work (Rasmussen and Craig, 1962; Tashjian, Ontjes and Munson, 1964). Cleavage with trypsin, however, resulted in complete inactivation. The purified PTH was shown to have two bands on disc electrophoresis, both of which had plasma-calcium-raising activity and similar amino acid compositions.

The recent developments in the field of purification have already been of great assistance in clarifying the problem of the site and mode of action of the hormone. Further exciting developments and re-appraisal of older work can be looked forward to in the near future.

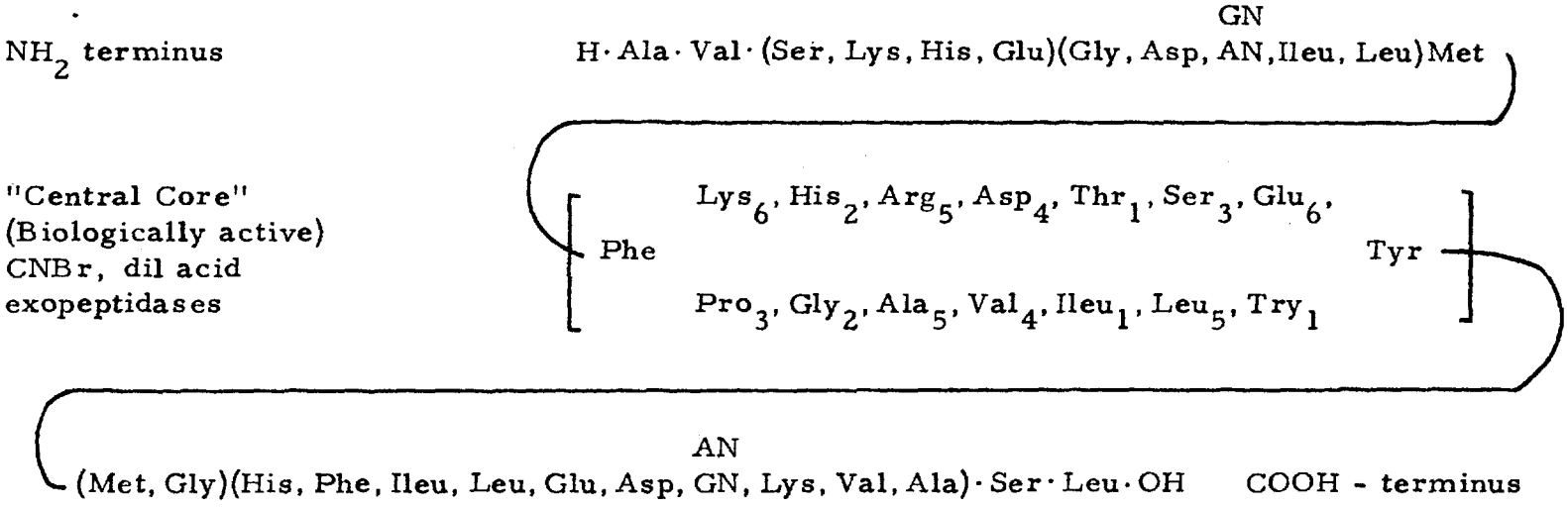


Figure 3. Incomplete amino acid sequence of parathyroid hormone showing biologically active central core. (Potts and Aurbach, 1965).

Sites of Action

PTH has a primary effect on bone, kidney and gut. The action on bone and kidney have been recognised for some years, but it was bitterly and emphatically debated as to whether the renal effects observed were secondary to bone changes (Albright and Ellsworth, 1929; Albright, 1941) or bone changes secondary to renal effects (Collip, 1925; Thompson and Collip, 1932. See also Albright and Reifenstein, 1948; Bartter, 1954). In retrospect it is comparatively easy to select a number of classical experiments which clearly show the effect on each of these systems is primary.

a) Bone: The case for direct PTH action on bone is now indisputable. Barnicot (1948), Chang (1951) and Gaillard (1955, 1961) have independently demonstrated that parathyroid tissue cultured in direct contact with bone results in both bone resorption and an increase in the observable osteoclast count. Rather more indirectly Raisz (1963) has shown that tissue fluid from parathyroid cultures under low calcium stimulation when placed in contact with bone fragments causes bone resorption, whereas fluid from parathyroid tissue cultured under high calcium conditions does not. This would suggest that these changes are due to a direct action of the hormone on bone rather than to a non-specific effect. Equally dramatic

have been in vivo experiments carried out in nephrectomized animals. Grollman (1954) working with dogs and Stoerk and Talmage and his collaborators (Stoerk, 1943; Talmage, Elliot and Enders, 1957) working with rats have clearly demonstrated that in nephrectomized animals, whose blood chemistries are controlled by intraperitoneal lavage, PTH increases blood calcium. They concluded from these experiments that the presence of the kidneys was not necessary for PTH action. Paralleling this work it was later found that parathyroidectomy in the rat reduced the osteoclast count in femurs and that the osteoclast count could be restored to normal by treatment with parathyroid extracts (Ingalls, Donaldson and Albright, 1943; Toft and Talmage, 1960). Finally a number of isotope experiments using both radiocalcium and radiophosphorus systems have conclusively shown that parathyroid extracts effect bone in vitro (Laskin and Engel, 1956; Laskin and Engel, 1960; Lekan, Laskin and Engel, 1960; Cohn and Forscher 1962; Lengemann, 1963; Egawa and Neuman, 1963; Cohn, 1964) as well as in vivo (Talmage, Lotz and Comar, 1953; Talmage, Toft and Buchanan, 1959; Jeffay and Bayne, 1964).

The mechanism by which PTH acts on bone is not known. An interesting hypothesis was proposed by Neuman (Neuman, 1956; Neuman and Dowse, 1961) who proposed that PTH acted by stimulating

the bone cells to produce organic acids. The change in local pH gradient could account for dissolution of bone by affecting the solubility constant coefficient for hydroxyapatite. Liberated citrate could also exchange with phosphate and complex with calcium to augment the effect. This theory has received considerable support by investigators who have shown that organic acids, in particular citrate, are increased in vivo following administration of parathyroid extracts (Martin, Firschein, Mulryan and Neuman, 1958; Firschein, Martin, Mulryan, Strates and Neuman, 1958; Kenny, Draskozy and Goldhaber, 1959; Lekan, Laskin and Engel, 1960; Kenny, 1961). Against this hypothesis has been the inability to demonstrate a reasonable correlation between calcium mobilized and the amount of organic acid produced (Raisz, Au and Tepperman, 1961; Vaes and Nichols, 1962). The theory also fails to explain the mobilization by PTH of mucoproteins and by-products of collagen breakdown, which have been reported (Engel, 1952; Bates, McGowen and Talmage, 1962; Bates, Awapapa and Talmage, 1964). The possibility that PTH deranges carbohydrate metabolism cannot be discounted at this time. Further studies will be necessary to resolve some of the present difficulties.

b) Kidney: The relationship of the parathyroids to renal handling of phosphorus has long been established (Greenwald, 1911).

For many years it was argued that the plasma calcium-raising effect of PTH was due to the phosphaturic effect which lowered the plasma phosphate concentration resulting in a compensatory shift in calcium in accord with the solubility coefficient (Albright and Ellsworth, 1929). The original conclusion was reached after observing the effect of parathyroid extract on a patient with hypoparathyroidism whose serum phosphate was high. Immediately following administration of PTH there was marked phosphaturia which was most pronounced after 2 hr. Chronic administration resulted in a fall in serum phosphate and a rise in calcium. Subsequent studies in animals revealed that this phosphaturic effect was most marked after parathyroidectomy (Harrison and Harrison, 1941; Tweedy, Chilcote and Patras, 1947; Albright and Reifenstein, 1948; Crawford, Osborne, Talbot, Terry and Morrill, 1950; Talmage and Kraintz, 1954; Talmage, 1956; Foulks and Perry, 1959).

For some years there was speculation that perhaps two separate hormones were secreted by the parathyroid: one which mobilized calcium from bone and another which affected the renal tubular handling of phosphate (Munson, 1955). Two observations appeared to support this possibility. First, variations in the ratio of hypercalcemic to phosphaturic activities were found in different parathyroid extracts (Kenny, Vine and Munson, 1959). Second, a rise in serum

calcium without a concomitant change in phosphate could be demonstrated in parathyroidectomized rats treated with extract (Munson, 1960). These observations suggested a dissociation of the two effects. The question was finally resolved by the demonstration that purified PTH produced phosphaturia when injected into the renal artery of the dog (Pullman, Lavender, Aho and Rasmussen, 1960; Lavender, Pullman, Rasmussen and Aho, 1962; Hirsch and Munson, 1964) and the chicken (Levinsky and Davidson, 1957).

The site of action of PTH in the kidney has been localized to the renal tubule. Nicholson (Nicholson and Shepherd, 1959; Nicholson, 1959) by selectively poisoning the first third and first two-thirds of the proximal tubule and the distal tubule in dogs has shown that PTH acts by increasing absorption of phosphate in the distal tubule. In similar experiments using stop-flow techniques in parathyroidectomized dogs, Samiy, Hirsch, Ramsay, Giordano and Merrill (1960) found that PTH not only affected phosphate reabsorption in the distal tubule, but in the proximal tubule as well. These investigators attribute the difference in their results to the increase in sensitivity they obtained by parathyroidectomy. Isotopic experiments using P^{32} have shown that parathyroid extracts cause a marked, sudden increase in total phosphate, inorganic phosphate, acid

soluble organic phosphate, phospholipid, nucleic acid phosphate and phosphoproteins in kidney tissue (De Verdier, 1957; Egawa and Neuman, 1964).

In addition to its effect on the excretion of phosphate, PTH also most likely affects the renal handling of divalent cations (Talmage and Kraintz, 1954; Kleeman, Bernstein, Rockney, Dowling and Maxwell, 1961). MacIntyre, Boss and Troughton (1963) have clearly shown that the purified hormone causes marked renal conservation of both calcium and magnesium without altering creatinine excretion.

c) Gastrointestinal tract: Attention was first drawn to the relation of the parathyroids to derangements in calcium by MacCallum and Voegtlin (1908) who noted that faecal calcium output was increased in parathyroidectomized dogs. This finding has been corroborated in the dog (Tweedy, Chilcote and Patras, 1947) and in the rat (Talmage and Elliott, in press). A partial explanation may be that removal of the parathyroids results in decreased gastric emptying time (Clark and Smith, 1964). PTH given to the intact animal has been shown to cause increased gastrointestinal absorption. Ca^{45} studies in the rat have demonstrated that not only is faecal calcium output decreased by PTH, but urinary excretion of calcium is increased (Tweedy, L'Heureux and Zorn, 1950). Similar verification of faecal calcium

decrease after PTH has been reported by Cramer et al (1961) in balance experiments in the rat.

Confirmation of the direct action of PTH on the gut has been shown in in vitro systems. Rasmussen (1959) demonstrated that everted sacs of the small intestine of the rat could actively transport Ca^{45} from mucosa to serosa against a concentration gradient. Parathyroidectomy performed before removal of the gut decreased uptake. Using a similar preparation from intact rats Kimberg, Schacter and Schenker (1961) showed that the calcium uptake took place in the small intestine where it was most marked in the duodenum. A partial clarification of some of the processes involved has come from recent studies with intestinal villi (Rasmussen, Waldorf, Dziewiatkowski and DeLuca, 1963). Villi prepared from intact rats incubated in media containing Ca^{45} have shown a more rapid uptake of calcium than have villi prepared from parathyroidectomized animals. Further studies with intestinal villi have suggested a three fold calcium exchange process: an active uptake dependent upon oxidative metabolism, a passive uptake process directly proportional to the calcium concentration of the incubating fluid and a temperature dependent release mechanism.

HOMEOSTATIC MECHANISMS

Homeostasis is the maintenance of the body's internal environment despite marked fluctuations in activity and external environment. Many of the early concepts of homeostasis were introduced by Claude Bernard and Walter B. Cannon. More recently the term "cybernetics" has been applied to the field of control and communication theory (Wiener, 1961) and many of the ideas have been applied to hormonal control of endocrine systems (Goldman, 1960; Bayliss, 1960; Defares and Sneddon, 1960).

Homeostasis is usually achieved by closed cycle control systems called servo systems. In a servo system a stimulus is translated into a response: the response, in turn, affects the stimulus. This process is called feedback. The stimulus in such a system is the error or difference between the desired and actual output. When the error causes an action to reduce the error the process is called negative feedback. These terms and their relations to one another are illustrated in Figure 4.

In 1961 McLean proposed that plasma calcium was controlled by such a servo system. In cybernetic terminology changes in plasma ionic calcium from normal levels produce an error signal which provides a control stimulus to the parathyroids or control signal transducer which actuates a feedback transducer. The feedback transducer in this system would be PTH which provides the corrective

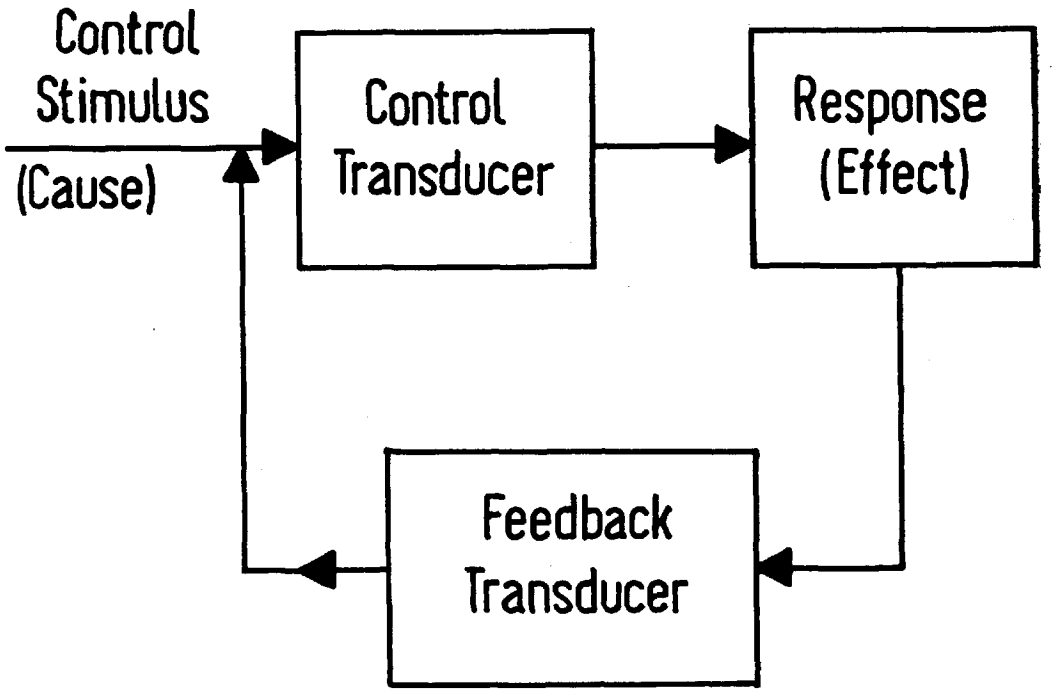


Figure 4. Schematic diagram of a control system (Goldman, 1960).

response. When stimulated by low plasma calcium PTH would be secreted by the parathyroids to cause the release of calcium from bone. Conversely, high levels of plasma calcium would depress PTH production and thereby depress bone resorption and lower plasma calcium.

Considerable evidence has accrued to support the contention that low levels of plasma calcium do indeed produce changes suggestive of hypersecretion of PTH by the parathyroids. In calcium depleted rats hyperplasia of the parathyroids is found (Ham, Littner, Drake, Robertson and Tisdall, 1940; Stoerk and Carnes, 1945). Osteoclast counts in the long bones become elevated in rats made hypocalcemic by peritoneal lavage (Toft and Talmage, 1960). Perfusate collected from thyro-parathyroid glands perfused with blood depleted of calcium by lead phosphate when injected into normal dogs produced hypercalcemia (Patt, Wallerstein and Luckhardt, 1942). Histological changes suggestive of variations in secretory activity occur in parathyroid cells incubated in media containing varying concentrations of calcium (Raisz, 1963; Roth and Raisz, 1964). These observations suggest that plasma calcium is maintained within normal limits by variations in the rate of PTH secretion.

Unfortunately such a servo system by itself cannot adequately prevent wide oscillations and overshoots in plasma calcium (MacIntyre,

Foster and Kumar, 1964) since the PTH effect on plasma calcium is long acting. The only alternative explanation is that an antagonist to PTH exists.

To study the dynamics of plasma calcium homeostasis Copp and Davidson (1961) devised a most ingenious technique to perfuse the thyroids and parathyroids in the dog with blood either high or low in calcium. They were able conclusively to show that systemic plasma calcium rose with low calcium stimulus and fell with high calcium stimulus to these glands. Extension of these experiments led to the exciting discovery that hypercalcemic perfusion of the thyro-parathyroids resulted in a fall in systemic calcium which was greater than that which could be accounted for by cessation of PTH production. This classical work led to the postulation of an antagonist to PTH, "Calcitonin" (Copp, Cameron, Cheney, Davidson and Henze, 1962; Copp and Cheney, 1962; Copp, Cheney, Davidson and Dube, 1962; Copp, 1963; Copp, 1963b).

The remainder of this thesis describes the studies which confirmed the existence of calcitonin, determined its thyroid origin and made possible its purification.

PART II

CALCITONIN

Chapter I

Confirmatory Evidence for the Existence of Calcitonin.

Summary

A technique was developed to perfuse the thyro-parathyroid apparatus of the dog with blood high in calcium. Modification of this method permitted alternating perfusions of blood high or low in calcium, while maintaining the animal in net calcium balance. These methods produced data in support of a plasma calcium-lowering hormone, calcitonin. This hormone was shown to be fast-acting, short lived and secreted in response to a high calcium stimulus.

The original experiments of Copp and his associates (Copp, Cameron, Cheney, Davidson and Henze, 1962) demonstrated that low calcium perfusion to the thyro-parathyroid apparatus in the dog induced a rise in plasma calcium. Conversely, a high calcium perfusion produced a fall in systemic calcium. The fall could be prevented by thyro-parathyroidectomy. To show these effects Copp et al (Copp and Davidson, 1961) employed an automated device which could add either calcium as calcium chloride or EDTA (ethyene-diamine tetraacetate) to the dog's own blood used to perfuse the animal's thyro-parathyroid apparatus. At the same time a comparable amount of the alternate reagent was added to a systemic vein to maintain the animal in net calcium balance. Three theoretical objections could be raised to this scheme. First, EDTA might have a toxic pharma-

cological effect on the glandular tissue perfused. Second, EDTA could bind divalent cations other than Ca^{++}. Third, even small pH changes at the cellular and subcellular level could effect minute but important changes in the ability of EDTA to bind calcium.

To avoid these difficulties an alternative technique was developed which used ion exchange resins to alter plasma inorganic cations (Kumar, Foster and MacIntyre, 1963). The results obtained by this new method supported Copp's conclusion that a second hormone was operative in calcium homeostasis. The data indicated that this hormone must be secreted by either the thyroid or parathyroid glands. Previous to this investigation no confirmatory evidence for calcitonin had been reported (Tashjian and Munson, 1963).

EXPERIMENTAL DETAILS

1) Preparation of Resins: Dowex - 50, a cross-linked polystyrene sulfonic acid cation exchange resin containing 8% divinyl benzene, 20-50 mesh, with an exchange capacity of approximately 5 mEq/g dry weight was used in all perfusion experiments. 500g batches were prepared by a triple washing with boiling 5 N-hydrochloric acid and 5 N-soldium carbonate, alternatively. The final sodium form was then thoroughly rinsed with an excess of distilled water

and the purified resin was stored until use either in a final rinse of distilled water or air dried.

2) Equilibration of Resins: A 30 x 1.6 cm column was filled with wet resin (approximately 15g) and the contents washed by slow percolation with 15 l of one of the following solutions:

a) High calcium resin solution:

140 mEq/l sodium as sodium chloride and sodium bicarbonate

3.8 mEq/l potassium as potassium chloride

4.5 mEq/l calcium as calcium chloride

1.2 mEq/l magnesium as magnesium chloride

b) Low calcium solution:

140 mEq/l sodium as sodium chloride and sodium bicarbonate

3.8 mEq/l potassium as potassium chloride

2.0 mEq/l calcium as calcium chloride

3.5 mEq/l magnesium as magnesium chloride.

Each solution was made up by dissolving 109g sodium chloride and 18.8g sodium bicarbonate in approximately 10 l of distilled water prior to the addition of the other salts to avoid calcium carbonate precipitation. The volume was adjusted to 15 l 5 percent carbon dioxide in oxygen was bubbled through to give a final pH of 7.4 (Dawson, Elliot, Elliot and Jones, 1959).

At the completion of equilibration, the resin was collected in an Erlenmyer flask with an excess of its equilibration solution until poured into the appropriate columns in the perfusion apparatus (see p. 57). For full details see Kumar (1964).

The precharged resin was designed to alter the perfusing blood in experimental animals so that deviations in plasma calcium in the order of $\pm$ 1.5 mEq/l could be made without otherwise affecting the concentrations of sodium, potassium and magnesium. The resin was charged with the above solutions on the assumption that it would equilibrate with them. Subsequent passage of blood through them would reproduce the original ionic concentrations by equilibration. This interchange would involve exchange of sodium for calcium and calcium for sodium in the resin in systems designed to increase or decrease the effluent calcium respectively. According to the law of Mass Action such an exchange involves the replacement of one Ca^{++} ion for two Na^{+} ions or the converse:

$$Ca\,R + 2\,Na \rightleftharpoons Na_2R + Ca^{++}$$

and

$$\frac{(Ca_s)\,(Na_r)}{(Na_s)\,(Ca_r)} = K$$

where R represents the acidic group on the resin and s and r designate solution or resin respectively. The successful

application of the technique in producing satisfactory effluents is summarized in Tables I - 1 and II - 2 (after Kumar, 1964).

3) Perfusion Equipment: Figure I - 1 schematically illustrates the components of the apparatus used in Experiment I. The tubing used throughout was 4.0 mm in diameter (6.0 mm outer diameter) silicone rubber tubing (Esco Rubber Ltd., 34 Somerford Grove, London, N.6). Terminal end pieces for cannulation of vessels were made from 2 cm sections cut from Simplastic Venous Catheters (No. 12 FG, G.U. Manufacturing Company Ltd.) slightly fused at both ends and inserted inside the silicone tubing and tied in place. All T and Y pieces were of polyvinyl chloride (Portex M-T2 and M-Y2, 4 mm internal diameter). The water jacket was also made from a length of flexible polyvinyl chloride tubing (Portex NT-23 19.05 mm bore) with a two hole bung at each end to accommodate tubing for blood flow and water supply. Water was circulated by means of a Tecam Tempunit (Techne Ltd., Duxford, Cambridge) from a reservoir maintained at 37°C. Details of the dripflow chamber with fluid level regulatory device and disposable filter discs punched from sheets of stainless steel (MSO/596 New Electronic Products Ltd., 358 Kensington Road, London, S.E.11) were specially made and are shown in Figure I - 2. These latter two items together with Y and T joints were treated with

Table I - 1. Biochemical measurements in resin equilibrating fluid, systemic blood and effluent blood in dogs. Last named values represent levels obtained after 3 hr. perfusion at 5 ml per min. through 20 ml Dowex 50W-X8 (After Kumar, 1964).

Exp. No.	Specimen	Ca mEq/l	Mg mEq/l	K mEq/l	Na mEq/l	pH	Phosphate mEq/l	Proteins g/100 ml
1.	Equilibrating fluid	3.2	0.4	3.8	145	-	-	-
	Systemic plasma	5.8	1.2	3.7	151	-	-	-
	Effluent plasma	5.9	0.7	3.7	150	-	-	-
2.	Equilibrating fluid	3.6	2.7	3.7	141	7.40	-	-
	Systemic plasma	5.9	1.5	4.1	151	7.39	-	-
	Effluent plasma	6.2	3.7	3.9	146	7.41	-	-
3.	Equilibrating fluid	2.0	1.3	3.8	140	-	-	-
	Systemic plasma	5.5	1.9	-	-	-	-	-
	Effluent plasma	4.1	1.9	-	-	-	-	-
4.	Equilibrating fluid	4.6	1.4	3.8	140	-	-	-
	Systemic plasma	5.6	1.9	-	-	-	-	-
	Effluent plasma	7.0	1.9	-	-	-	-	-
5.	Equilibrating fluid	3.0	1.2	3.9	137	7.40	-	-
	Systemic plasma	4.6	1.4	4.4	147	7.38	-	-
	Effluent plasma	4.6	1.7	4.0	143	7.36	-	-
6.	Equilibrating fluid	5.0	0	-	-	-	0	0
	Systemic plasma	5.4	1.6	-	-	-	2.3	7.2
	Effluent plasma	9.0	0.4	-	-	-	2.3	7.1
7.	Equilibrating fluid	2.0	3.5	-	-	-	0	0
	Systemic plasma	5.4	1.6	-	-	-	2.3	7.2
	Effluent plasma	3.6	4.8	-	-	-	2.2	7.3

Table I - 2. Relationship between calcium and magnesium in resin equilibrating fluid and effluent blood. The resin used was Dowex 50W-X8. Equilibrating fluid in addition to Ca and Mg contained Na 140 mEq/l and K 3.8 mEq/l. (After Kumar, 1964).

Equilibrating Fluid		Effluent Plasma	
Ca mEq/l	Mg mEq/l	Ca mEq/l	Mg mEq/l
2.0	0	3.5	0.5
2.0	1.0	3.5	1.7
2.0	3.3	3.5	4.5
2.8	0	5.2	0.5
3.0	1.1	5.2	1.7
3.0	4.0	5.2	4.5
4.0	0	7.0	0.5
4.5	1.2	7.0	1.7
4.9	3.8	7.0	4.5

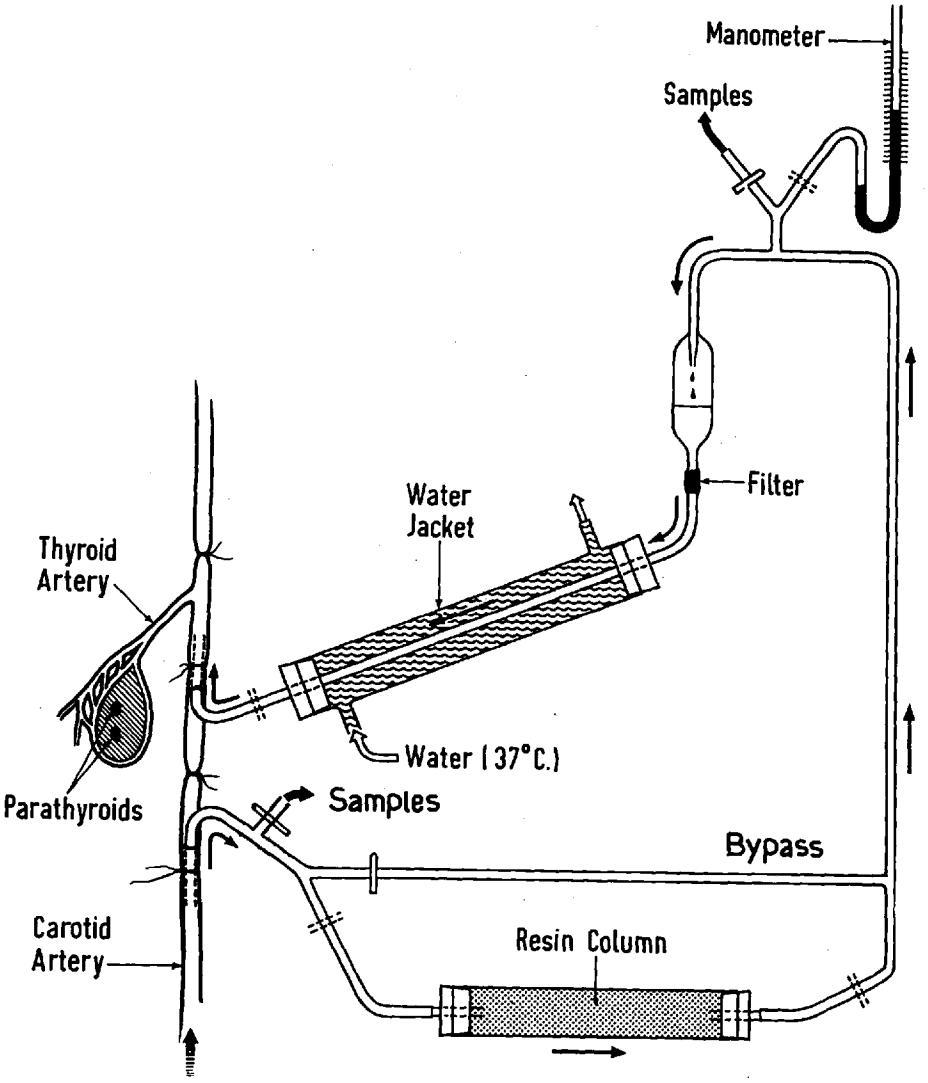


Figure 1 - 1. Single resin column perfusion apparatus. Blood from a heparinized animal was directed through a cation exchange column pre-equilibrated to produce effluents either high or low in calcium to perfuse the gland area.

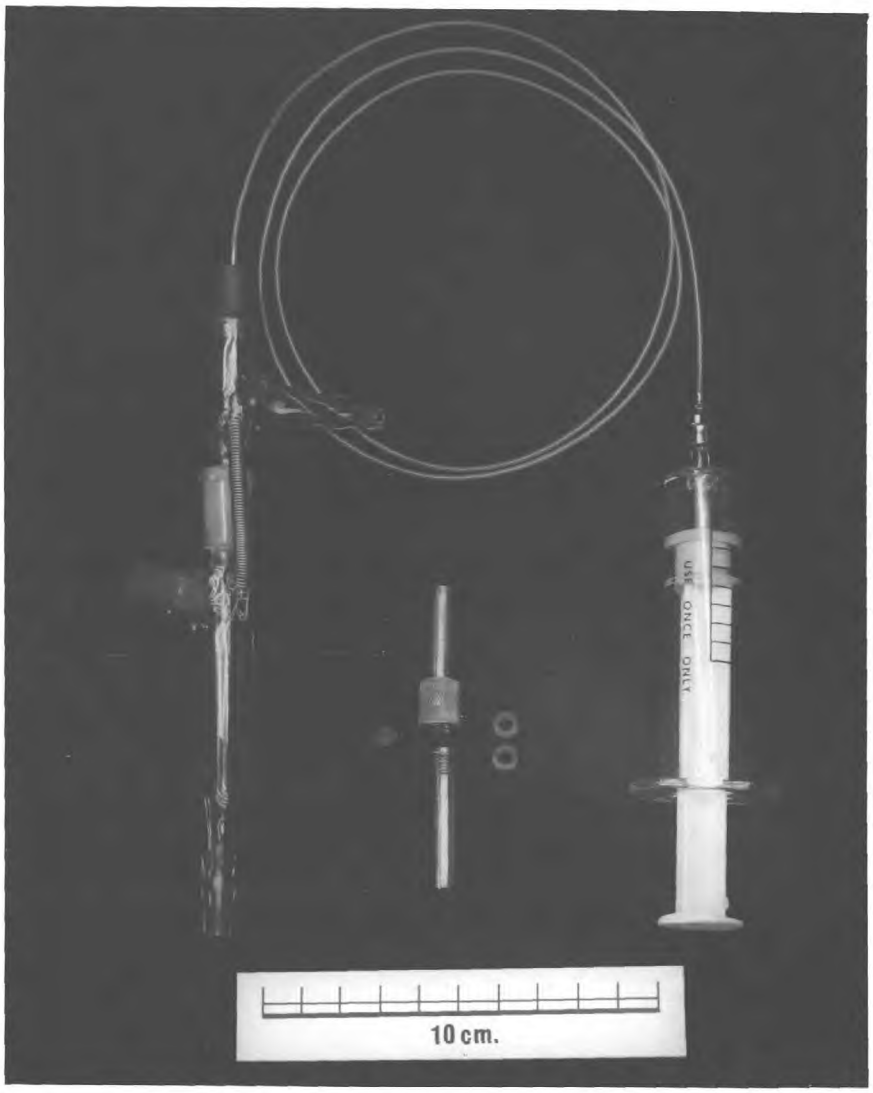


Figure 1 - 2. Detail of (1) drop counting device with tubing and syringe attached to regulate blood level (side pieces) and (2) stainless steel adapter used to hold disposable stainless steel mesh filter with polyethylene washers (centre pieces).

2 percent silicone in acetone before use. They were cleaned in boiling 10 percent sodium hydroxide, rinsed thoroughly and dried before re-use. All other tubing including cannulae and resin column jackets were discarded after each experiment.

The resin column was placed in an upright position with the end with tubing tipped with a cannula down. The upper bung was then removed and the column filled with equilibrating electrolyte solution. The tubing was allowed to fill and was thoroughly cleared of any air bubbles and clamped. Resin was poured into the column until it was filled to within an inch of the top. During pouring it was important that no air bubbles be trapped in the resin. The remaining portion of the column was then filled with equilibrating fluid, the bung replaced and adjacent tubing clamped.

The apparatus was designed to enable arterial blood from a dog's carotid artery to be shunted either through a by-pass or through a resin column precharged to deliver a high calcium effluent for perfusing the gland area. The tubing circuit had suitable outlets for sampling blood before and after passage through the resin. A mercury manometer, interposed in the circuit, allowed continual monitoring of the animal's blood pressure. Manipulation of interposable clamps (Fischer, Scientific Company) simplified sampling and control over direction of blood flow.

Apparatus used in Experiment II is shown in Figures I - 3 and I - 4. The purpose of this experiment was to divide equally the carotid blood flow. Half was passed through a resin column pretreated to deliver an effluent high in calcium; the other half was directed through a second resin column to produce a low calcium effluent. The effluents from one column could then be directed for perfusion of the gland area while blood from the second column could be returned to a systemic vein.

The design of the apparatus was essentially the same as described for Experiment I. The major modification was the introduction of a second circuit to accommodate the second resin column. This circuit was designed with its own manometer, drip flow chamber, filter, sampling tap and cannula. When in use manipulation of a screw clamp valve on the tubing proximal to the outlet regulated the resistence to the venous outflow. By adjusting equal flow rates through both columns equal amounts of blood, high and low in calcium, could be delivered to the animal, thus maintaining net calcium balance. Interchange of the two clamps just distal to the resin columns made it possible to alternate the effluent to the gland area.

4) Animals: Healthy mongrel dogs of either sex and between 10-21 kg were used. Animals were fasted overnight. Anaesthesia

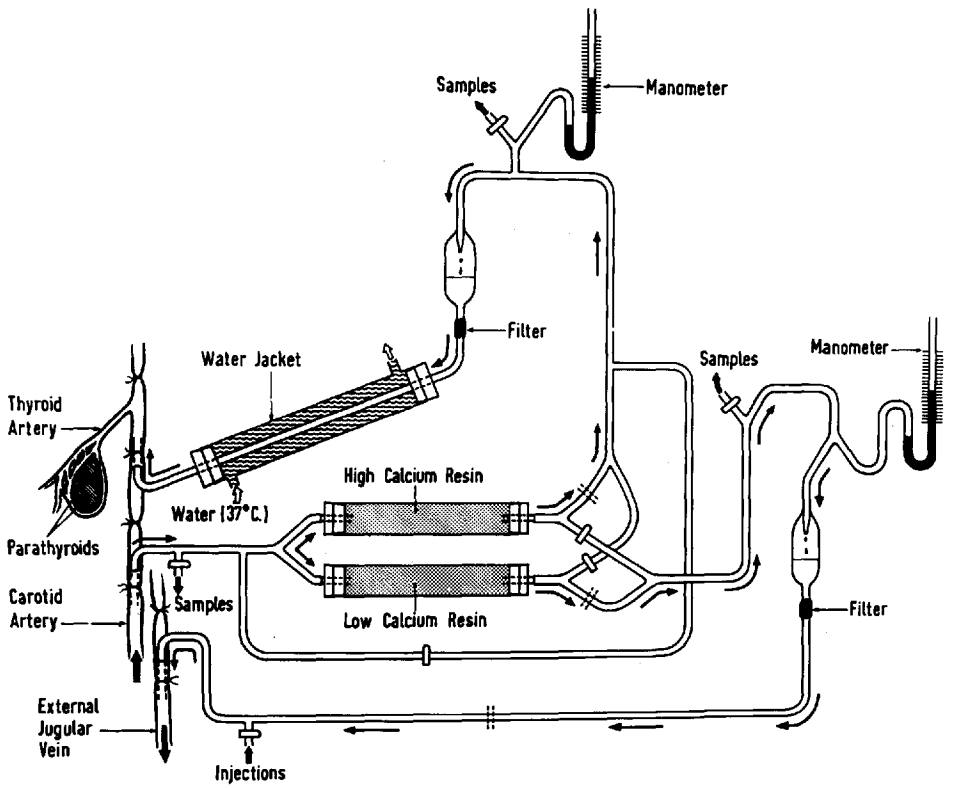


Figure 1 - 3. Double column perfusion apparatus. The blood flow from a heparinized animal's carotid artery was equally divided. Half was passed through a resin column previously equilibrated to deliver a high calcium effluent. The other half was directed through a second resin column to produce a low calcium effluent. Blood from one column was used to perfuse the gland area; blood from the second column was returned to a systemic vein. In this way the animal was maintained in net calcium balance.



Figure 1 - 4. Double column perfusion apparatus showing (1) constant temperature water bath supplying water to (2) water jacket enclosing tubing returning blood to perfusion area, (3) screw valve to adjust rate of blood being returned to vein, and (4) device for regulation of blood level in drop counter. Arrows indicate the direction of blood flow.

was induced with pentobarbitone (Abbott's Veterinary Nembutal) 30-35 mg/kg intravenously and subsequently maintained with booster doses of 25-50 mg as needed. During all experimental procedures the animals were restrained in a supine position on an animal board. Their airways were kept patent by means of an inflated endotracheal tube. Insertion of small bore polyethylene tubing into a dorsal forepaw vein facilitated intravenous injections. The tubing was filled with a slightly heparinized saline solution prior to insertion to prevent clot formation. The end was fitted with a three-way stopcock.

5) Experimental Procedures:

Experiment I - Thyro-parathyroid perfusion with hypercalcemic blood.

In one group of five dogs the right thyro-parathyroid apparatus was removed through a longitudinal exposure. Pretracheal facia was dissected manually. The right thyroid gland with its attendant parathyroids was found lying lateral to the trachea just below the lower level of the carotid cartilege and underlying the lateral neck strap muscles. The gland was dissected free of its underlying attachments, clamped at its superior and inferior pedicles without injury to thyroid tissue and cut free. The left thyro-parathyroid apparatus and its attendant thyroid artery arising from the carotid

artery was similarly dissected and all branches arising from the thyroid artery ligated. The animal was heparinized with 4000 units/kg (Evans Sodium Heparin) and thereafter maintained on 1000 units/kg given hourly. The left common carotid artery was dissected free of its associated structures within the carotid sheath. At a point proximal to the origin of the thyroid artery the cannula nearest the resin column was inserted toward the heart and tied in place. The remaining cannula was inserted distal to the first, pointing ~~cranial~~ TO THE HEAD and proximal to the thyroid artery. The tubing passing through the water jacket was filled with blood by retrograde flow by opening the sampling tap near the manometer. Tubing between the water jacket and the cannula was clamped and carefully inspected to insure no air bubbles were present. The by-pass tubing was next allowed to fill by releasing the clamp nearest the carotid artery and the sampling tap near the manometer. When all the tubing to the drip flow chamber was filled with blood the sample tap was closed and all other clamps removed. This allowed for circulation through the by-pass shunt, but not through the resin column. The carotid artery was then ligated distal to the origin of the thyroid artery.

A blood sample (2-3 ml) was collected from the proximal sample tap every 10 min. during a half-hour control period. The

by-pass was then clamped closed and the blood was allowed to circulate through the high-calcium resin precharged to deliver an effluent of approximately 7.0 mEq/l. Blood samples were collected from the proximal sample tap every 15 min. for the first hour and every 30 min. thereafter. In addition at least three post-resin blood samples were collected during the perfusion procedure to insure that correct calcium concentrations were being delivered to the perfused area.

In every experiment blood pressure was recorded each time blood was withdrawn. If the blood pressure fell below 60mmHg the experiment was deleted from the reported series. This was necessary since blood pressures lower than 50mmHg invariably resulted in elevations of plasma calcium, magnesium and phosphate. Undoubtedly these changes reflected the passage of intracellular ions out into plasma as seen in irreversible shock.

A second group of five dogs, matched for weight, served as controls. In the control animals the identical protocol was followed as in the experimental series excepting all thyro-parathyroid tissue was removed. The muscular branches of the thyroid artery on the side to be perfused were left patent to allow blood flow through the external circuit. The flow rate was adjusted to match that of the experimental animal. The perfusions involved the administration of 1.5 - 15 μEq. calcium/min.

Experiment 2 - Successive hypocalcemic and hypercalcemic thyro-parathyroid perfusions in the same animal.

The two resin column apparatus was used in this study. One column was filled with resin equilibrated to deliver a high calcium effluent of 7.0 mEq/l and the other with a resin to deliver a low calcium effluent of 4.0 mEq/l. Right thyro-parathyroidectomy was performed and the left carotid artery doubly ligated as previously described. In addition, the external jugular vein was cannulated in the direction of venous return with tubing from one of the resin columns (see Figure I - 4).

Blood was circulated through the by-pass circuit for 30 min. In two animals the left thyro-parathyroid apparatus was then perfused for $2\frac{1}{2}$ hr. with blood high in calcium while blood comparably low in calcium was returned to the jugular vein. The tubing clamps were then changed. For another $2\frac{1}{2}$ hr. blood low in calcium was used to perfuse the gland area and the high calcium blood was returned to the jugular vein. In two other animals the order of perfusion was reversed. Throughout all procedures the flow rates through both column systems were kept equal. In this way calcium added by one resin column system was compensated for by deletion by the second column.

An additional four dogs were studied as controls. Right thyro-parathyroidectomy was done at the onset of each experiment

and left thyro-parathyroidectomy at the beginning of the perfusion period. Perfusion of the neck strap muscles via the muscular branches from the thyroid artery was carried out as described in the preceding controls in Experiment 1. In all four animals the 30 min. control period was followed by a $2\frac{1}{2}$ hr. perfusion period with high calcium blood and a second $2\frac{1}{2}$ hr. low calcium perfusion period. This order was observed to avoid confusion with the gradual fall in plasma calcium seen 2-3 hr. following thyro-parathyroidectomy.

Analytical Methods: All blood specimens collected were immediately centrifuged. 0.2 ml of plasma were diluted within 30 min., capped with parafilm and later analysed for calcium with the Zeiss flame spectrophotometer PMM12 (MacIntyre, 1963).

Theoretical Note on Flame Spectrophotometry.

Flame photometry entails the spraying of the diluted plasma in a hot oxygen-acetylene flame and comparing the emission at 422.7 mμ to standard solutions of calcium. During spraying the water of solution is vaporized. The remaining salt molecules are dissociated into atoms, some of which become ionized. The various components of the flame's milieu may absorb energy from the flame and pass into an excited state. When an atom passes

into an excited state one of its outer valency electrons becomes displaced to an orbit further from the nucleus. In resuming the stable state energy may be released as light. In the relationship

$$E_j - E_o = h\nu$$

where E_j is the higher energy level, E_o the lower energy level, h Plank's constant and ν the frequency of radiation, each transition is associated with light emission of a certain wave length. Ions and molecules in the excited state also may emit light. The sum total of light emitted from a mixed population of particles extends through a spectral range. In the analysis of calcium a wave length in the blue part of the spectrum is recorded to avoid interference from sodium. Since the excitation potential of calcium is relatively high, a hot flame is used. Excess phosphate is added with the diluting fluid in the preparation of both standards and samples to overcome difficulties due to formation of calcium-phosphate complexes not readily volatilized. In an excess of phosphate the equilibrium is shifted in the direction of more complex formation in both standards and sample. The emission is then independent of the original phosphate content of the sample (MacIntyre, 1963).

RESULTS

Experiment 1 - Thyro-parathyroid perfusion with hypercalcemic blood.

The results are shown in Figure I - 5 and Tables I - 3 through I - 5. In the experimental group the systemic plasma calcium fell 7 percent during the $2\frac{1}{2}$ hr. perfusion of the thyro-parathyroid apparatus. In contrast essentially no change in plasma calcium was observed during a similar period in the control group. The small increase in blood calcium in the control group was due to the positive balance from high calcium perfusion; the subsequent fall was secondary to parathyroidectomy.

Experiment 2 - Successive hypercalcemic and hypocalcemic perfusion of the thyro-parathyroids.

Hypercalcemic perfusion of the gland area resulted in a rapid fall in systemic calcium: hypocalcemic perfusion produced a rise. The fall in plasma calcium observed with hypercalcemic perfusion was 13 percent and occurred irrespective of the order of perfusion (Figures I - 6 and I - 7 and Tables I - 6 through I - 9). The difference in magnitude between this fall and the 7 percent fall in Experiment 1 is readily accounted for. In the two column experiment the animals were maintained in net calcium balance, whereas in Experiment 1 the fall was minimized by calcium added during perfusion.

Of the four control experiments, little or no fall in plasma calcium was observed during high calcium perfusion in three, A

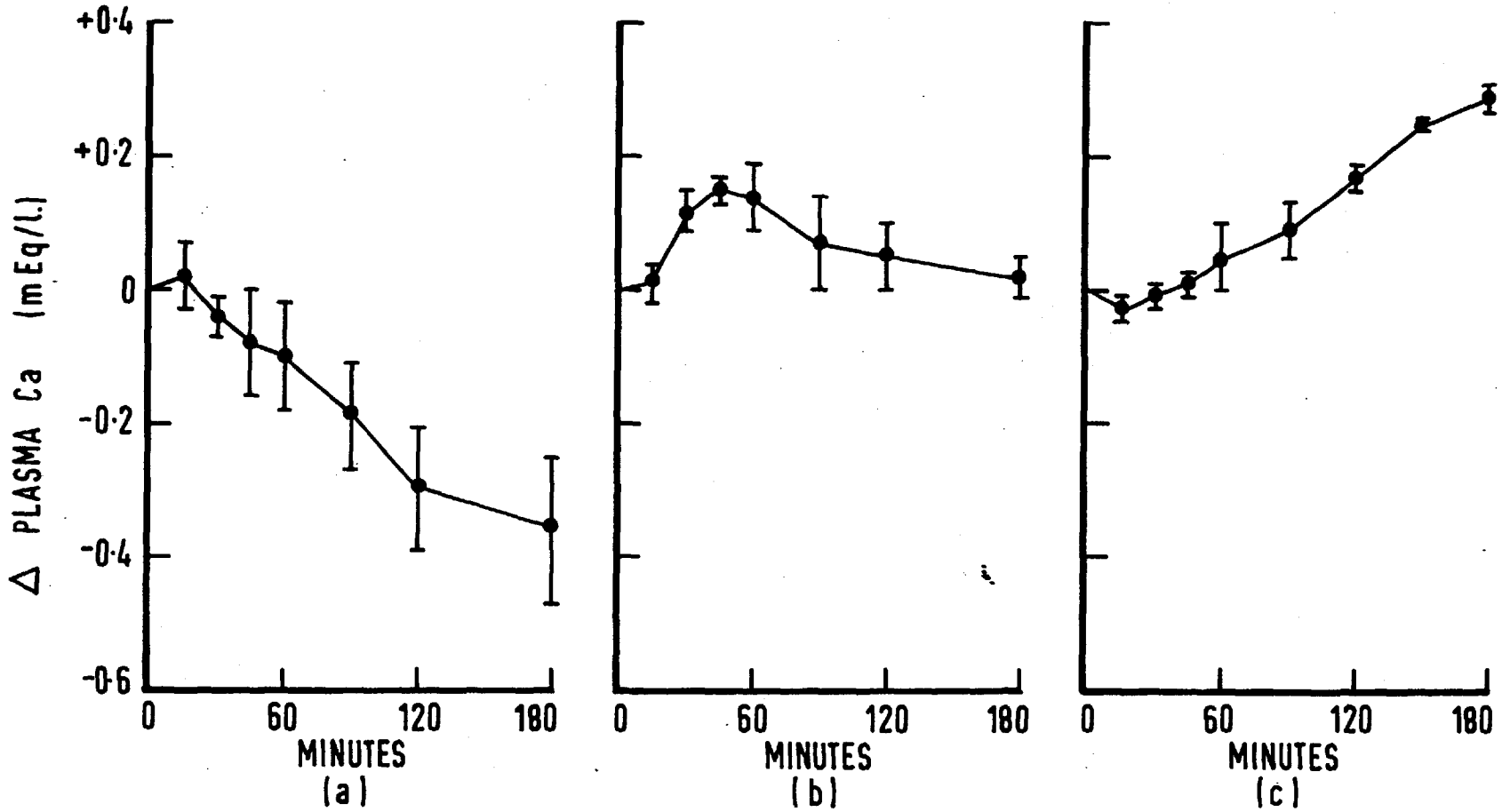


Figure 1 - 5. Changes in systemic plasma calcium observed in dogs during (a) unilateral hypercalcemic perfusion of the thyro-parathyroid apparatus after contralateral thyro-parathyroidectomy (b) hypercalcemic perfusion of the neck strap muscles after bilateral thyro-parathyroidectomy and (c) unilateral hypocalcemic perfusion of the thyro-parathyroid apparatus after contralateral thyro-parathyroidectomy. Calcium concentrations of 7 and 4 mEq/l used for hyper- and hypocalcemic perfusions respectively were produced by passage of carotid arterial blood through a resin column previously equilibrated to give the desired effluent. Points indicate mean values for five animals and the vertical bars the standard errors of the mean.

Table I - 3. Changes in plasma calcium observed during unilateral hypercalcemic perfusion of the thyroid and parathyroid glands in the dog.

All thyroid and parathyroid tissue was removed on one side at the start of each experiment. The remaining glands on the contralateral side were perfused with blood enriched with calcium (approximately 7 mEq/l) by passage through a pre-equilibrated resin column.

Time (minutes)	Dog No. 31		Dog No. 36		Dog No. 35		Dog No. 34		Dog No. 52		S.E. of change in plasma Ca
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period											
-30	5.70		-		5.60		5.65		5.44		
-20	5.56		5.60		5.60		5.76		5.56		
-10	5.45		5.90		5.60		5.65		5.40		
0	5.56		5.80		5.55		5.63		5.50		
Hyper-calcemic perfusion											
15	5.50	-0.06	5.80	0	5.60	+0.05	5.53	-0.10	5.70	+0.20	$+0.02 \pm 0.05$
30	5.50	-0.06	5.70	-0.10	5.60	+0.05	5.53	-0.10	5.50	0	-0.04 ± 0.03
45	5.56	0	5.70	-0.10	5.60	+0.05	5.25	-0.38	5.50	0	-0.08 ± 0.08
60	5.25	-0.31	5.70	-0.10	5.70	+0.15	5.38	-0.25	5.50	0	-0.10 ± 0.08
90	5.38	-0.18	5.70	-0.10	5.50	-0.05	5.13	-0.50	5.40	-0.10	-0.19 ± 0.08
120	5.25	-0.31	5.60	-0.20	5.40	-0.15	5.00	-0.63	5.30	-0.20	-0.30 ± 0.09
150	5.25	-0.31	5.60	-0.20	5.40	-0.15	4.88	-0.75	5.10	-0.40	-0.36 ± 0.11

Table I - 4. Changes in plasma calcium observed during hypercalcemic perfusion of the neck strap muscles in the thyro- parathyroidectomized dog.

Unilateral thyro-parathyroidectomy was performed at the start of each experiment. The remaining contralateral thyroid and parathyroid tissue was removed at the end of the control period. Hypercalcemic perfusion of the neck strap muscles carried out via the thyroid artery on one side with blood enriched with calcium (approximately 7 mEq/l) by means of a pre-equilibrated resin column.

Time (minutes)	Dog No. 32		Dog No. 37		Dog No. 40		Dog No. 42		Dog No. 55		Mean ± S.E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period											
-30	5.40		5.50		5.00		5.38		5.77		
-20	5.50		5.40		5.00		5.44		5.82		
-10	5.56		5.50		5.10		5.38		5.71		
0	5.44		5.40		5.00		5.44		5.77		
Hyper-calcemic perfusion											
15	5.56	+0.12	5.40	0	5.00	0	5.38	-0.06	5.77	0	+0.01 ± 0.03
30	5.56	+0.12	5.50	+0.10	5.00	0	5.63	+0.19	5.94	+0.17	+0.12 ± 0.03
45	5.56	+0.12	5.60	+0.20	5.10	+0.10	5.56	+0.12	6.00	+0.23	+0.15 ± 0.02
60	5.63	+0.19	5.70	+0.30	5.00	0	5.56	+0.12	5.88	+0.11	+0.14 ± 0.05
90	5.44	0	5.60	+0.20	5.00	0	5.44	0	5.94	+0.17	+0.07 ± 0.05
120	5.44	0	5.60	+0.20	5.00	0	5.38	-0.06	5.88	+0.11	+0.05 ± 0.05
150	5.44	0	5.50	+0.10	5.00	0	5.38	-0.06	5.82	+0.05	+0.02 ± 0.03

Table I - 5. Changes in plasma calcium observed during unilateral hypocalcemic perfusion of the thyroid and parathyroid glands in the dog.

Thyro-parathyroidectomy was performed on one side at the start of each experiment. The remaining thyroid and parathyroid glands on the contralateral side were perfused with blood partially depleted of calcium (4 mEq calcium/l) by passage through a pre-equilibrated resin column.

Time (minutes)	Dog No. 176		Dog No 177		Dog No. 178		Dog No. 179		Mean ± S. E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period									
-30	5.22		5.65		5.22		6.05		
-20	5.22		5.65		5.22		6.05		
-10	5.22		5.65		5.22		6.05		
0	5.22		5.65		5.22		6.05		
Hypo-calcemic perfusion									
15	5.22	0	5.59	-0.06	5.22	0	6.00	-0.05	-0.03 ± 0.02
30	5.26	+0.04	5.61	-0.04	5.22	0	6.00	-0.05	-0.01 ± 0.02
45	5.22	0	5.67	+0.02	5.28	+0.06	6.00	-0.05	+0.01 ± 0.02
60	5.28	+0.06	5.83	+0.18	5.24	+0.02	6.00	-0.05	+0.05 ± 0.05
90	5.32	+0.10	5.83	+0.18	5.29	+0.07	6.05	0	+0.09 ± 0.04
120	5.39	+0.17	5.89	+0.24	5.35	+0.13	6.19	+0.14	+0.17 ± 0.02
150	5.50	+0.28	5.89	+0.24	5.47	+0.25	6.29	+0.24	+0.25 ± 0.01
180	5.56	+0.34	5.89	+0.24	5.53	+0.31	6.33	+0.28	+0.29 ± 0.02

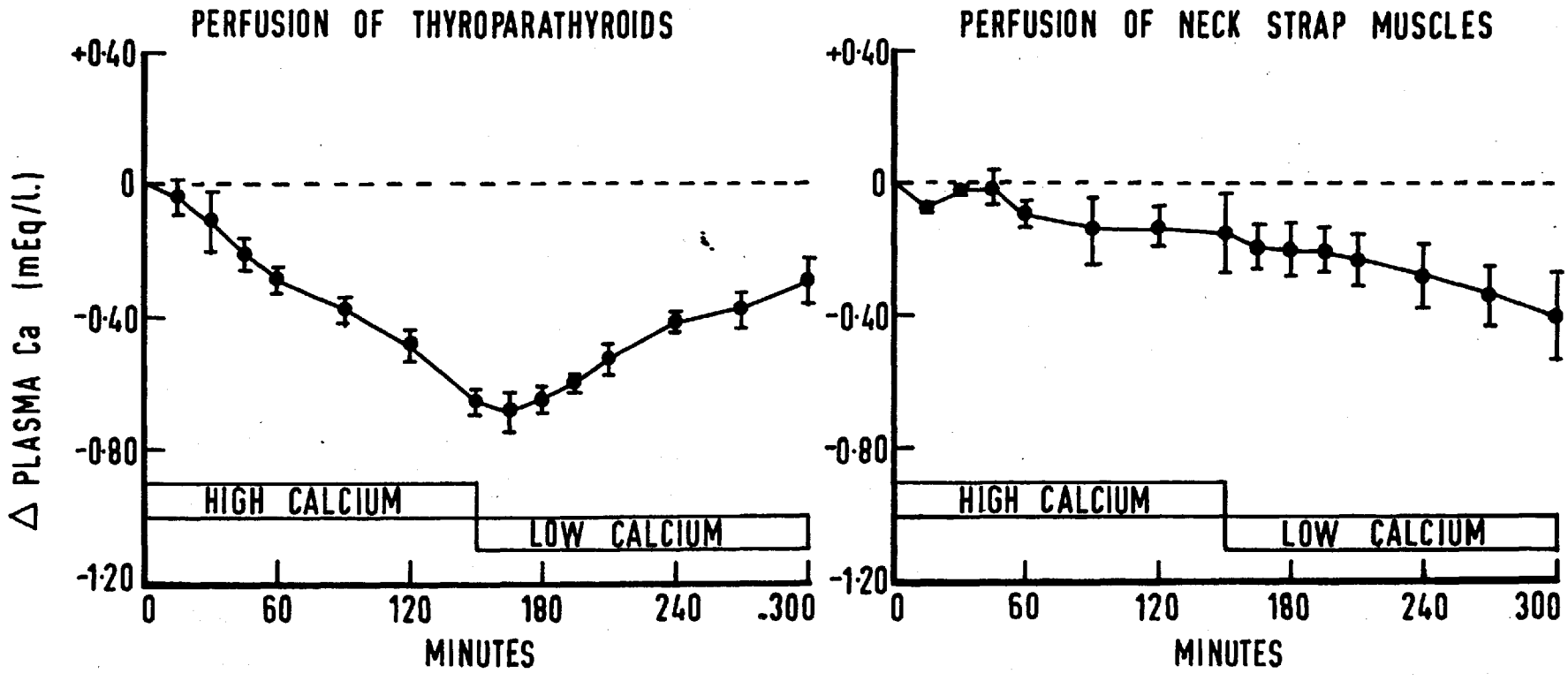


Figure 1 - 6. Changes in plasma calcium observed during successive hyper- and hypocalcemic perfusion of (a) the remaining thyro-parathyroids in four unilaterally thyroparathyroidectomized dogs and (b) the neck strap muscles in four bilaterally thyro-parathyroidectomized dogs. All animals were maintained in net calcium balance by means of two resin columns. Single points indicate mean values and vertical lines the standard errors of the mean.

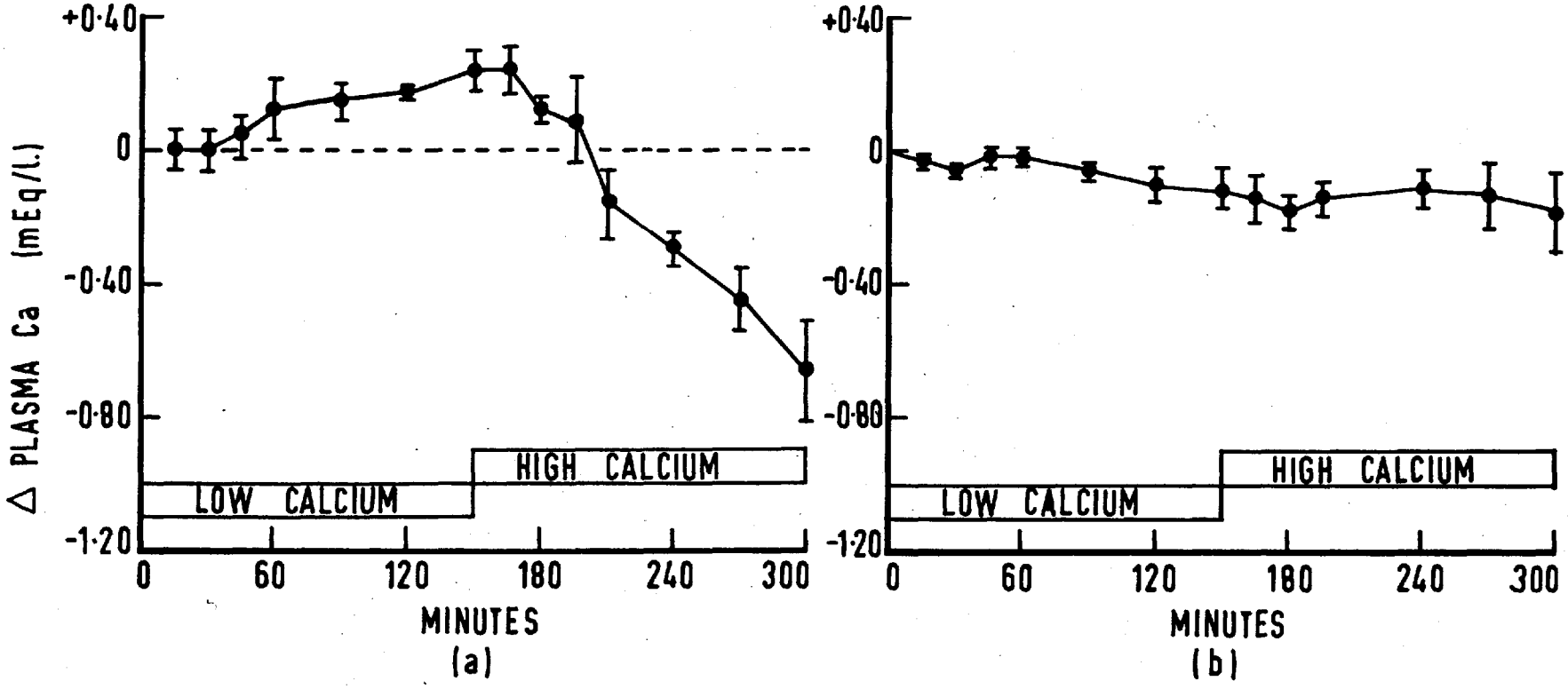


Figure 1 - 7. Changes in plasma calcium observed during successive hypo- and hypercalcemic perfusions of (a) the remaining thyro-parathyroids in four unilaterally thyro-parathyroidectomized dogs and (b) the neck strap muscles in four bilaterally thyro-parathyroidectomized dogs. All animals were maintained in net calcium balance by means of two resin columns. Single points indicate mean values and vertical lines the standard errors of the means.

Table I - 6. Effect on systemic plasma calcium of successive hyper- and hypocalcemic perfusion of the thyroid and parathyroid glands in the dog.

Unilateral thyro-parathyroidectomy was performed at the onset of each experiment. Two resin columns were used to maintain each animal in net calcium balance. Normocalcemic blood perfused the contralateral gland area for 30 minutes, hypercalcemic blood (approx. 7 mEq calcium/l) for the next 150 minutes and hypocalcemic blood (approx. 4mEq calcium/l) for the last 150 minutes.

Time (minutes)	Dog No. 45		Dog No. 50		Dog No. 74		Dog No. 99		Mean ± S.E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period									
-30	6.00		5.75		5.47		5.35		
-20	6.00		5.70		5.47		5.35		
-10	6.00		5.75		5.44		5.35		
0	6.00		5.68		5.44		5.35		
Hyper-calcemic perfusion									
15	5.88	-0.12	5.78	+0.10	5.37	-0.07	5.30	-0.05	-0.04±0.05
30	5.68	-0.32	5.78	+0.10	5.32	-0.12	5.25	-0.10	-0.11±0.09
45	5.68	-0.32	5.58	-0.10	5.16	-0.28	5.20	-0.15	-0.21±0.05
60	5.63	-0.37	5.44	-0.24	5.11	-0.33	5.15	-0.20	-0.29±0.04
90	5.50	-0.50	5.33	-0.35	5.06	-0.38	5.05	-0.30	-0.38±0.04
120	5.40	-0.60	5.33	-0.35	4.90	-0.54	4.89	-0.46	-0.49±0.05
150	5.26	-0.74	5.00	-0.68	4.79	-0.65	4.79	-0.56	-0.66±0.04
Hypo-calcemic perfusion									
165	5.13	-0.87	5.00	-0.68	4.79	-0.65	4.79	-0.56	-0.69±0.06
180	5.26	-0.74	5.00	-0.68	4.84	-0.60	4.79	-0.56	-0.65±0.04
195	5.40	-0.60	5.00	-0.68	4.84	-0.60	4.84	-0.51	-0.60±0.03
210	5.40	-0.60	5.28	-0.40	4.84	-0.60	4.84	-0.51	-0.53±0.05
240	5.60	-0.40	5.33	-0.35	4.95	-0.49	4.90	-0.45	-0.42±0.03
270	5.60	-0.40	5.44	-0.24	5.00	-0.44	4.90	-0.45	-0.38±0.05
300	5.67	-0.33	5.61	-0.07	5.05	-0.39	5.00	-0.35	-0.29±0.07

Table I - 7. Effect on systemic plasma calcium of successive hyper- and hypocalcemic perfusion of the neck strap muscles of the dog after thyro-parathyroidectomy.

Unilateral thyro-parathyroidectomy was performed at the onset of each experiment. After a 30 minutes normocalcemic perfusion of the contralateral gland area, all the remaining thyroid and parathyroid tissue was removed. Hypercalcemic blood (approx. 7 mEq calcium/l) was used to perfuse the muscular branches of the thyroid artery on one side for 150 minutes, followed by hypocalcemic perfusion with blood containing approximately 4 mEq calcium/l for an additional 150 minutes.

Time (minutes)	Dog No. 60		Dog No. 62		Dog No. 65		Dog No. 66		Mean ± S.E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period									
-30	5.45		5.17		5.55		5.80		
-20	5.44		5.06		5.70		5.85		
-10	5.50		5.06		5.70		5.85		
0	5.42		5.06		5.70		5.75		
Hypercalcemic perfusion									
15	5.37	-0.05	5.00	-0.06	5.60	-0.10	5.70	-0.05	-0.07±0.01
30	5.40	-0.02	5.00	-0.06	5.75	+0.05	5.70	-0.05	-0.02±0.02
45	5.35	-0.07	5.00	-0.06	5.85	+0.15	5.70	-0.05	-0.01±0.05
60	5.45	+0.03	4.94	-0.12	5.60	-0.10	5.60	-0.15	-0.09±0.04
90	5.45	+0.03	4.94	-0.12	5.50	-0.20	5.50	-0.25	-0.14±0.10
120	5.45	+0.03	5.00	-0.06	5.50	-0.20	5.47	-0.28	-0.13±0.06
150	5.40	-0.02	5.00	-0.06	5.40	-0.30	5.47	-0.28	-0.15±0.12
Hypocalcemic perfusion									
165	5.35	-0.07	5.00	-0.06	5.40	-0.30	5.42	-0.33	-0.19±0.07
180	5.35	-0.07	5.00	-0.06	5.30	-0.40	5.47	-0.28	-0.20±0.08
195	5.30	-0.12	5.00	-0.06	5.40	-0.30	5.42	-0.33	-0.20±0.07
210	5.30	-0.12	5.00	-0.06	5.40	-0.30	5.33	-0.42	-0.23±0.08
240	5.20	-0.22	5.00	-0.06	5.40	-0.30	5.22	-0.53	-0.28±0.10
270	5.00	-0.42	4.94	-0.12	5.40	-0.30	5.22	-0.53	-0.34±0.09
300	5.00	-0.42	4.89	-0.17	5.45	-0.25	5.00	-0.75	-0.40±0.13

Table I - 8. Effect on systemic plasma calcium of successive hypo- and hypercalcemic perfusion of the thyroid and parathyroid glands in the dog.

Unilateral thyro-parathyroidectomy was performed at the onset of each experiment. Two resin columns were used to maintain each animal in net calcium balance. Normocalcemic blood perfused the contralateral gland area for 30 minutes, hypocalcemic blood (approx. 4 mEq calcium/l) for the next 150 minutes, and hypercalcemic blood (approx. 7 mEq calcium/l) for the last 150 minutes.

Time (minutes)	Dog No. 42		Dog No. 49		Dog No. 71		Dog No. 87		Mean ± S.E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period									
-30	5.71		6.17		5.44		4.67		
-20	5.82		6.17		5.50		4.78		
-10	5.71		6.17		5.50		4.83		
0	5.77		6.11		5.50		4.89		
Hypocalcemic perfusion									
15	5.77	0	6.22	+0.11	5.33	-0.17	4.94	+0.05	0 ± 0.06
30	5.78	+0.01	6.22	+0.11	5.33	-0.17	4.95	+0.06	0 ± 0.06
45	-	-	6.25	+0.14	5.44	-0.06	4.95	+0.06	+0.05 ± 0.06
60	-	-	6.30	+0.19	5.44	-0.06	5.11	+0.22	+0.12 ± 0.09
90	5.83	+0.06	6.40	+0.29	5.56	+0.06	5.06	+0.17	+0.15 ± 0.05
120	5.89	+0.12	6.33	+0.22	5.72	+0.22	5.06	+0.17	+0.18 ± 0.02
150	6.00	+0.23	6.45	+0.34	6.00	+0.50	5.17	+0.28	+0.34 ± 0.06
Hypercalcemic perfusion									
165	5.83	+0.06	6.45	+0.34	6.11	+0.61	-		+0.34 ± 0.07
180	5.61	-0.16	6.35	+0.24	5.78	+0.28	-		+0.12 ± 0.04
195	5.61	-0.16	6.35	+0.24	5.67	+0.17	-		+0.08 ± 0.12
210	5.39	-0.38	6.00	-0.11	5.50	0	-		-0.16 ± 0.11
240	5.39	-0.38	5.80	-0.31	5.28	-0.22	-		-0.30 ± 0.05
270	-	-	5.60	-0.51	5.11	-0.39	-		-0.45 ± 0.09
300	-	-	5.30	-0.81	5.00	-0.50	-		-0.66 ± 0.15

Table I - 9. Effect on systemic plasma calcium of successive hypo- and hypercalcemic perfusion of the neck strap muscles of the dog after thyro-parathyroidectomy.

Unilateral thyro-parathyroidectomy was performed at the onset of each experiment. After a 30 minute normocalcemic perfusion of the contralateral gland area, all the remaining thyroid and parathyroid tissue was removed. Hypocalcemic blood (approx. 4 mEq calcium/l) was used to perfuse the muscular branches of the thyroid artery on one side for 150 minutes followed by hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l for an additional 150 minutes.

Time (minutes)	Dog No. 59		Dog No. 61		Dog No. 68		Dog No. 69		Mean ± S.E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period									
-30	5.25		5.50		5.17		5.79		
-20	5.19		5.50		5.39		5.83		
-10	5.19		5.40		5.39		5.83		
0	5.13		5.35		5.39		5.83		
Hypocalcemic perfusion									
15	5.13	0	5.30	-0.05	5.39	0	5.78	-0.05	-0.03 ± 0.02
30	5.06	-0.07	5.30	-0.05	5.37	-0.02	5.72	-0.11	-0.06 ± 0.02
45	5.13	0	5.30	-0.05	5.32	-0.07	5.89	+0.06	-0.02 ± 0.03
60	5.06	-0.07	5.35	0	5.22	-0.07	5.88	+0.05	-0.02 ± 0.03
90	5.00	-0.13	5.30	-0.05	5.22	-0.07	5.83	0	-0.06 ± 0.03
120	5.00	-0.13	5.25	-0.10	5.11	-0.28	5.94	+0.11	-0.10 ± 0.10
150	5.06	-0.07	5.25	-0.10	5.00	-0.39	5.90	+0.07	-0.12 ± 0.10
Hypercalcemic perfusion									
165	5.00	-0.13	5.25	-0.10	5.06	-0.33	5.83	0	-0.14 ± 0.07
180	4.94	-0.19	5.25	-0.10	5.06	-0.33	5.94	+0.11	-0.18 ± 0.05
195	5.00	-0.13	5.25	-0.10	5.11	-0.28	5.89	+0.06	-0.14 ± 0.05
210	5.00	-0.13	5.25	-0.10	5.11	-0.28	5.78	-0.05	-0.14 ± 0.05
240	5.13	0	5.30	-0.05	5.11	-0.28	5.71	-0.12	-0.11 ± 0.06
270	5.25	+0.12	5.30	-0.05	5.06	-0.33	5.59	-0.24	-0.13 ± 0.10
300	-	-	5.35	0	5.14	-0.24	5.35	-0.48	-0.18 ± 0.12

slight fall was obtained in the fourth. A fall during the second $2\frac{1}{2}$ hr. period was seen as anticipated and could be accounted for by parathyroidectomy.

An average fall of 1 percent occurred during hypercalcemic perfusion following thyro-parathyroidectomy. In contrast a 13 percent fall was observed during high calcium perfusion of the thyro-parathyroid apparatus. These studies show the fall obtained in the experimental series was real and not due to high calcium inhibition of PTH production. A 12 percent fall greater than that which could be attributed to removal of the parathyroids alone was demonstrated.

DISCUSSION

The results are in essential agreement with those of Copp et al (1962). They confirm that (1) PTH production is stimulated by low calcium, (2) a plasma calcium-lowering hormone is elaborated in response to high calcium, (3) it is fast-acting and (4) this hormonal substance must be secreted by either the thyroids or parathyroids.

The data conclusively shows that the servo system originally proposed by McLean and Urist (1961) to account for calcium homeostasis is incomplete. Confirmation of the existence of a second calcium regulatory hormone of opposite effect to PTH necessitates

proposing a double feedback mechanism. In such a system low plasma calcium would stimulate the secretion of PTH and high plasma calcium the secretion of calcitonin. Since PTH is a relatively slow acting hormone with prolonged effect (Rasmussen, 1961) the relatively rapidly acting calcitonin would be responsible for preventing overshoots or oscillations in plasma calcium. Copp et al (1963) have shown that variations in plasma calcium of ± 5 percent are sufficient to stimulate corrective responses in either direction.

Further support for an antagonist to PTH is found in experiments in animals in which the thyroids have been removed. The effect of PTH following thyro-parathyroidectomy is more marked in the dog (Foster, unpublished) and in the rat (Hirsh, personal communication; Cameron and Copp, 1963) than in the intact animal. The experiments illustrate the "overshoots" that might be seen if a second hormone was not present.

The studies reported in this chapter do not show whether the hormone is secreted by the thyroid or parathyroids. At the time this work was done it seemed reasonable to believe the parathyroids were most likely involved. The evidence was three-fold:

First, Copp et al (1962) reported that the calcium-lowering effect could not be demonstrated following parathyroidectomy. Elevated plasma calcium values produced in dogs by low calcium perfusion of the thyro-parathyroids could not be depressed by high calcium perfusion of the thyroid after the parathyroids were removed. Later inquiry revealed that in this experiment what had been reported as parathyroidectomies had been thyro-parathyroidectomies (Copp, personal communication).

Second, in extracorporeal high calcium perfusion experiments thyroid perfusates produced no fall in the hemi-thyro-parathyroidectomized dog, whereas thyro-parathyroid perfusates did (Copp et al , 1962). The falls reported, however, were only of the order of 5 percent in 2 hr. compared with previous falls of greater than 10 percent.

Third, transitory small falls in plasma calcium had been observed in dogs after intravenous administration of massive amounts of crude preparations of PTH (Copp and Cameron, 1961; Copp et al, 1962). These falls were seldom greater than 5 percent and were not always

reproducible. Subsequent studies in our laboratory using similar extracts in similar doses were found to produce marked hypotension and occasionally death without showing significant hypocalcemia (See Table II - 8).

Suffice it to say that prior to the studies to be presented in the next three chapters, objective evaluation of Copp's original work and the Hammersmith confirmation favoured a parathyroid origin for calcitonin (editor, B.M.J., 1963).

Chapter II

Presumptive Evidence for the Thyroid Origin of Calcitonin

Summary

The following three experiments supported the possibility of a thyroid source of calcitonin

(1) Damage to rats' thyroid by electro-cautery produced a fall in plasma calcium.
(2) Acid extracts of dog thyroid when injected intravenously into dogs produced a prompt and marked hypocalcemic response.
(3) High calcium perfusion of the dog thyroid after parathyroidectomy produced a systemic fall in plasma calcium, greater than that observed following parathyroidectomy without thyroid perfusion. The possibility of performing isolated parathyroid perfusion was investigated and found not feasible.

A hypocalcemia producing substance from the parathyroid was excluded by finding no calcium-lowering activity in fractionated PTH extracts as assayed in the rat.

Having confirmed the existence of calcitonin, it was necessary to establish its source. Evidence for a parathyroid origin has already been reviewed at the end of the last chapter.

Late in 1963 the possibility of a calcium-lowering thyroid factor was proposed by Hirsch, Gauthier and Munson (1963). These investigators reported the hypocalcemic response of rats to parathyroidectomy differed significantly depending on whether ablation was performed by cauterization or surgery. With parathyroidectomy by cautery, serum calcium levels fell 40 percent in 5 hr.

If the parathyroids were removed surgically or total thyro-parathyroidectomy was performed, only 25 percent falls were seen. Interestingly, falls in plasma calcium occurred when the thyroids were cauterized even without parathyroidectomy. These results strongly suggested that cauterization released a calcium-lowering substance from the thyroid. In addition, when rats parathyroidectomized by cautery were thyroidectomized 3 hr. later, a rise of 10 percent occurred during the following 3 hr. This finding was compatible with the removal of the source of the calcium-lowering factor. Finally, these workers observed that the serum calcium declined 20 percent 5 hr. following unilateral parathyroidectomy by cautery, but no fall was seen in similar experiments in which the parathyroids were surgically removed.

Later the same year it was reported that crude acid extracts of thyroid produced hypocalcemia in the rat (Hirsch, Gauthier and Munson, 1963) and in the dog (Foster, Kumar and MacIntyre, 1964). Because of the possibility that the thyroid hypocalcemia producing factor might prove to be identical with calcitonin, the Harvard group tentatively proposed the name "thyrocalcitonin" (Hirsch, Gauthier and Munson, 1963).

The following experiments confirm that electro-cautery of the thyroid produces a fall in blood calcium in the rat, show that a plasma

calcium-lowering factor can be extracted from dog thyroid and, finally, demonstrate that the plasma calcium-lowering factor is released from the thyroid under hypercalcemic conditions.

Experiment 1 - Verification of hypocalcemia produced in the rat by cauterization.

Methods: Male 200-230 g Hammersmith strain piebald rats, fed on a normal house diet were fasted overnight prior to use. Anaesthesia was induced with sodium pentobarbitone given subcutaneously (5 mg/rat Abbott's Veterinary Nembutal) and maintained with ether. The necks were exposed by longitudinal incision. Subcutaneous tissue and salivary glands were freed by blunt probe dissection and displaced laterally. The strap muscles of the neck were separated exposing the two thyroid lobes lying on either side of the trachea just below the thyroid cartilage. The lower poles were cauterized with an electric needle bilaterally to the same extent had parathyroidectomy been done by this technique. Since the parathyroids are invariably associated with the upper poles, there was little chance to damage them during this procedure. The rats were allowed to recover. Aortic blood samples were drawn at 30, 90, 180 and 360 min. following cauterization. 5-8 animals were assayed at each time interval. In addition, 7 rats were not cauterized and served as controls.

Results: The results are shown in Figure II - 1 and Table II - 1. A fall in plasma calcium was evident within 30 min., reached a maximum of 10 percent in 3 hr. and had returned to normal after 6 hr. The data confirms the findings of Hirsch et al (1963), but the observed falls were neither as marked or as prolonged. Falls as great as 35 percent in 3 hr. had been produced by the Harvard group using animals in which parathyroidectomy had accompanied cautery. This difference may be explained as follows:

First, the rats used in the present experiment were older and presumably had smaller exchangeable bone calcium pools than those used by Hirsch.

Second, falls were minimized in these animals in which the parathyroids had been left intact and which were capable of secreting parathyroid hormone in response to low blood calcium.

In summary, the results support a thyroid plasma calcium-lowering factor which is released by injury. They further delineate the time course of the resulting hypocalcemia.

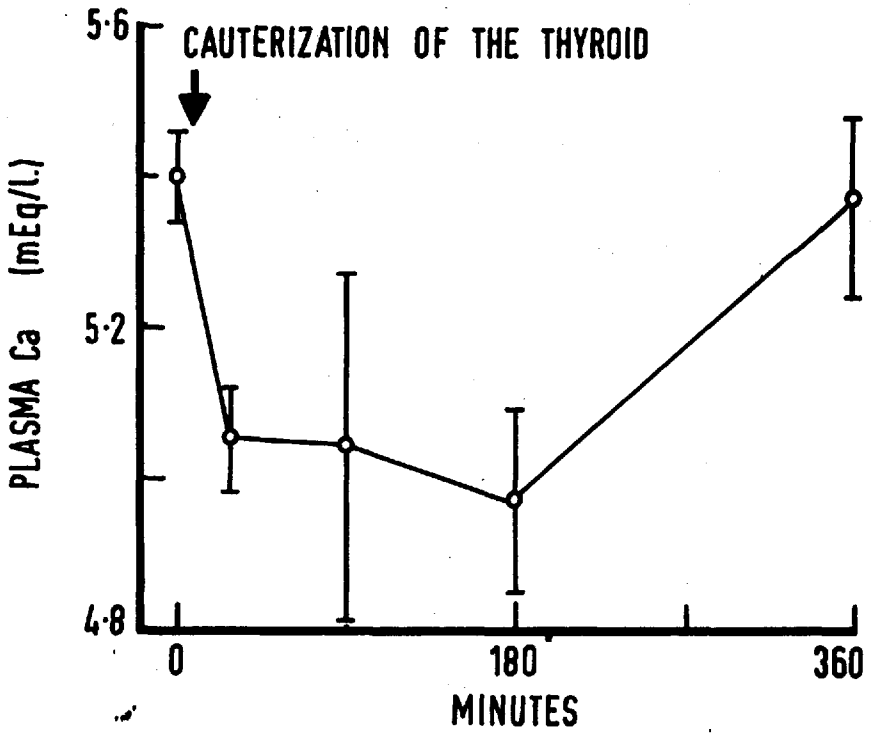


Figure II - 1. Changes in plasma calcium observed after bilateral cauterization of the lower poles of the rat thyroid. Each point represents the mean average of 5-8 animals. The vertical lines indicate the standard error of the mean.

Table II - 1. Plasma calcium and phosphate values following cauterization of the lower poles of the thyroid gland in the rat.

Time (min.)	No. rats	Plasma Calcium (mEq/l) mean ± S.E. (range)	Plasma Phosphate (mEq/l) mean ± S.E. (range)
0	7	5.40 ± 0.06 (5.25-5.65)	3.58 ± 0.06 (3.36-3.76)
30	5	5.05 ± 0.07 (4.90-5.25)	3.72 ± 0.13 (3.39-4.13)
90	6	5.04 ± 0.23 (4.55-5.70)	3.46 ± 0.23 (2.76-4.29)
180	7	4.97 ± 0.12 (4.45-5.35)	3.61 ± 0.18 (3.08-4.03)
360	8	5.36 ± 0.12 (5.10-6.10)	3.53 ± 0.13 (3.10-3.95)

Experiment 2 - Demonstration of extraction of calcium-lowering activity from dog thyroids.

Methods:

(1) Animal preparation. These studies were carried out in two normal healthy dogs, fasted overnight. The animals were anaesthetized with sodium pentobarbitone (30-35 mg/kg) intravenously and restrained on a dissecting board. A cut-down of a forepaw vein was cannulated with small bore polythylene tubing. All subsequent injections were given by this route. Anaesthesia was maintained with doses of 25-50 mg. of pentobarbitone as needed. A longitudinal incision was made in the neck and blunt dissection of the common carotid artery on one side was carried out as previously described. The opposite thyro-parathyroid apparatus was removed and the tissue saved. 1,000 units of sodium heparin (Evans Ltd.) was given initially and repeated hourly. 10 min. following heparinization, the dissected common carotid artery was cannulated with a 15 cm long by-pass circuit. The tubing used was silicone tubing fitted at either end with plastic cannulae as described in the previous chapter. Appropriate outlets were fitted to allow for sample taking and for attachment of a mercury

manometer to allow for blood pressure readings during the course of the experiment.

(2) Extract. The thyro-parathyroid apparatus removed from the same animal was carefully stripped of its external capsule and attending fat. Two well defined parathyroids were removed. 1.18 g of tissue was cut from the central portion of the remaining thyroid. This was minced with a scalpel blade and ground in a mortar in the presence of 10 ml of 0.1M-hydrochloric acid. The homogenized digest was heated in a water bath at 75° for 5 min. Digestion was carried out at room temperature for another 2 hr. and the residue removed by centrifugation. The resulting crude extract had a bright yellow colour and was free of obvious particular matter. The extract was administered intravenously to two intact dogs in doses of 0.1 ml which contained less than 0.6 mg protein nitrogen. To ascertain if the effect could be due to contaminating thyroxine, 120 µg of l-thyroxine was given to a third dog.

Results: The results are seen in Figures II - 2, II - 3 and II - 4 and Tables II - 2, II - 3 and II - 4. A single dose of thyroid extract lowered the plasma calcium 5 percent within 30 min. in one animal (Figure II - 2 and Table II - 2). Thereafter the plasma calcium began

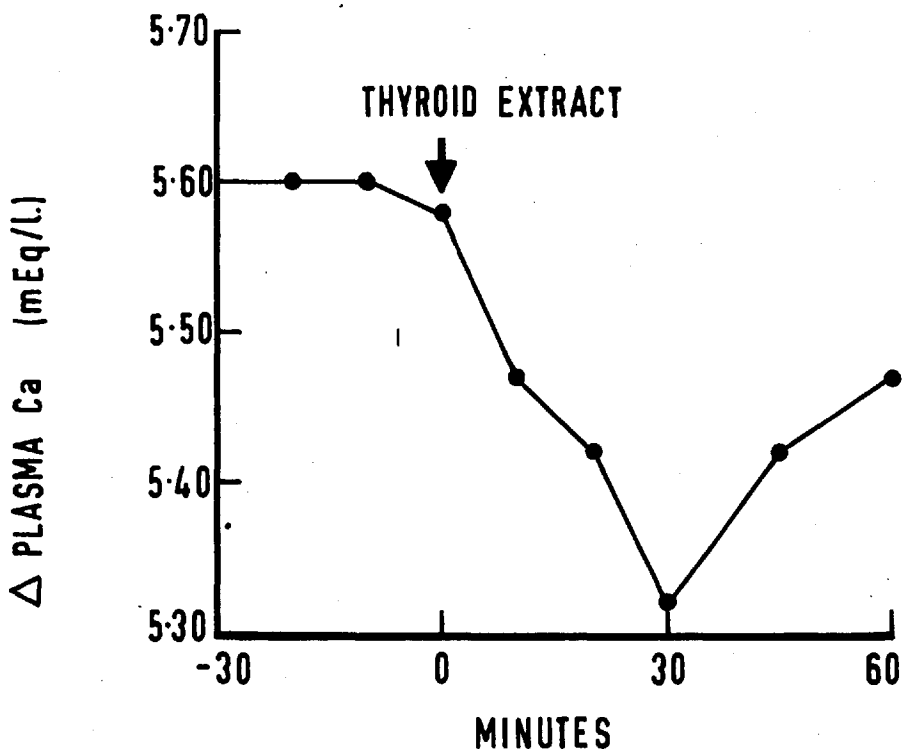


Figure II - 2. Effect on plasma calcium of dog thyroid extract in a 17.9 kg intact dog (No. 84). The extract was prepared by digestion of fresh frozen thyroid with 0.2N-hydrochloric acid. The dose given was prepared from 0.018 g of tissue and was administered intravenously.

Table II - 2. Effect on plasma calcium of extract of dog thyroid in the intact dog (No. 84).

At time zero 0.1 ml of an 0.2N-hydrochloric acid extract prepared from 0.018 g of fresh frozen dog thyroid was given intravenously.

Time (min.)	Plasma Calcium mEq/l
- 30	5.60
- 20	5.60
- 10	5.60
0	5.58
+ 10	5.47
+ 20	5.42
+ 30	5.32
+ 45	5.42
+ 60	5.47

to return to normal. In a second animal (Figure II - 3 and Table II - 3) the same dose was given every 10 min. for 4 doses. The plasma calcium fell approximately 10 percent reaching its maximum depression 90 min. after the initial dose. The plasma calcium remained depressed even after $2\frac{1}{2}$ hr. The falls in plasma calcium could not be attributed to thyroxine. As can be seen in Figure II - 4 and Table II - 4, 120 µg of l-thyroxine, administered intravenously, failed to produce any significant change.

These preliminary experiments showed for the first time that the calcium-lowering factor was extractable from dog thyroid. The results suggested that the material extracted was probably the same as that released by electrocautery of the rat thyroid.

Experiment 3 - Hypocalcemia produced by high calcium perfusion of the thyroid in the dog.

This experiment was designed to show if the thyroid, in the absence of the parathyroids, could produce a systemic plasma calcium-lowering effect.

Methods: The animals used were healthy mongrel dogs of both sexes varying in weight between 12 - 17 kg and fasted overnight. Anaesthesia, heparinization, and employment of the double resin column apparatus were the same as described in Experiment 2 in the last chapter. Similarly, each experiment consisted of a half

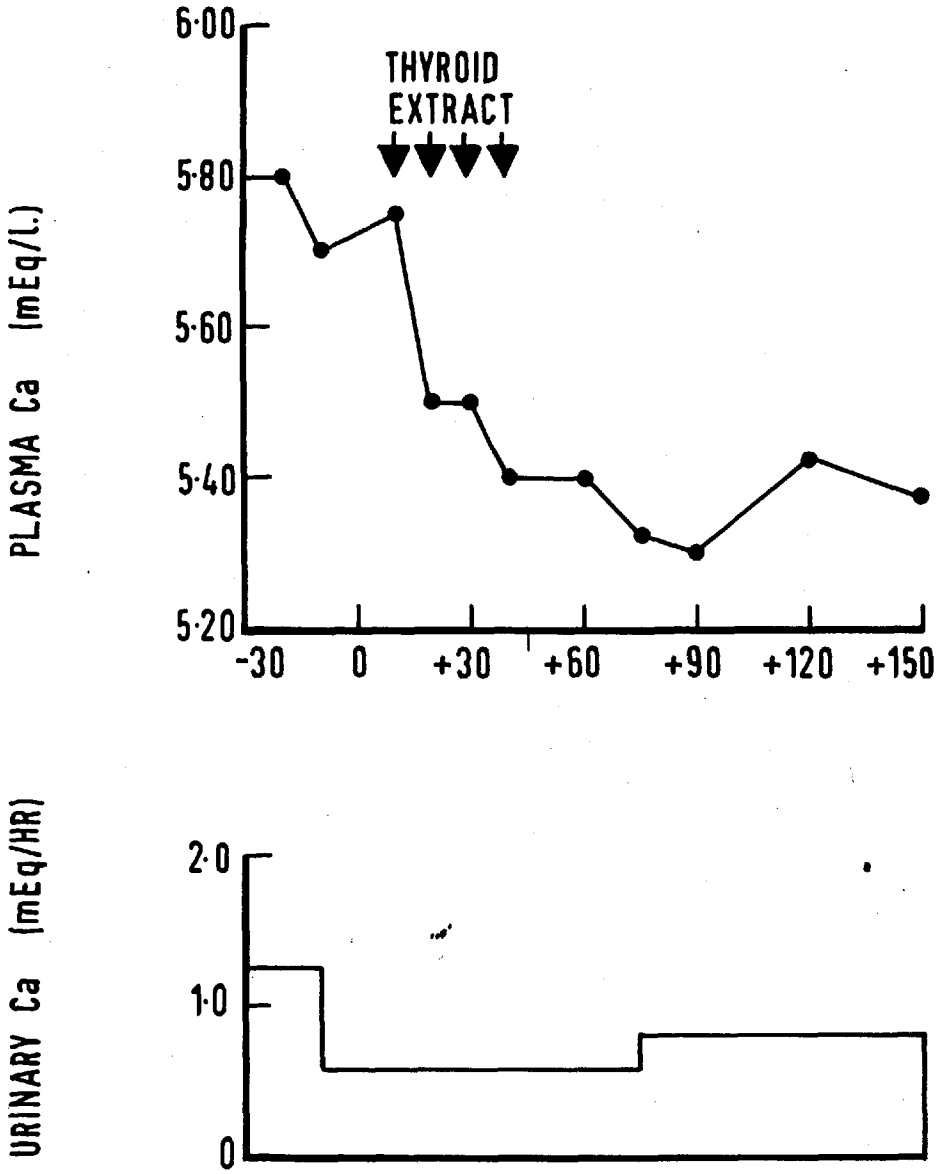


Figure II - 3. Effect on plasma and urinary calcium of multiple intravenous injections of dog thyroid extract in a 12.9 kg intact dog (No. 85). Each injection contained activity from 0.018 g of fresh frozen extract digested in 0.2N-hydrochloric acid.

Table II - 3. Effect on calcium of multiple injections of extract of dog thyroid in the intact dog (No. 85).

At time 0, 15, 30 and 45 min. 0.1 ml of an 0.2N-hydrochloric acid extract prepared from 0.018g of fresh frozen dog thyroid was given intravenously.

BLOOD	
Time (min.)	Plasma Calcium mEq/l
-30	5.80
-20	5.80
-10	5.70
0 (injection)	5.75
+15 (injection)	5.50
+30 (injection)	5.50
+45 (injection)	5.40
+60	5.40
+75	5.35
+90	5.30
+120	5.45
+150	5.35

URINE	
Period	mEq calcium per hr.
Pre-injection -30 - 0 min.	1.24
Post-injection 0 - 75 min.	.57
75 - 150 min.	.80

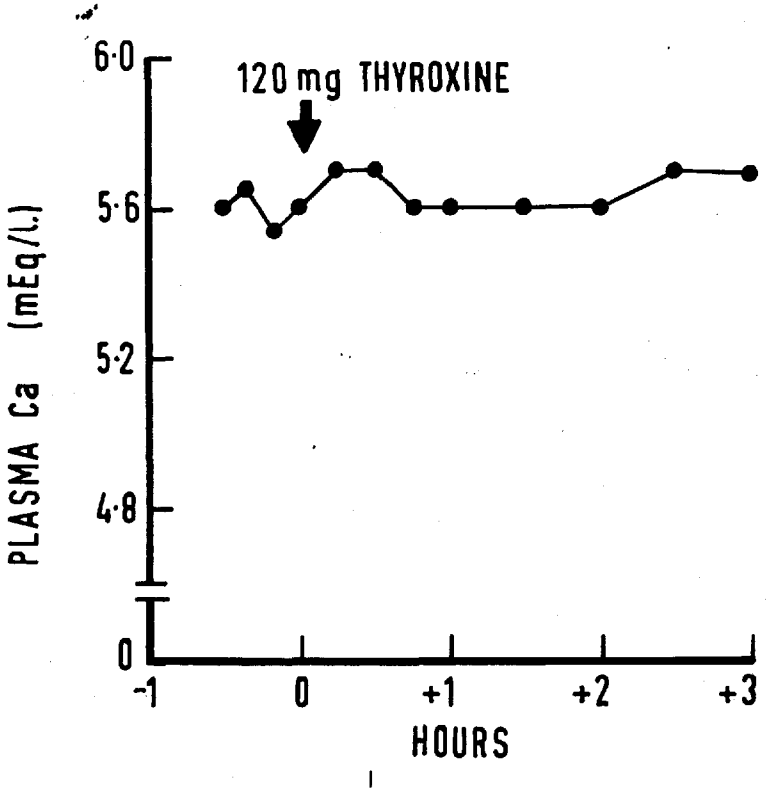


Figure II - 4. Effect of intravenous administration of sodium l-thyroxine to an intact dog.

Table II - 4. Plasma calcium values observed in a 16 kg dog following 120 μg thyroxine administered intravenously.

Time (min.)	Plasma calcium mEq/l
- 30	5.60
- 20	5.65
- 10	5.55
0	5.60
15	5.70
30	5.70
45	5.60
60	5.60
90	5.60
120	5.60
150	5.70
180	5.70

hour control period, followed by a $2\frac{1}{2}$ hr. high calcium perfusion and finally a $2\frac{1}{2}$ hr. low calcium perfusion. Blood pressure was monitored throughout all experiments. Animals which developed hypotension (blood pressure below 60 mm Hg) were not included in the series. Blood samples were collected and assayed as previously described.

Three groups of seven animals each were studied. In the first group the neck strap muscles alone were perfused via the muscular branches from the thyroid artery. The contralateral thyro-parathyroid was removed before perfusing with high calcium. In the second series the remaining parathyroids alone were removed prior to high calcium perfusion leaving only the thyroid. Parathyroidectomy was done by raising a parathyroid from the surrounding thyroid matrix with forceps or a small clamp and tying it below at its base. It was then cut free with little danger of injuring the thyroid tissue itself. No animals were included in this group in which two discrete parathyroids could not be readily identified and removed without question. Finally, in the third group the remaining thyroid and its adjacent parathyroids were left intact and perfused as such.

Results: The three diagrams in Figure II - 5 (Tables II - 5, II - 6 and II - 7) show the plasma calcium changes during perfusions in the

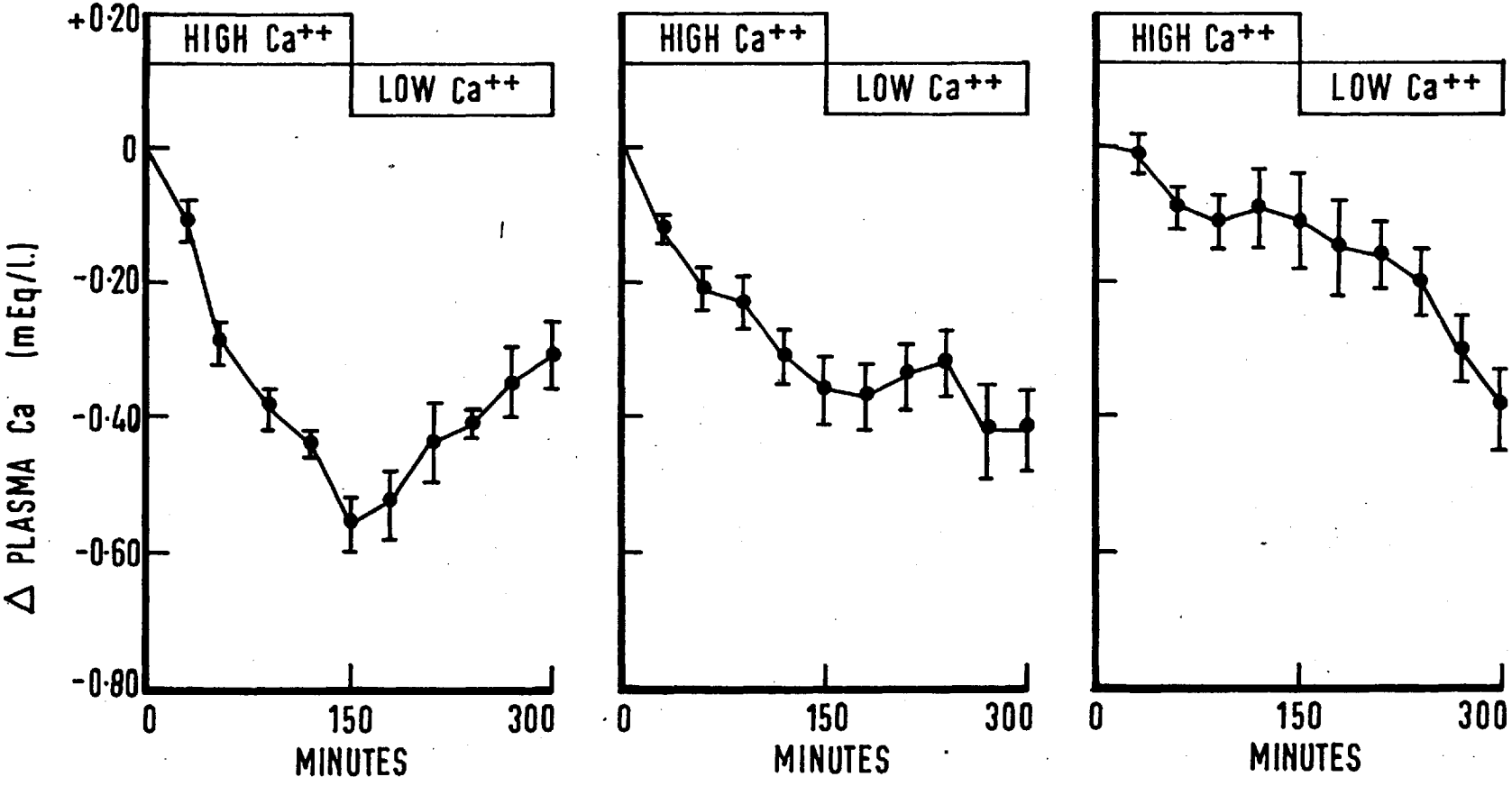


Figure II - 5. Change in plasma calcium observed in the dog during unilateral hypercalcemic perfusion of (a) the intact thyro-parathyroid body, (b) the thyroid alone after surgical removal of the adjacent parathyroids and (c) the neck strap muscles after removal of the thyro-parathyroids. In each animal the contralateral glands had been removed prior to perfusion. The mean of changes in plasma calcium for each time for five animals in each group is represented as a single point and the standard error of the mean as a vertical bar. Dogs were maintained in net calcium balance in all experiments.

Table II - 5. Changes in plasma calcium observed during successive hyper- and hypo-calcemic perfusion of the neck strap muscles in the dog.

Unilateral thyro-parathyroidectomy was performed at the onset of each experiment. Thyroid and parathyroid tissue on the contralateral side was removed at the conclusion of a 30 min. normocalcemic perfusion of the muscular branches of the thyroid artery. There followed a 150 min. hypercalcemic perfusion (approx. 7 mEq calcium/l) and an additional 150 min. hypocalcemic perfusion (approx. 4 mEq calcium/l).

Time (minutes)	No. 60		No. 62		No. 65		No. 66		No. 67		No. 102		No. 104		Mean ± S. E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period															
0	5.45		5.17		5.55		5.80		5.80		5.47		5.10		
10	5.44		5.06		5.70		5.85		5.85		5.42		5.10		
20	5.50		5.06		5.70		5.85		5.85		5.42		5.10		
30	5.42		5.06		5.70		5.75		5.80		5.47		5.10		
Hypercalcemic perfusion															
45	5.37	-0.05	5.00	-0.06	5.60	-0.10	5.70	-0.05	5.82	+0.02	5.47	0	5.00	-0.10	-0.05 ± 0.02
60	5.40	-0.02	5.00	-0.06	5.75	+0.05	5.70	-0.05	5.91	+0.11	5.42	-0.05	5.00	-0.10	-0.01 ± 0.03
75	5.35	-0.07	5.00	-0.06	5.85	+0.15	5.70	-0.05	5.82	+0.02	5.35	-0.12	5.00	-0.10	-0.03 ± 0.03
90	5.45	+0.03	4.94	-0.12	5.60	-0.10	5.60	-0.15	5.82	+0.02	5.37	-0.10	4.90	-0.20	-0.09 ± 0.03
120	5.45	+0.03	4.94	-0.12	5.50	-0.20	5.50	-0.25	5.82	+0.02	5.42	-0.05	4.90	-0.20	-0.11 ± 0.04
150	5.45	+0.03	5.00	-0.06	5.50	-0.20	5.47	-0.28	5.90	+0.10	5.47	0	4.85	-0.25	-0.09 ± 0.06
180	5.40	+0.02	5.00	-0.06	5.40	-0.30	5.47	-0.28	5.90	+0.10	5.53	+0.06	4.80	-0.30	-0.11 ± 0.07
Hypocalcemic perfusion															
165	5.35	-0.07	5.00	-0.06	5.40	-0.30	5.42	-0.05	5.80	0	5.40	-0.07	4.75	-0.35	-0.12 ± 0.05
180	5.35	-0.07	5.00	-0.06	5.30	-0.40	5.47	0	5.80	0	5.35	-0.12	4.70	-0.40	-0.15 ± 0.07
195	5.30	-0.12	5.00	-0.06	5.40	-0.30	5.42	-0.05	5.80	0	5.40	-0.07	4.75	-0.35	-0.14 ± 0.05
210	5.30	-0.12	5.00	-0.06	5.40	-0.30	5.33	-0.14	5.80	0	5.37	-0.10	4.70	-0.40	-0.16 ± 0.05
240	5.20	-0.22	5.00	-0.06	5.40	-0.30	5.22	-0.25	5.70	-0.10	5.37	-0.10	4.70	-0.40	-0.20 ± 0.05
270	5.00	-0.42	4.94	-0.12	5.40	-0.30	5.22	-0.25	5.50	-0.30	5.26	-0.21	4.60	-0.50	-0.30 ± 0.05
300	5.00	-0.42	4.89	-0.17	5.45	-0.25	5.00	-0.47	5.40	-0.40	5.15	-0.32	4.40	-0.70	-0.39 ± 0.06

Table II - 6. Changes in plasma calcium observed during successive hyper- and hypo-calcemic perfusion of the thyroid gland in the dog.

Unilateral thyro-parathyroidectomy was performed at the onset of each experiment. At the conclusion of a 30 min. normocalcemic perfusion to the contralateral gland area, the remaining parathyroids were removed. Perfusion of the remaining thyroid was carried out with hypercalcemic blood (approx. 7mEq calcium/l) for 150 min., followed by hypocalcemic blood (approximately 4 mEq calcium/l) for an additional 150 min. In all experiments the animals were maintained in net calcium balance.

Time (minutes)	No. 56		No. 92		No. 93		No. 94		No. 95		No. 101		No. 106		Mean ± S.E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period															
-30	5.18		5.05		4.70		4.83		5.11		5.50		5.25		
-20	5.12		5.05		4.80		4.83		5.11		5.50		5.30		
-10	5.12		5.05		4.65		4.83		5.11		5.50		5.26		
0	5.18		5.00		4.75		4.89		5.06		5.50		5.25		
Hypercalcemic perfusion															
15	5.12	-0.06	4.89	-0.11	4.70	-0.05	4.83	-0.06	5.06	0	5.33	-0.17	5.20	-0.05	-0.07 ± 0.02
30	5.12	-0.06	4.84	-0.16	4.55	-0.20	4.83	-0.06	4.94	-0.12	5.32	-0.18	5.20	-0.05	-0.12 ± 0.02
45	5.12	-0.06	4.68	-0.32	4.60	-0.15	4.83	-0.06	4.89	-0.17	5.21	-0.29	5.15	-0.10	-0.16 ± 0.04
60	5.10	-0.08	4.74	-0.26	4.50	-0.25	4.72	-0.17	4.83	-0.23	5.17	-0.33	5.11	-0.14	-0.21 ± 0.03
90	5.00	-0.18	4.63	-0.37	4.50	-0.25	4.78	-0.11	4.83	-0.23	5.16	-0.34	5.11	-0.14	-0.23 ± 0.04
120	4.88	-0.30	4.50	-0.50	4.50	-0.25	4.72	-0.17	4.78	-0.28	5.16	-0.34	4.95	-0.30	-0.31 ± 0.04
150	4.82	-0.36	4.42	-0.58	4.50	-0.25	4.61	-0.28	4.72	-0.34	5.05	-0.45	5.00	-0.25	-0.36 ± 0.05
Hypocalcemic perfusion															
165	4.75	-0.43	4.37	-0.63	4.50	-0.25	4.61	-0.28	4.78	-0.28	5.06	-0.44	4.95	-0.30	-0.37 ± 0.05
180	4.75	-0.43	4.42	-0.58	4.50	-0.25	4.50	-0.39	4.82	-0.24	5.06	-0.44	5.00	-0.25	-0.37 ± 0.05
195	4.88	-0.30	4.47	-0.53	4.50	-0.25	4.50	-0.39	4.76	-0.30	5.00	-0.50	5.00	-0.25	-0.36 ± 0.04
210	4.88	-0.30	4.39	-0.61	4.50	-0.25	4.50	-0.39	4.82	-0.24	5.11	-0.39	5.05	-0.20	-0.34 ± 0.05
240	4.94	-0.24	4.37	-0.63	4.55	-0.20	4.50	-0.39	4.89	-0.17	5.11	-0.39	4.95	-0.30	-0.32 ± 0.05
270	4.82	-0.36	4.42	-0.58	4.50	-0.25	4.44	-0.45	4.89	-0.17	5.06	-0.44	4.53	-0.72	-0.42 ± 0.07
300	4.71	-0.47	4.42	-0.58	4.50	-0.25	4.39	-0.50	4.94	-0.12	5.00	-0.50	4.74	-0.51	-0.42 ± 0.06

Table II - 7. Changes in plasma calcium observed during successive hyper- and hypo-calcemic perfusion of the thyroid and parathyroid glands in the dog.

Unilateral thyro-parathyroidectomy was performed at the onset of each experiment. The glands on the contralateral side were perfused with normocalcemic blood for 30 min., and subsequently with hypercalcemic blood (approx. 7 mEq calcium/l) for 150 min., followed by hypocalcemic blood (approx. 4 mEq calcium/l) for a similar period. In all experiments the animals were maintained in net calcium balance.

Time (minutes)	No. 45		No. 50		No. 74		No. 99		No. 100		No. 103		No. 105		Mean ± S.E.
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period															
-30	6.00		5.75		5.47		5.35		5.45		5.47		5.68		
-20	6.00		5.70		5.47		5.35		5.45		5.47		5.79		
-10	6.00		5.75		5.44		5.35		5.50		5.42		5.74		
0	6.00		5.68		5.44		5.35		5.50		5.42		5.74		
Hypercalcemic erfusion															
15	5.88	-0.12	5.78	+0.10	5.37	-0.07	5.30	-0.05	5.40	-0.10	5.37	-0.05	5.70	-0.04	-0.05 ± 0.03
30	5.68	-0.32	5.78	+0.10	5.32	-0.12	5.25	-0.10	5.30	-0.20	5.39	-0.03	5.63	-0.11	-0.11 ± 0.03
45	5.68	-0.32	5.58	-0.10	5.16	-0.28	5.20	-0.15	5.20	-0.30	5.22	-0.22	5.53	-0.21	-0.23 ± 0.03
60	5.63	-0.37	5.44	-0.24	5.11	-0.33	5.15	-0.20	5.10	-0.40	5.17	-0.25	5.53	-0.21	-0.29 ± 0.03
90	5.50	-0.50	5.33	-0.35	5.06	-0.38	5.05	-0.30	5.05	-0.45	5.11	-0.31	5.44	-0.30	-0.37 ± 0.03
120	5.40	-0.60	5.33	-0.35	4.90	-0.54	4.89	-0.46	5.05	-0.45	5.05	-0.37	5.44	-0.30	-0.44 ± 0.04
150	5.26	-0.74	5.00	-0.68	4.79	-0.65	4.79	-0.56	5.00	-0.50	5.00	-0.42	5.39	-0.35	-0.56 ± 0.05
Hypocalcemic perfusion															
165	5.13	-0.87	5.00	-0.68	4.79	-0.65	4.79	-0.56	5.00	-0.50	5.00	-0.42	5.44	-0.30	-0.57 ± 0.07
180	5.26	-0.74	5.00	-0.68	4.84	-0.60	4.79	-0.56	5.00	-0.50	5.00	-0.42	5.50	-0.24	-0.53 ± 0.06
195	5.40	-0.60	5.00	-0.68	4.84	-0.60	4.84	-0.51	5.10	-0.40	4.94	-0.48	5.50	-0.24	-0.50 ± 0.06
210	5.40	-0.60	5.28	-0.40	4.84	-0.60	4.84	-0.51	5.15	-0.35	5.10	-0.32	5.44	-0.30	-0.44 ± 0.05
240	5.60	-0.40	5.33	-0.35	4.95	-0.49	4.90	-0.45	5.10	-0.40	5.10	-0.32	5.28	-0.46	-0.41 ± 0.02
270	5.60	-0.40	5.44	-0.24	5.00	-0.44	4.90	-0.45	5.10	-0.40	5.35	-0.07	5.32	-0.42	-0.35 ± 0.05
300	5.67	-0.33	5.61	-0.07	5.05	-0.39	5.00	-0.35	5.10	-0.40	5.25	-0.17	5.28	-0.46	-0.31 ± 0.05

following order: the neck strap muscles, a single thyroid, and an intact thyroid and parathyroid. During the $2\frac{1}{2}$ hr. high calcium perfusion of the strap muscles a fall of approximately 3 percent was observed. During the subsequent $2\frac{1}{2}$ hr. the fall was more marked. These falls are caused by parathyroid removal and represent anticipated changes due to the absence of PTH. In the absence of all thyroid and parathyroid tissue the perfusion of neck strap muscles with varying concentrations of calcium would not be expected to cause any changes.

In comparison to the control group the changes seen during perfusion of the intact thyro-parathyroid apparatus were dramatic. A fall of 12 percent was observed during high calcium perfusion. This fall was quite comparable to those obtained in Experiment 2 in the preceding chapter. The fall is presumably due to:

(1) inhibition of PTH production

and (2) secretion of calcitonin.

The viability of the parathyroids was demonstrated by the subsequent rise in plasma calcium during low calcium stimulation. This rise was far more rapid than could be expected after massive injections of PTH. One explanation is that the rise was due to inhibition of calcitonin secretion. A second explanation is that during high calcium perfusion plasma calcium was deposited in the exchangeable

calcium bone pool. During the following low calcium perfusion period when PTH production was stimulated, more calcium was available to be released from bone than under normal circumstances. Possibly the calcium most recently deposited in tissues is more readily displaced by PTH than calcium that has had more time to become "fixed".

Finally, hypercalcemic perfusion of the isolated thyroid without question produced a calcitonin effect. The fall was in the order of 7 percent. Two explanations can account for the fall being less than that noted during thyro-parathyroid perfusion. First, surgical trauma during parathyroidectomy may have caused local vasoconstriction. This could have caused a diminution in calcitonin production. Second, parathyroidectomy itself may have caused the release of PTH which would have had the effect of raising the plasma calcium.

The objection might be raised that the fall in this experiment was due to the presence of residual parathyroid tissue within the thyroid matrix. This possibility is most unlikely. Low calcium perfusion to the thyroid did not produce a rise as would be anticipated had PTH been secreted in response to this stimulus. Kumar and De Bats (Foster, Kumar, Baghdiantz, Soliman, Slack, De Bats and MacIntyre, 1965) investigated the possibility of ectopic parathyroid tissue within the thyroid of the dog. They removed all visible external parathyroid tissue from

twenty consecutive thyroid lobes. In only one instance did they find evidence of discrete parathyroid tissue within the thyroid.

In conclusion, the thyroid was shown to be at least partially responsible for the fall in plasma calcium observed during hypercalcemic stimulation. The experiment did not exclude the possibility that the parathyroid played some role.

Experiment 4 - Investigation of possibility of parathyroid perfusion in the dog.

Unfortunately, the close association of the parathyroids with the thyroid in the dog prevented parathyroid perfusion studies in this animal using the previously described method. The possibility of developing a microtechnique to accomplish such a study was investigated.

Methods: A healthy mongrel dog which had been used for extract assay studies was sacrificed. A segment of the common carotid above and below the thyroid artery was isolated with silk ties. The segment was injected with 2 ml of a suspension of India Ink in a solution of gelatine (Reid and Heard, 1963). See Figure II - 6. The dye traversed the thyroid artery and filled the vessels of the thyro-parathyroid apparatus. The gland's pedicles were tied off and the thyro-parathyroid body was removed and stored at 40°. The soft tissue was subsequently cleared by Dawson's technique (Culling, 1963) to show the position of the internal blood vessels.

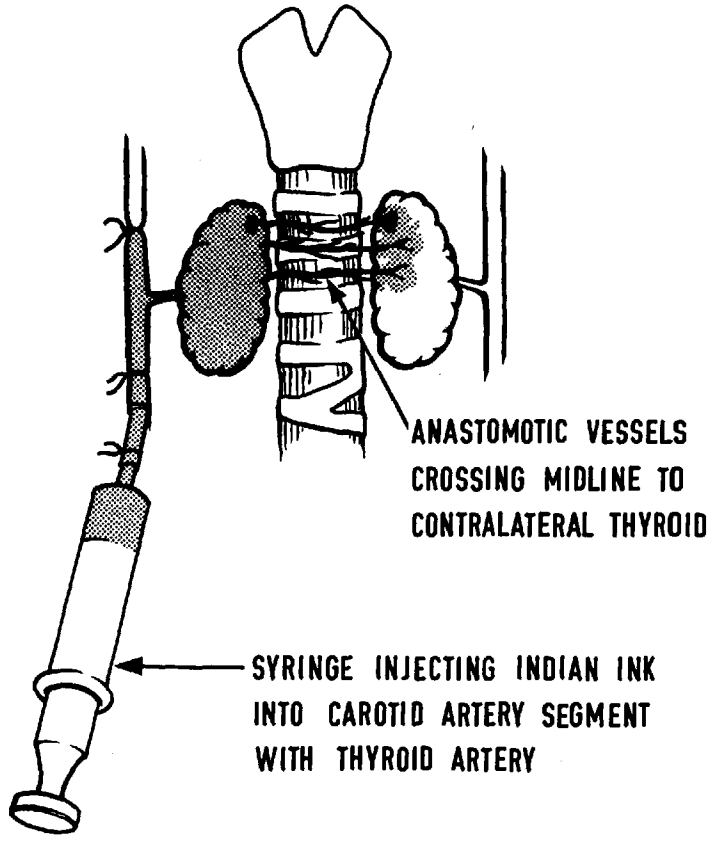


Figure II - 6. Schematic representation of dye injection study which demonstrated that colateral blood vessels cross the midline from one thyroid lobe to the other in the dog.

Results: Figure II - 7 is a schematic representation of the vascular supply to both glands. The thyroid artery lay along the medial surface of the thyroid gland. The blood supply to both the internal and external parathyroids consisted of small branches from the thyroid artery. These vessels partially surrounded the parathyroid glands in an arcuate fashion and gave rise to numerous capillaries which entered the parathyroids. This intimate relationship of the thyroid and parathyroid blood supply precluded the possibility of developing a feasible technique to perfuse differentially the parathyroid gland in the dog.

Experiment 5 - Investigation of possible calcium-lowering activity in parathyroid extracts.

Preliminary studies with crude acid extracts of bovine and canine parathyroid tissue failed to produce significant hypocalcemia in dogs or rats (Table II - 8). These extracts when given in large doses to dogs produced marked hypotension and even death. In order to assess if calcium-lowering activity might exist though masked by PTH, crude parathyroid extract was fractionated and each fraction assayed.

Methods: Fresh frozen bovine parathyroid glands were extracted and the resulting extract fractionated by Dr. Iain MacIntyre using the method of Rasmussen (1964). Precipitates were collected and

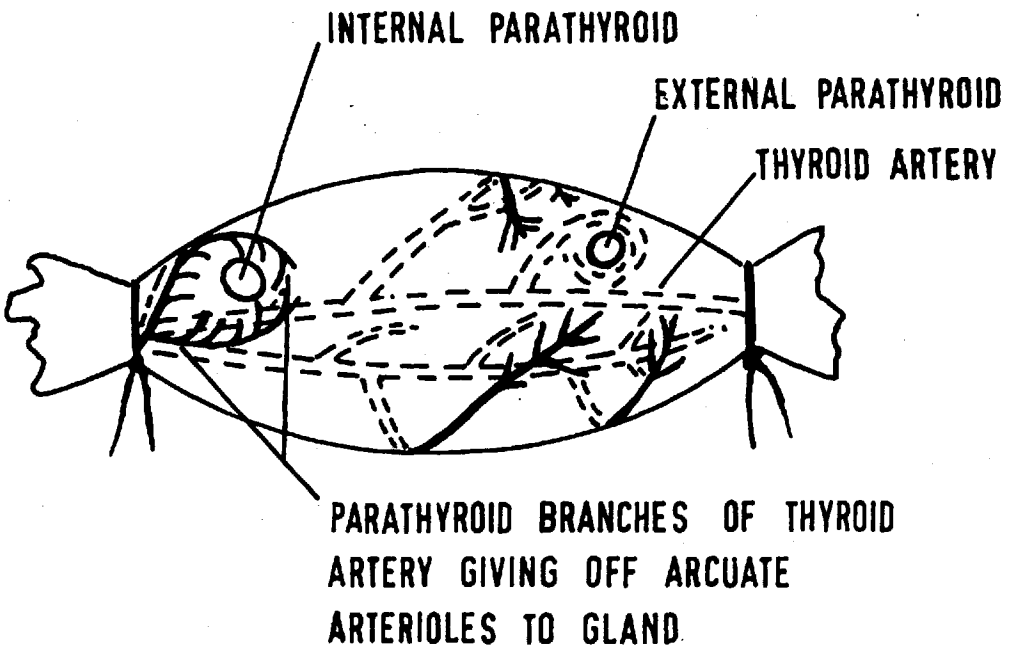


Figure II - 7. Drawing of dye injected arterial tree of dog thyroid illustrating intimate relation of vessels supplying both thyroid and parathyroids.

Table II - 8. Preliminary studies on the effect of parathyroid extracts on blood plasma constituents. Minced fresh frozen beef parathyroid was extracted in a ratio of 1 g wet weight to 10 ml of solvent. Digestion was carried out at 70-90° for 5 minutes and the mixture allowed to stand for 1 hour at room temperature. Residue was washed with an equal volume of water before centrifugation. Each dog received 10 ml of extract intravenously unless otherwise noted and each rat 0.2 - 0.3 ml by intravenous infusion over a 1 hr. period. Values for rats represent mean of deviation of 4 - 5 animals from 4 - 5 control animals not receiving extract.

	DOGS								RATS			
Solvents	Animal No.	B.P. mmHg	Δ Ca mEq/l	Δ Mg mEq/l	Δ P_i mEq/l	Δ Na N/100 ml	Δ K N/100 ml	Δ Protein g/100ml	Δ Ca mEq/l	Δ Mg mEq/l	Δ P_i mEq/l	Δ Protein g/100ml
50% ethanol 0.2N-HCl	111	-80	0	-0.27	+5.35	0	0		0	0		-0.22
	*114	-80		-0.25	+1.71	0	-0.4	0				
0.2N-HCl	117	-30	0	-0.14	-0.36	0	0	0	0	0	-0.96	-1.04
4.5% saline 50% ethanol	118	-45	0	-0.10	0	0	-0.5	0	+0.18 -0.20			
0.2N-Na_2CO_3	124	-25	0	-0.14	+1.09	0	0	0	0		-0.64	+0.24

* Dog 114 received 5 ml of extract.

This table is to be compared to Table VI - 1 for contrast of the ability of the same solvents to extract hypocalcemic producing activity from pork thyroid.

dried and supernatents lyophilized. All fractions were subsequently dissolved in 0.1N-hydrochloric acid to prepare solutions containing approximately 150 mg protein nitrogen/100 ml.

Assays were carried out as described in Chapter V. 0.4 ml of each extract was administered as an intravenous infusion over a 1 hr. period in a tail vein of a 150 g male Hammersmith strain piebald rat. Following the infusion aortic blood was collected and the plasma calcium determined as described in Chapter I. All assays were done in quadruplicate and control animals received 0.4 ml of 0.1N-hydrochloric acid.

Results: As shown in Figure II - 8 no calcium-lowering activity was found in crude extract, the ether precipitate, the acetic acid-sodium chloride precipitate or supernatant, the trichloro-acetic acid precipitate or supernatant or the fractions from Sephadex G-100. Marked PTH calcium-raising activity was found in the eluant from the third peak from Sephadex and some calcium-raising in the fourth peak.

Since hypocalcaemia could not be produced by any of the fractions it was concluded that calcitonin-like activity could not be extracted from parathyroid tissue.

The experiments to be described in the following chapter confirmed the thyroid origin of calcitonin and demonstrated that the parathyroids, when perfused alone, did not contribute to the hypocalcemic effect.

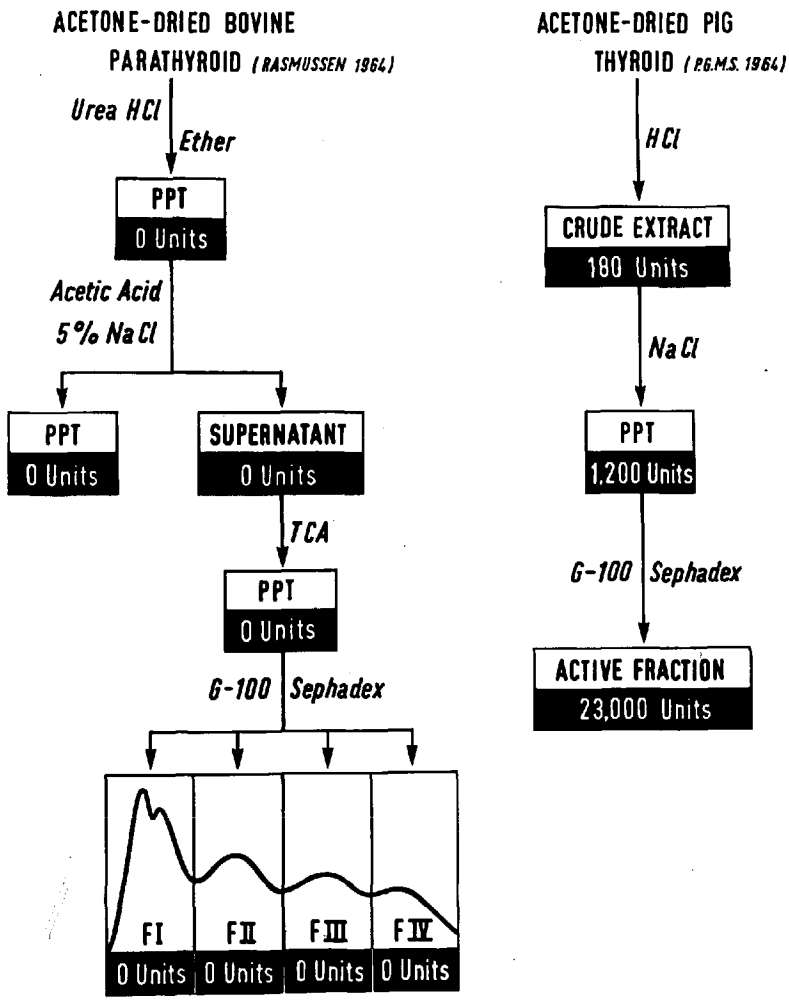


Figure II - 8. On the left is shown the purification protocol for parathyroid hormone (F III from Sephadex G-100). All fractions were assayed for calcitonin activity in the rat with negative results as indicated in the black boxes. For comparison the relative ease of extracting calcium-lowering activity from thyroid tissue is shown on the right (See Chapters VI and VIII).

Chapter III

Confirmation of the Thyroid Origin of Calcitonin

Summary

Hypercalcemic perfusions carried out in the goat demonstrated that calcitonin is secreted by the thyroid and not by the parathyroids. Two groups of five animals were studied. In one the external parathyroid alone was perfused. In the other the thyroid was included in the perfusion. No fall in systemic plasma calcium occurred during parathyroid perfusion. In the presence of the thyroid a marked fall was observed. A crude extract was prepared from goat thyroid which had marked plasma calcium-lowering activity. A brief review of additional evidence favouring the thyroid origin is included in the discussion.

Having shown that separate parathyroid perfusion was not feasible in the dog (Experiment 4, Chapter II), a more suitable species was sought in which the parathyroids could be isolated and the effects of separate thyroid and parathyroid perfusion studied. Of the common domestic animals the horse, ox, pig, sheep and goat have external parathyroid glands with blood supplies separate from the thyroid (Grau and Dellmann, 1958; Kraus, 1953; Bartz, 1902; Reimen, 1926; Vermeulen, 1917). The horse and ox were excluded on the basis of excessive cost and inconvenience. The pig was found to have an arterial blood supply to the thyroid originating

from a vessel arising from the aortic arch. This necessitated removal of the sternum which raised technical problems in surgery. Preliminary studies in the sheep showed this species extremely liable to hypotension, attributed to the anaesthetics used. The goat, however, was found satisfactory. In the goat the external pair of parathyroid glands have a separate blood supply arising from the carotid artery near its bifurcation. The internal pair of glands are vestigal and inseparable from the lower pole of the thyroid (Care, personal communication). They receive their blood supply from the superior or inferior thyroid arteries.

Experiment 1 - Evidence for calcitonin secretion by the thyroid.

Methods. Ten healthy male goats (21-82 kg) were used. All fermentable food except for hay bedding was withheld the night before an experiment. Anaesthesia was induced with pentobarbitone (20-30 mg/kg) intravenously Veterinary preparations which contained glycol were not used since they induce hemolysis. The animals were restrained on an operating table in the supine position with the chest high and feet and neck low (Figure III - 1). This position insured salivary drainage and at the same time prevented the stomach from herniating through the diaphragm and impairing respiration. The animals were intubated with an endotracheal tube with an inflatable cuff to ensure an unrestricted airway. Anaesthesia was maintained

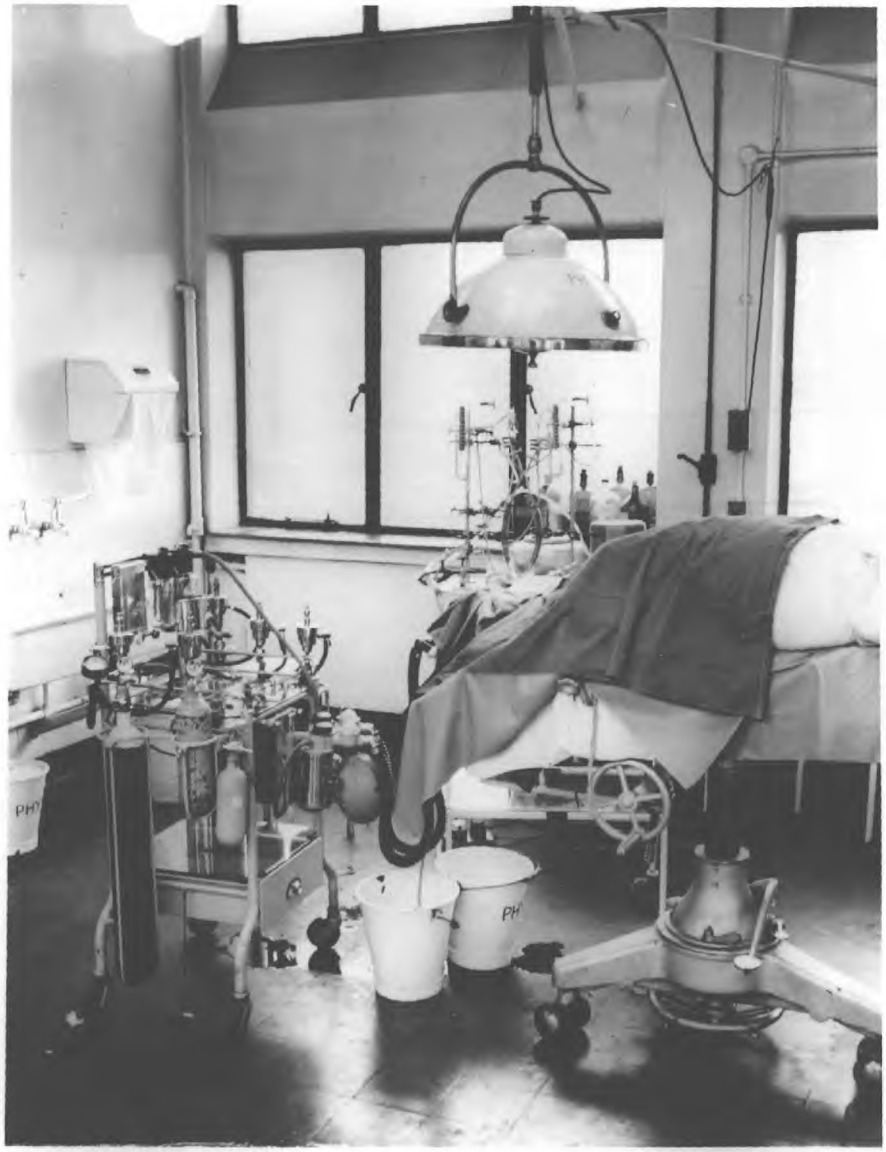


Figure III - 1. A goat perfusion experiment in progress. The animal is supine with chest high and feet and neck low. This position insures salivary drainage and at the same time prevents herniation of the stomach through the diaphragm.

with 10-20 percent cyclopropane and oxygen in a close-circuit apparatus. A gastric tube was passed into the stomach to prevent gastric distention which is prevalent in ruminants undergoing prolonged surgical procedures.

The neck was exposed by a longitudinal incision (Figure III - 2). Blunt dissection facilitated locating the thyroids and parathyroids. As shown in Figure III - 3 the thyroids are located just below the level of the thyroid cartilage. Two separate 3.0-5.0 x 1.0-1.5 cm lobes are found on either side of the trachea. The superior thyroid artery enters the upper pole and the inferior thyroid artery the lower. Venous drainage was variable, but usually one or more veins were seen in association with the arterial supply. The external parathyroids were usually found at the level of the superior pole of the thyroid just lateral or dorsal to the common carotid artery from which it receives its blood supply (Figure III - 4). Their pinkish colour made them readily identifiable from adjacent structures.

In all experiments the external parathyroid and thyroid lobe with its contained internal parathyroid were removed on one side. A section of the common carotid artery on the opposite side was dissected free from the carotid sheath from well above the origin of the external parathyroid artery to well below the inferior thyroid artery. Goats were heparinized with 8,000 units/kg sodium heparin



Figure III - 2. Exposure of the trachea at the level of the thyroid cartilage in the goat. The thyroid lobes are found underlying the sterno-cephalicus muscles. (Animal's head is in the direction of the bottom of the page.)



Figure III - 3. The goat's thyroid lobes have been laterally displaced to show their position with respect to the thyroid cartilage which appears as the shining ill-defined mass in the lower angle of the incision.

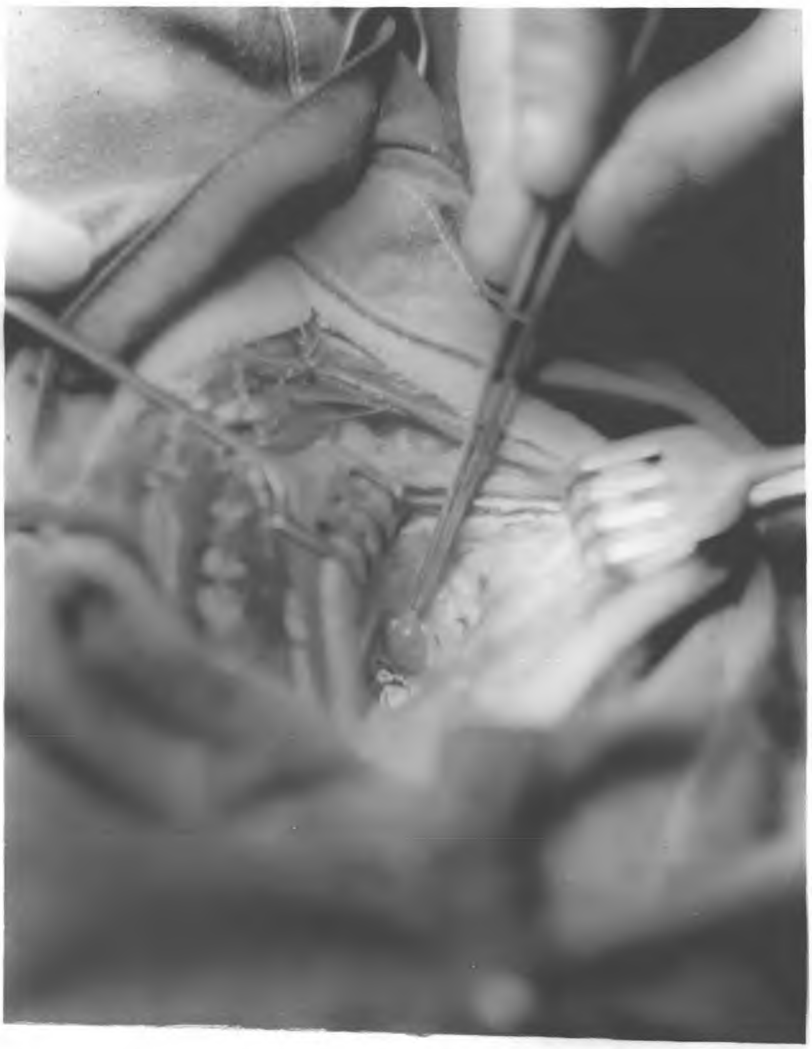


Figure III - 4. An external parathyroid is seen near the tip of the hemastat. The common carotid artery and ipsilateral thyroid lobe have been displaced medially. In the goat the external parathyroid is usually found at the level of the upper pole of the thyroid, just lateral or dorsal to the carotid artery.

(Evans) initially and maintained on 2,000 units/kg hourly thereafter. The common carotid artery was cannulated and perfusion carried out using the two resin column technique as described in Chapter I. Blood pressure was monitored throughout. Animals which developed hypotension below 60 mm Hg were not included in the reported series. The gland area in all experiments was perfused with blood high in calcium (7 mEq/l plasma). Bloodlow in calcium (4 mEq/l plasma) was returned to one of the major vessels of the neck before its entry into the jugular. In this way calcium intake and output by the resin systems were approximately equal throughout the experimental period.

Two groups of goats were studied. In one group of five goats, consisting of two castrates and three billies, perfusion of both thyroid and parathyroids was carried out. In a second group of five animals, consisting again of two castrates and three billies, the thyroid lobe on the perfused side was removed at the same time as removal of the glands on the contralateral side. In this group the external parathyroid was perfused alone. A scheme of the gland area perfused is shown in Figure III - 5.

In all experiments, after an initial 30 min. control period, high calcium perfusion to the gland area was carried out for 2½ hr. Serial arterial blood specimens were collected as previously described. Plasma was separated within 30 min. of sampling. Calcium and

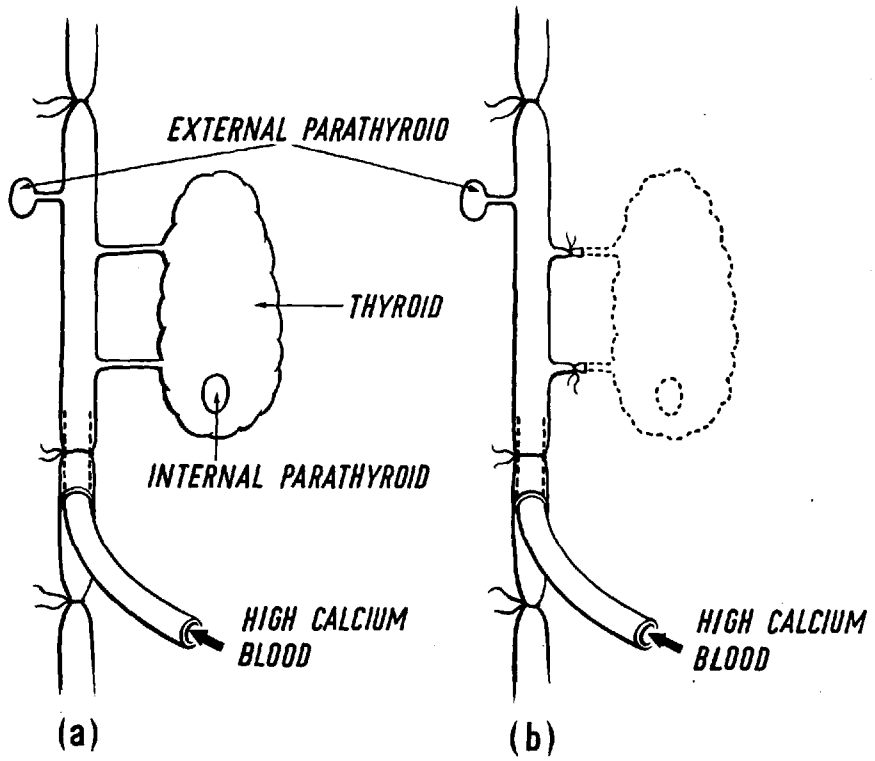


Figure III - 5. Gland areas perfused in the goat. In all animals the contralateral thyroid and parathyroids were removed. In one series (a) the remaining thyroid and parathyroids were perfused together. In a second series (2) the thyroid together with its internal parathyroid was extirpated and the external parathyroid perfused alone.

magnesium were determined by flame spectrophotometry (MacIntyre, 1961), protein by biuret reaction (Wootton, 1964) and phosphate by the method of Delsal and Manhouri (1958).

Results. Figure III - 6 and Table III - 1 and III - 2 show the results obtained. High calcium perfusion of a single external parathyroid gland after removal of all other thyroid and parathyroid tissue produced no change in systemic calcium, strong evidence against a parathyroid origin of calcitonin. In contrast, inclusion of the thyroid in the perfused area resulted in a dramatic 15 percent fall during the same period. These experiments show first, that calcitonin exists in the goat and second, that the thyroid is essential for the production of the calcitonin effect.

The changes in magnesium, phosphate and protein are also shown in Figures III - 7, III - 8, III - 9, and Tables III - 3, III - 4, III - 5, III - 6, III - 7 and III - 8. The minor variations observed in protein were insufficient to account for the falls in systemic plasma calcium. Plasma magnesium was not altered by hypercalcemic perfusion of the parathyroid. When the thyroid was included in the perfusion a small but significant fall ($p < 0.01$) was noted. This would suggest calcitonin affects magnesium as does PTH (MacIntyre, Boss and Troughton, 1963). No significant difference in plasma phosphate levels was detected. In both groups of goats production of PTH was

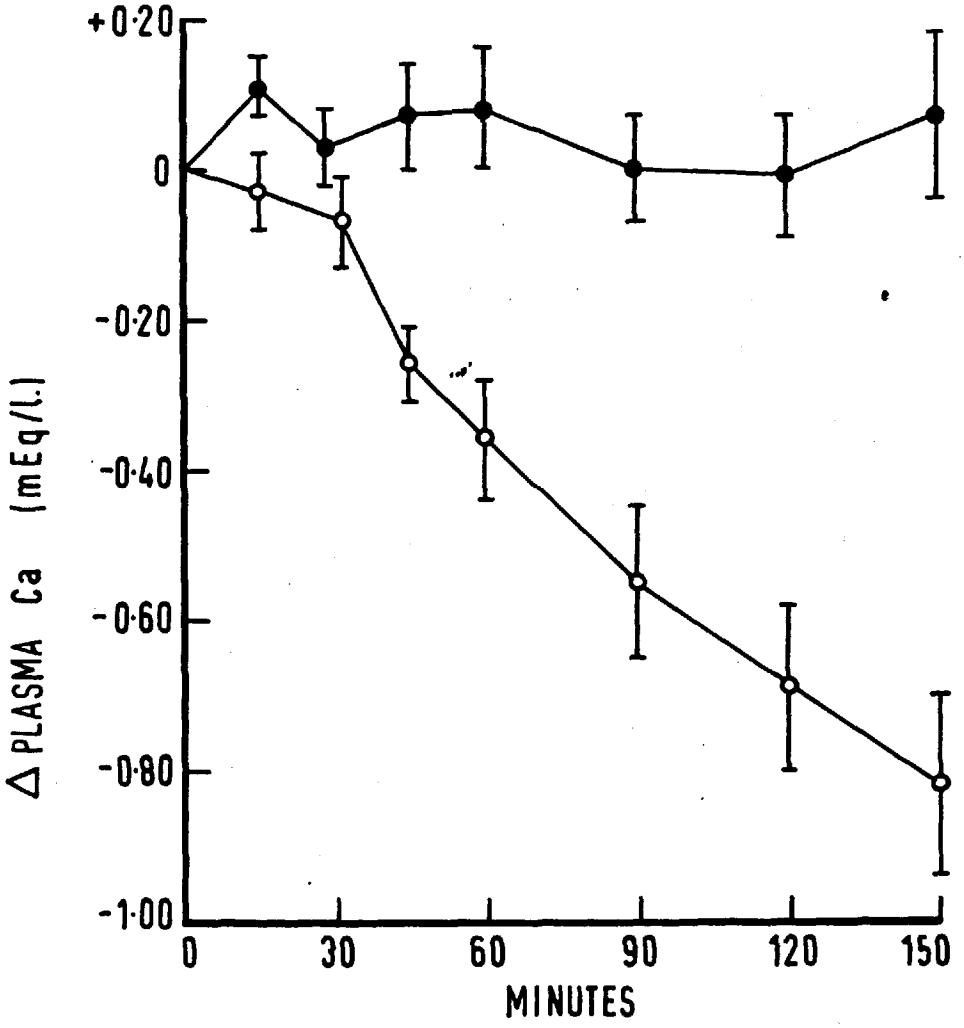


Figure III - 6. Changes in plasma calcium in the goat observed during hypercalcemic perfusion of the external parathyroid alone (●——●) and during perfusion of both parathyroid and thyroid tissue (0——0). Five animals were studied in each group. Single points indicate means and vertical bars standard errors of the means.

Table III - 1. Changes in plasma calcium observed during hypercalcemic perfusion of external parathyroid of the goat.

All thyroid and parathyroid tissue, other than the external parathyroid to be perfused, was removed prior to the beginning of the control period. Hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 1		No. 3		No. 11		No. 15		No. 20		S.E. of change in plasma Ca
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period											
-30	4.85		5.44		5.15		5.60		5.60		
-20	4.68		5.44		4.98		5.55		5.65		
-10	4.85		5.41		5.15		5.40		5.70		
0	4.80		5.47		5.08		5.35		5.60		
Hyper-calcemic perfusion											
15	4.85	+0.05	5.72	+0.25	5.15	+0.07	5.45	+0.10	5.70	+0.10	$+0.11^{+}_{-}0.04$
30	4.85	+0.05	5.61	+0.14	5.18	+0.10	5.38	+0.03	5.45	-0.15	$+0.03^{+}_{-}0.05$
45	4.85	+0.05	5.68	+0.21	5.25	+0.17	5.45	+0.10	5.42	-0.18	$+0.07^{+}_{-}0.07$
60	4.75	-0.05	5.79	+0.32	5.25	+0.17	5.40	+0.05	5.50	-0.10	$+0.08^{+}_{-}0.07$
90	4.76	-0.04	5.72	+0.25	5.10	+0.02	5.29	-0.06	5.45	-0.15	$0^{+}_{-}0.07$
120	4.60	-0.20	5.67	+0.20	5.12	+0.04	5.15	-0.20	5.70	+0.10	$-0.01^{+}_{-}0.08$
150	4.73	-0.07	5.67	+0.20	5.39	+0.31	5.20	-0.15	-	-	$+0.07^{+}_{-}0.11$

Table III - 2. Changes in plasma calcium observed during hypercalcemic perfusion of the thyroid and parathyroids of the goat. At the onset of every experiment all thyroid and parathyroid tissue was removed on the side opposite to that to be perfused. Hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 5		No. 9		No. 10		No. 13		No. 17		S.E. of change in plasma Ca
	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	Plasma Ca mEq/l	Change in plasma Ca	
Control period											
-30	5.35		5.25		5.60		5.60		5.55		
-20	5.38		5.23		5.53		5.55		5.50		
-10	5.20		5.30		5.53		5.52		5.47		
0	5.20		5.35		5.50		5.57		5.50		
Hyper calcemic perfusion											
15	5.35	+0.15	5.30	-0.05	5.33	-0.17	5.60	+0.03	5.40	-0.10	-0.03 ± 0.05
30	5.35	+0.15	5.25	-0.10	5.30	-0.20	5.50	-0.07	5.35	-0.15	-0.07 ± 0.06
45	5.05	-0.15	5.15	-0.20	5.30	-0.20	5.20	-0.37	5.10	-0.40	-0.26 ± 0.05
60	4.75	-0.45	5.11	-0.24	5.30	-0.20	4.95	-0.62	5.21	-0.29	-0.36 ± 0.08
90	4.45	-0.75	4.80	-0.55	5.23	-0.27	4.80	-0.77	5.10	-0.40	-0.55 ± 0.10
120	4.38	-0.82	4.70	-0.65	5.10	-0.40	4.53	-1.04	4.95	-0.55	-0.69 ± 0.11
150	4.25	-0.95	4.65	-0.70	5.00	-0.50	4.35	-1.22	4.75	-0.75	-0.82 ± 0.12

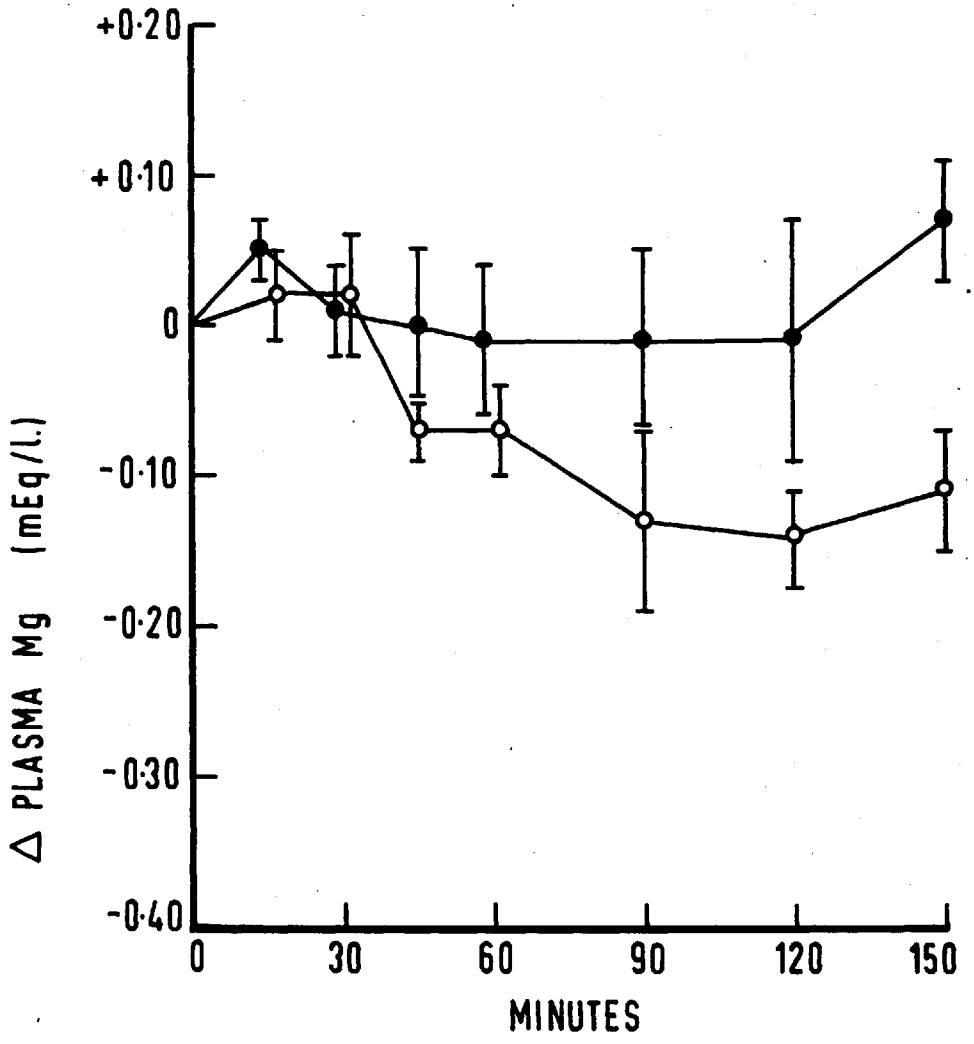


Figure III - 7. Changes in plasma magnesium in the goat observed during hypercalcemic perfusion of the external parathyroid alone (●——●) and during perfusion of both parathyroid and thyroid tissue (0——0). Five animals were studied in each group. Single points indicate means and vertical bars standard errors of the means.

Table III - 3. Changes in plasma magnesium observed during hypercalcemic perfusion of external parathyroid of the goat.

All thyroid and parathyroid tissue, other than the external parathyroid to be perfused, was removed prior to the beginning of the control period. Hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 1		No. 3		No. 11		No. 15		No. 20		S.E. of change in
	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	
Control period											
-30	2.00		2.14		1.82		2.56		2.12		
-20	1.93		2.14		1.66		2.52		2.17		
-10	1.96		2.07		1.71		2.48		2.25		
0	1.92		2.07		1.58		2.53		2.08		
Hyper-calcemic perfusion											
15	2.00	+0.08	2.14	+0.07	1.63	+0.05	2.48	-0.05	2.17	+0.09	$+0.05^{+}_{-}0.02$
30	1.96	+0.04	2.14	+0.07	1.63	+0.05	2.42	-0.11	2.08	0	$+0.01^{+}_{-}0.03$
45	2.00	+0.08	-	-	1.66	+0.08	2.38	-0.15	2.08	0	$0^{+}_{-}0.05$
60	1.93	+0.01	2.14	+0.07	1.54	-0.04	2.38	-0.15	2.16	+0.08	$-0.01^{+}_{-}0.05$
90	1.88	-0.04	2.07	0	1.63	+0.05	2.33	-0.20	2.24	+0.16	$-0.01^{+}_{-}0.06$
120	1.81	-0.11	2.21	+0.14	1.68	+0.10	2.33	-0.20	2.56	-	$-0.01^{+}_{-}0.08$
150	1.88	-0.04	2.21	+0.14	1.72	+0.14	2.57	+0.04	-	-	$+0.07^{+}_{-}0.04$

Table III - 4. Changes in plasma magnesium observed during hypercalcemic perfusion of the thyroids and parathyroid in the goat.

At the onset of every experiment all thyroid and parathyroid tissue was removed on the side opposite to that to be perfused. Hypercalcemic perfusion with blood containing approximately 7.0 mEq/l calcium was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 5		No. 9		No. 10		No. 13		No. 17		S.E. of change in plasma Mg
	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	Plasma Mg mEq/l	Change in plasma Mg	
Control period											
-30	2.86		2.52		2.29		2.00		2.75		
-20	2.81		2.58		2.37		1.96		2.71		
-10	2.81		2.70		2.37		1.92		2.71		
0	2.82		2.62		2.37		1.92		2.69		
Hyper-calcemic perfusion											
15	2.93	+0.11	2.61	-0.01	2.33	-0.04	2.00	+0.08	2.63	-0.06	$+0.02 \pm 0.03$
30	2.78	-0.04	2.58	-0.04	2.33	-0.04	2.00	+0.08	2.67	-0.02	$+0.02 \pm 0.04$
45	2.73	-0.09	2.53	-0.09	2.24	-0.13	1.92	0	2.63	-0.06	-0.07 ± 0.02
60	2.70	-0.12	2.45	-0.17	2.33	-0.04	1.92	0	2.67	-0.02	-0.07 ± 0.03
90	2.68	-0.14	2.27	-0.35	2.29	-0.08	1.88	-0.04	2.64	-0.05	-0.13 ± 0.06
120	2.75	-0.07	2.36	-0.26	2.19	-0.18	1.83	-0.09	2.58	-0.11	-0.14 ± 0.03
150	2.75	-0.07	2.41	-0.21	2.15	-0.22	1.88	-0.04	2.69	0	-0.11 ± 0.04

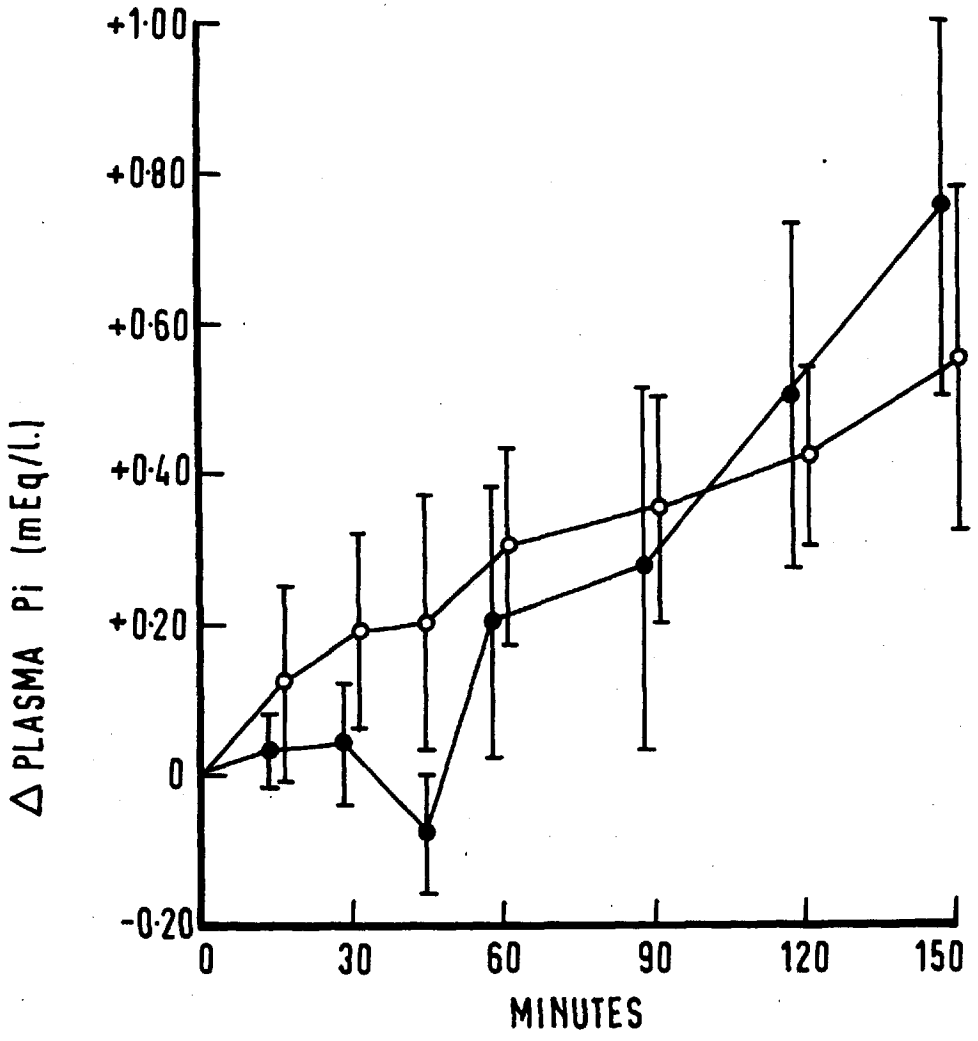


Figure III - 8. Changes in plasma inorganic phosphate in the goat observed during hypercalcemic perfusion of the external parathyroid alone (●——●) and during perfusion of both parathyroid and thyroid tissue (0——0). Five animals were studied in each group. Single points indicate means and vertical bars standard errors of the means.

Table III - 5. Changes in plasma inorganic phosphate observed during hypercalcemic perfusion of external parathyroid of the goat.

All thyroid and parathyroid tissue, other than the external parathyroid to be perfused, was removed prior to the beginning of the control period. Hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 1		No. 3		No. 11		No. 15		No. 20		S.E. of change in plasma P_i
	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	
Control period											
-30	4.25		-		2.79		5.57		5.05		
-20	4.18		3.99		2.72		5.45		5.59		
-10	4.18		4.02		2.79		5.57		5.79		
0	4.02		4.02		2.98		5.45		5.79		
Hyper-calcemic perfusion											
15	3.99	-0.03	4.09	+0.07	2.85	-0.13	5.57	+0.12	5.92	+0.13	$+0.03 \pm 0.05$
30	4.09	+0.07	4.27	+0.25	2.66	-0.32	5.45	0	5.99	+0.20	$+0.04 \pm 0.08$
45	3.55	-0.47	4.30	+0.28	2.66	-0.32	5.54	-0.09	5.99	+0.20	-0.08 ± 0.08
60	3.81	-0.21	4.41	+0.39	2.85	-0.13	5.61	+0.16	6.59	+0.80	$+0.20 \pm 0.18$
90	3.90	-0.12	4.48	+0.46	2.83	-0.15	5.45	0	6.93	+1.14	$+0.27 \pm 0.24$
120	3.76	-0.26	4.76	+0.74	3.30	+0.32	6.02	+0.57	6.93	+1.14	$+0.50 \pm 0.23$
150	3.90	-0.12	4.93	+0.91	3.69	+0.71	6.30	+0.85	7.20	+1.41	$+0.75 \pm 0.25$

Table III - 6. Changes in plasma inorganic phosphate observed during hypercalcemic perfusion of the thyroid and parathyroid of the goat.

At the onset of every experiment all thyroid and parathyroid tissue was removed on the side opposite to that to be perfused. Hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 5		No. 9		No. 10		No. 13		No. 17		S.E. of change in plasma P_i
	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	Plasma P_i mEq/l	Change in plasma P_i	
Control period											
-30	4.95		4.62		4.81		5.63		5.37		
-20	5.20		4.76		5.30		5.76		5.70		
-10	5.19		4.72		5.04		5.88		5.93		
0	5.33		4.76		5.30		5.82		5.83		
Hyper-calcemic perfusion											
15	5.94	+0.61	4.65	-0.11	5.35	+0.05	5.82	0	5.86	+0.03	$+0.12 \pm 0.13$
30	6.00	+0.67	4.92	+0.16	5.43	+0.13	5.69	-0.13	5.93	+0.10	$+0.19 \pm 0.13$
45	6.21	+0.88	4.76	0	5.40	+0.10	5.82	0	5.86	+0.03	$+0.20 \pm 0.17$
60	5.94	+0.61	4.76	0	5.79	+0.49	5.82	0	6.22	+0.39	$+0.30 \pm 0.13$
90	6.03	+0.70	4.76	0	5.95	+0.65	-	-	6.22	+0.39	$+0.35 \pm 0.15$
120	5.94	+0.61	4.76	0	5.95	+0.65	6.26	+0.44	6.22	+0.39	$+0.42 \pm 0.12$
150	5.94	+0.61	4.68	-0.08	5.95	+0.65	-	-	6.85	+1.02	$+0.55 \pm 0.23$

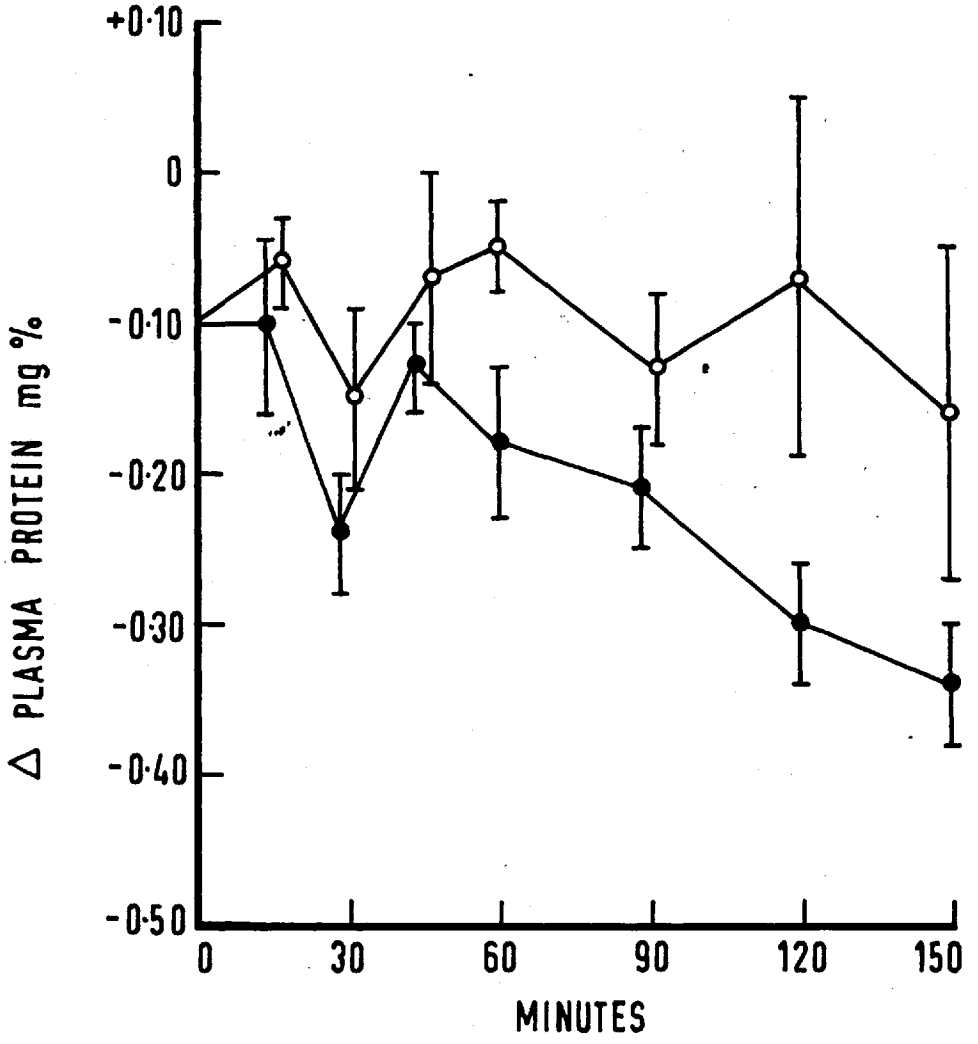


Figure III - 9. Changes in plasma protein in the goat observed during hypercalcemic perfusion of the external parathyroid alone (●——●) and during perfusion of both parathyroid and thyroid tissue (0——0). Five animals were studied in each group. Single points indicate means and vertical bars standard errors of the means.

Table III - 7. Changes in plasma protein observed during hypercalcemic perfusion of the external parathyroid of the goat.

All thyroid and parathyroid tissue, other than the external parathyroid to be perfused, was removed prior to the beginning of the control period. Hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 3		No. 11		No. 15		No. 20		S.E. of change in plasma protein
	Plasma protein mg %	Change in plasma protein	Plasma protein mg %	Change in plasma protein	Plasma protein mg %	Change in plasma protein	Plasma protein mg %	Change in plasma protein	
Control period									
-30	6.24		6.01		6.01		6.17		
-20	6.24		6.50		5.94		6.17		
-10	6.20		6.40		5.94		6.35		
0	6.12		6.47		6.01		6.24		
Hyper-calcemic perfusion									
15	6.12	0	6.22	-0.25	5.88	-0.13	6.24	0	-0.10 ± 0.06
30	6.08	-0.04	5.86	-0.61	5.94	-0.07	6.00	-0.24	-0.24 ± 0.04
45	6.08	-0.04	6.01	-0.46	6.01	0	6.24	0	-0.13 ± 0.03
60	6.12	0	5.79	-0.68	5.94	-0.07	6.07	-0.17	-0.18 ± 0.05
90	6.20	+0.08	6.01	-0.46	5.98	-0.03	5.83	-0.41	-0.21 ± 0.04
120	6.12	0	5.86	-0.61	5.88	-0.13	5.79	-0.45	-0.30 ± 0.04
150	6.00	-0 12	5.86	-0.61	5.88	-0.13	5.79	-0.45	-0.34 ± 0.04

Table III - 8. Changes in plasma protein observed during hypercalcemic perfusion of the thyroid and parathyroid of the goat.

At the onset of every experiment all thyroid and parathyroid tissue was removed on the side opposite to that to be perfused. Hypercalcemic perfusion with blood containing approximately 7.0 mEq calcium/l was carried out in animals maintained in net calcium balance.

Time (minutes)	No. 5		No. 9		No. 10		No. 13		No. 17		S.E. of change in plasma protein
	Plasma protein mg %	Change in plasma protein	Plasma protein mg %	Change in plasma protein	Plasma protein mg %	Change in plasma protein	Plasma protein mg %	Change in plasma protein	Plasma protein mg %	Change in plasma protein	
Control period											
-30	5.96		6.34		6.54		5.96		6.07		
-20	5.96		6.23		6.43		5.75		6.18		
-10	5.96		6.23		6.29		5.68		6.25		
0	-		6.37		6.15		5.61		6.18		
Hyper-calcemic perfusion											
15	5.82	-0.14	6.37	0	6.15	0	5.50	-0.11	6.11	-0.07	-0.06 ± 0.03
30	5.60	-0.36	6.30	-0.07	6.15	0	5.50	-0.11	5.96	-0.22	-0.15 ± 0.06
45	5.67	-0.29	6.62	+0.25	6.18	+0.03	5.40	-0.21	6.11	-0.07	-0.07 ± 0.07
60	5.56	-0.40	6.62	+0.25	6.00	-0.15	5.85	+0.24	6.00	-0.18	-0.05 ± 0.03
90	5.71	-0.25	6.34	-0.03	5.97	-0.18	5.61	0	6.00	-0.18	-0.13 ± 0.05
120	5.56	-0.40	6.34	-0.03	6.00	-0.15	5.96	+0.35	6.07	-0.11	-0.07 ± 0.12
150	5.49	-0.47	6.48	+0.11	5.86	-0.29	5.40	-0.21	6.25	+0.07	-0.16 ± 0.11

prevented by high calcium perfusion. Since PTH normally causes phosphaturia a plasma rise could be anticipated in its absence.

Experiment 2 - Extraction of calcitonin from goat thyroid.

This experiment was undertaken to demonstrate that the calcium-lowering principle released during high calcium stimulation could be extracted.

Methods. A healthy 21 kg billy goat was anaesthetized, restrained and intubated as described in the preceding experiment. The animal was heparinized and the carotid blood shunted extracorporeally as previously described (Experiment 2 in Chapter II). After surgical exposure of the neck unilateral thyro-parathyroidectomy was carried out.

An extract was prepared from the upper pole of the removed thyroid to avoid contamination with the internal parathyroid found in the lower pole. 1 g of tissue was minced and macerated in a hand mortar. Extraction was carried out in 10 ml of 0.2$\underline{\underline{N}}$-hydrochloric acid. The digest was heated for 5 min. at 70-90^{o} and centrifuged to remove particulate matter. After a 30 min. control period the crude extract was injected intravenously into a leg vein. Blood samples were collected and calcium and magnesium determinations were carried out as before.

Results. The results are contained in Figure III - 10 and Table III - 9 A 16 percent fall in plasma calcium occurred in $2\frac{1}{2}$ hr. following the injection of the extract. No appreciable change in plasma magnesium was observed. This was not considered contradictory to the previous experiment since magnesium changes were not observed in every perfused animal. The minimal fall in plasma protein could not account for the change in calcium.

The results of this study showed that a crude extract prepared from 1.0 g of thyroid produced a fall in plasma calcium comparable to that observed during high calcium stimulation of the thyroid gland over the same $2\frac{1}{2}$ hr period.

DISCUSSION

In summary the experiments described in this chapter show:

(1) perfusion of the external parathyroid in the goat with hypercalcemic blood produces no alteration in systemic plasma calcium, whereas if the thyroid is included in the perfusion a marked fall ensues and

(2) a calcium-lowering factor is readily extractable from the thyroid of the goat.

The data clearly demonstrates the thyroid origin of calcitonin. Undoubtedly calcitonin and thyrocalcitonin are one and the same substance.

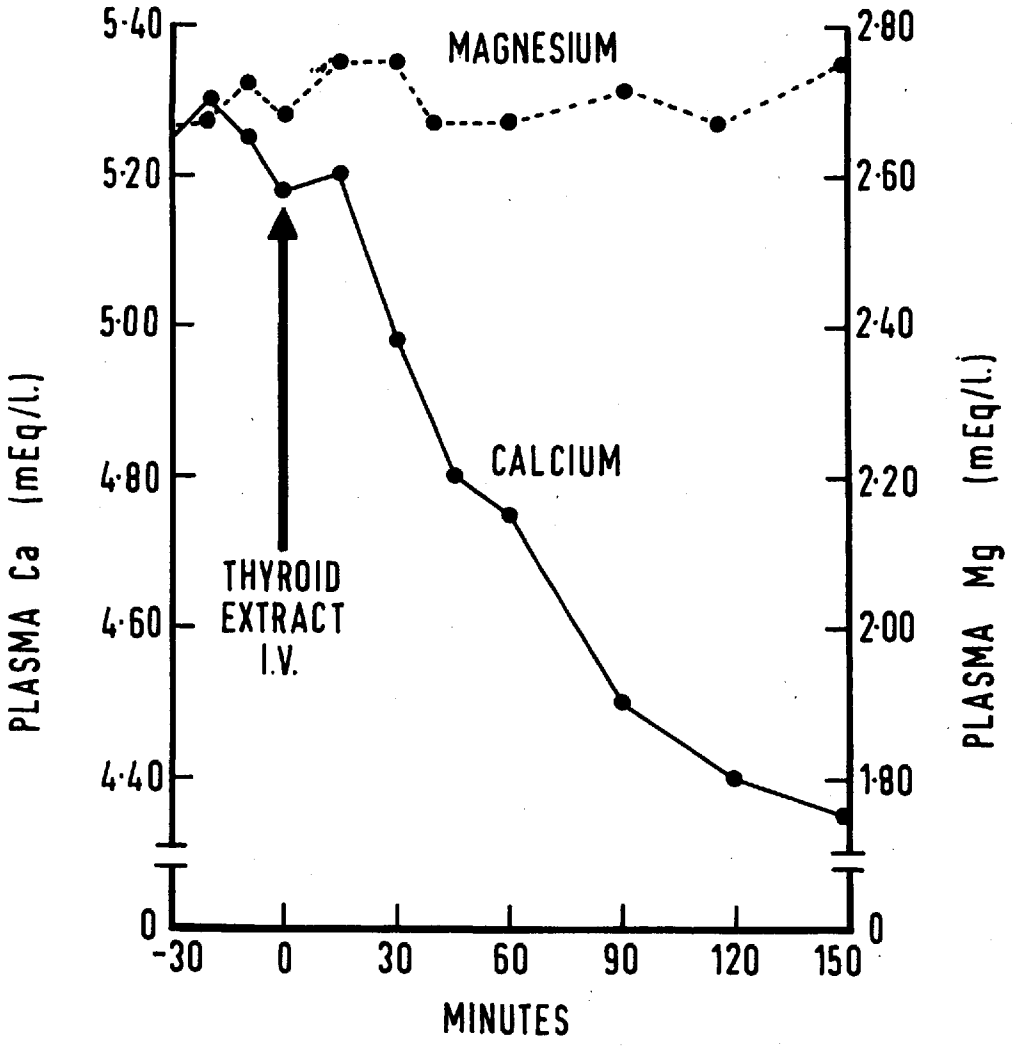


Figure III - 10. Changes in plasma calcium and plasma magnesium observed in the goat following intravenous administration of autogenous thyroid extract. 1.0 ml of an 0.2N-hydrochloric acid extract (0.47 mg protein nitrogen) from the animal's own thyroid was injected two hr. after hemi-thyroidectomy. The amount used was that extractable from one-twentieth of the animal's total thyroid tissue.

Table III - 9. Effect on plasma calcium, magnesium and protein of an intravenous injection of a crude autogenous hydrochloric acid extract of goat thyroid (Goat No. 19). 1 ml of extract containing 0.47 mg protein nitrogen was given at zero time.

Time (minutes)	Plasma Ca (mEq/l)	Plasma Mg (mEq/l)	Plasma Protein (g/100ml)
-30	5.25	2.67	6.80
-20	5.30	2.68	6.80
-10	5.25	2.72	6.80
0	5.18	2.68	6.80
+15	5.20	2.75	6.80
+30	4.98	2.75	6 80
+45	4.80	2.67	6.80
+60	4.75	2.67	6.73
+90	4.50	2.71	6.70
+120	4.40	2.67	6.70
+150	4.35	2.75	6.70

Since publication of the work in this and the preceding chapter (Foster, 1964; Foster, Kumar, Baghdiantz, Soliman, Slack, De Bats and MacIntyre, 1965; Foster, Baghdiantz, Kumar, Slack, Soliman and MacIntyre, 1964), Copp and Henze (1964) and Care and Keynes (1965) have reported results in direct disagreement. In perfusion experiments in sheep they showed that hypercalcemic perfusion of the external parathyroid was followed by a small fall in systemic calcium. The contralateral thyroid was present in most of their preparations. In man, anastomatic vessels cross the mid-line in the neck from one carotid artery to another (Gray, 1964). Dye injection studies in our laboratory in the dog (Figure II - 6) and in the goat have shown that similar vessels exist in these animals as well. It is likely they occur in sheep as well. The falls reported by Copp and Henze and by Care and Keynes can readily be explained by high calcium perfusion of the contralateral thyroid.

Recently confirmatory evidence for a thyroid origin of calcitonin has been reported from four independent laboratories:

(1) Bernstein, Kleeman and Pine (1965) noted in dogs that hypocalcemic perfusion of either the thyroid or thyro-parathyroids caused a fall in serum calcium. The fall observed was equal in both instances and was greater than that seen following parathyroidectomy.

(2) Similarly Care (1965) has demonstrated a fall in plasma calcium during hypercalcemic perfusion of the thyroid of the pig. The fall was reversed during extracorporeal collection of thyroid venous blood. When re-injected into a second animal, the venous effluent produced a hypocalcemic response.

(3) Talmage, Neuschwander and Kraintz (1965) have shown that removal of the thyroids made animals more susceptible to hypercalcemia. These investigators thyro-parathyroidectomized rats and transplanted the parathyroids into the scrotum. Peritoneal infusions of calcium into these "thyroidectomized" animals raised plasma levels higher than in intact controls. Recovery from intravenous infusion of calcium was more rapid in intact than in thyroidectomized rats.

(4) Copp (personal communication) has been successful in showing plasma calcium falls in sheep during hypercalcemic perfusion of the thyroid.

These studies indicated that the hypocalcemia-producing principle is secreted by the thyroid in response to hypercalcemia in the rat, pig and sheep, as well as in the goat and the dog.

Further evidence that calcitonin is not secreted by the parathyroids has been reported recently by Dale, Roth and Garcia (1965). These investigators perfused parathyroid transplants in the exterior chamber of the eye of parathyroidectomized rats with solutions containing high and low concentrations of calcium. High calcium perfusions produced a fall in serum calcium but the fall was less than that found when the parathyroid transplants were removed. The authors concluded that the fall produced by the high calcium perfusion was fully accounted for by a depression in the secretion of PTH. This view is supported by recent work in the dog, ox and sheep (Sherwood, Care, Mayer and Aurbach, 1966; Care, Sherwood, Potts and Aurbach, 1966) which clearly shows that PTH production is depressed in hypercalcemic conditions.

In addition to dog and goat, calcitonin activity has been extracted from thyroid of pig, ox, rabbit, rat and monkey by Hirsch, Gauthier and Munson (1963). Calcium-lowering activity has been confirmed in thyroid extracts from pig (Kenny, 1964; Foster, Kumar, Baghdiantz, Soliman, Slack, De Bats and MacIntyre, 1965), rat (Milhaud and Mouktar, 1965), and ox (Foster, Kumar, Baghdiantz, Soliman, Slack, De Bats and MacIntyre, 1965).

The existence of this new thyroid hormone is now generally accepted (Lancet, 1965; British Medical Journal, 1965; Lancet, 1966)

despite an unsettled nomenclature. Hirsch, Gauthier and Munson (1963) suggested that the thyroid might be the source of Copp's calcium-lowering principle. They did not present experimental evidence for this view and tentatively suggested the thyroid hormone be called "thyrocalcitonin". With the evidence presented here that calcitonin is of thyroid origin I have retained Copp's original shorter name. It is clear that "calcitonin" and "thyrocalcitonin" are the same.

Chapter IV

Thyroid Cell Origin of Calcitonin

Summary

Dog thyroid and parathyroid glands, perfused *in vitro* with blood high and low in calcium, were studied histochemically. Dog thyroid tissue was shown to be made up of two metabolically different cells: the principal cells and mitochondrion-rich cells. The latter were characteristically found in nodes or groups between follicles as well as occasionally lining the follicles. Staining techniques showed them to have markedly higher levels of ∝-glycerophosphate dehydrogenase than adjacent cells. It was found that they were sensitive to high concentrations of calcium ions as reflected by changes in acid phosphatase, ∝-glycerophosphate dehydrogenase and indoxyl esterase. Low calcium perfusion produced vacuolization. Parathyroid glands similarly perfused were unaffected.

Normal thyroid tissue is composed of closely packed small sacs called acini or follicles. Within the follicle is found colloid which may comprise as much as 70 percent of the total mass of the thyroid (Williams and Bakke, 1962). The follicles are variable in size and reflect the activity of their luminal cells. In the human 20-40 follicles are bound together by connective tissue to form a lobule. Each lobule functions as a separate unit (Taylor, 1960). The cells lining the follicles are called acinar or follicular cells. They normally are closely packed cuboidal cells, but under thyrotropin

stimulation undergo three characteristic morphological changes concurrent with an increase uptake of iodine:

(1) Microvilli proliferate along the luminal borders greatly increasing the cell surface area of the follicle. The increase in surface area has been directly correlated with the uptake of isotopic I^{131} in studies in the rat (Wollman, 1960).

(2) Cysternae of ergoplasm form within the cytoplasm, migrate to the borders and there discharge their contents (Herman, 1960).

(3) Mitrochondria swell.

Follicular cells are of two types: the principal cells and the mitochondrion-rich cells (Seecof, 1927). The distribution of mitochondria in both types is distinctly different. In the principal cells the mitochondria are predominantly on the luminal side of the nucleus. In contrast, in the mitochondrion-rich cell they are equally distributed throughout. The mitochondrion-rich cells in the dog are found not only lining the follicles, but more characteristically in nodes or groups between follicles. For this reason they are frequently referred to as parafollicular cells. Because of their position they were formerly thought to be tangentially cut acinar cells. Studies by Tremblay and Pearse (1960) have shown them to have many times the

oxidative enzyme activity found in neighbouring principal cells. These investigators suggested that they might carry out some secretory function other than the production of thyroglobulin and the classical thyroid hormones. Previously Seecof (1927) had drawn attention to the parallel between the cell types in the thyroid and the α and β cells found in the pituitary and pancreas.

It was decided to study thyroid tissue histochemically to evaluate if the mitochondrion-rich cells were responsible for the production of calcitonin. Prior to the onset of this investigation, Pearse had speculated that these cells were capable of secreting a polypeptide on the basis of their staining characteristics. The studies reported below were carried out in the dog (Foster, MacIntyre and Pearse, 1964; Foster, MacIntyre and Pearse, 1965). The dog, like the human, contains follicular cells of both types.

Methods.

Gland Preparation: Four healthy mongrel dogs (23 - 35 kg) of both sexes were used. Anaesthesia, heparinization and perfusion using a single resin column were done as described in Chapter I. At the onset of the operative procedure the thyroids and parathyroids on one side were removed to serve as control tissue. In two dogs the remaining thyro-parathyroid tissue was perfused with blood high in calcium (7 mEq/l). In two other dogs perfusion was carried out with blood low in calcium (4 mEq/l). The changes represented variations of approximately

± 27 percent of normal plasma calcium. Perfusion of the glands was carried out for 3 hr. instead of $2\frac{1}{2}$ hr. as in previous experiments. At the conclusion of the perfusion period the glands were surgically removed, quenched in liquid nitrogen (-185°) and stored at -90° until cutting.

Staining technique: 4-8 μ sections were prepared on a compact type Slee cryostat at -20°. These were simultaneously incubated for demonstration of the following enzymes: acid phosphatase, alkaline phosphatase, α-glycerophosphate dehydrogenase (menadione), NADH-diaphorase and non-specific (α-napthal and indoxyl) esterases. The techniques used are those given by Pearse (1960). Selected blocks were freeze-dried in a thermoelectrically controlled apparatus (Pearse, 1963) and subjected to formalin vapour treatment for 1 hr. at 80° (Flack, Hillarp, Thieme and Torp, 1962). The blocks were used to examine the distribution and activity of adrenergic nerves and of fluorescent material within the cells. The position of the parafollicular cells relative to the basement membrane was established and confirmed in periodic acid-Schiff-stained sections (Culling, 1963). Hematoxylin and eosin were used to demonstrate the general cytological morphology (Culling, 1963).

Results.

The distribution of mitochondrion-rich cells in a normal, unperfused, dog thyroid are shown in Figure IV - 1. The cells are readily distinguishable from the predominant principal cells by their high level of mitochondrial α-glycerophosphate dehydrogenase shown by the dark stain. They are found occasionally bordering the lumens of follicles but more characteristically they are seen as isolated groups in parafollicular regions. At higher magnification (Figure IV-2) a variable proportion of the parafollicular cells appear to have relatively low levels of mitochondrial α-glycerophosphate dehydrogenase. These "mitochondrion-poor" cells may be a distinct cell type or possibly a stage in the development of the "mitochondrion-rich" cells. Elevated levels of α-glycerophosphate dehydrogenase are found in the "mitochondrion-rich" cells of other endocrine glands known to secretepolypeptide hormones. Figure IV - 3 is a section of rat pancreas prepared by Pearse which shows increased enzyme levels in the β cells of an islet of Langerhans. Cells of the anterior pituitary and the principal cells of the parathyroid likewise have similar elevated levels of α-glycerophosphate dehydrogenase.

The changes in thyroid enzyme levels following perfusion with hyper- or hypocalcemic blood are summarized in Table IV - 1. No changes were observed in parathyroid principal cells examined either

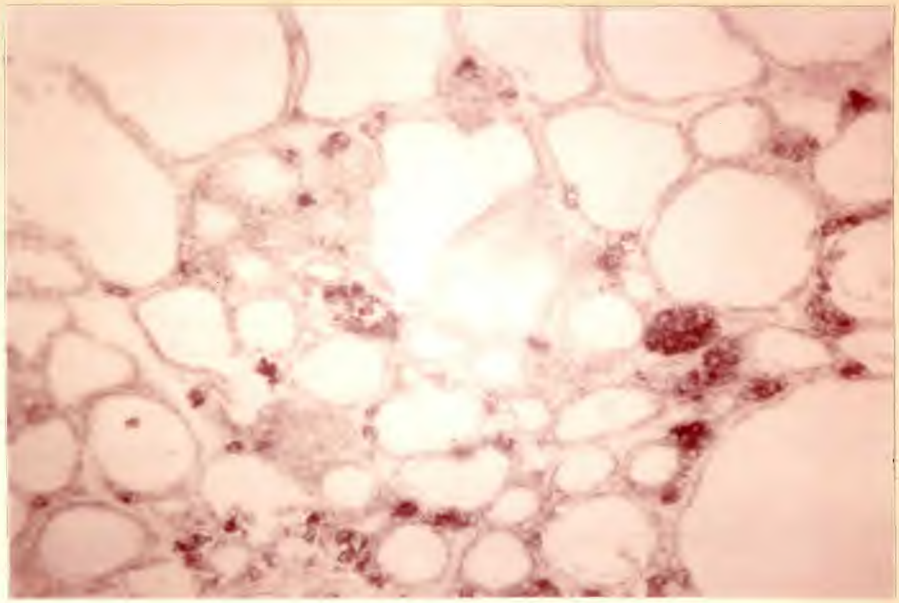


Figure IV - 1. 8 μ cryostat section of unperfused dog thyroid showing high mitochondrial ∝-glycerophosphate dehydrogenase in the "mitochondrion-rich" cells (X 200).

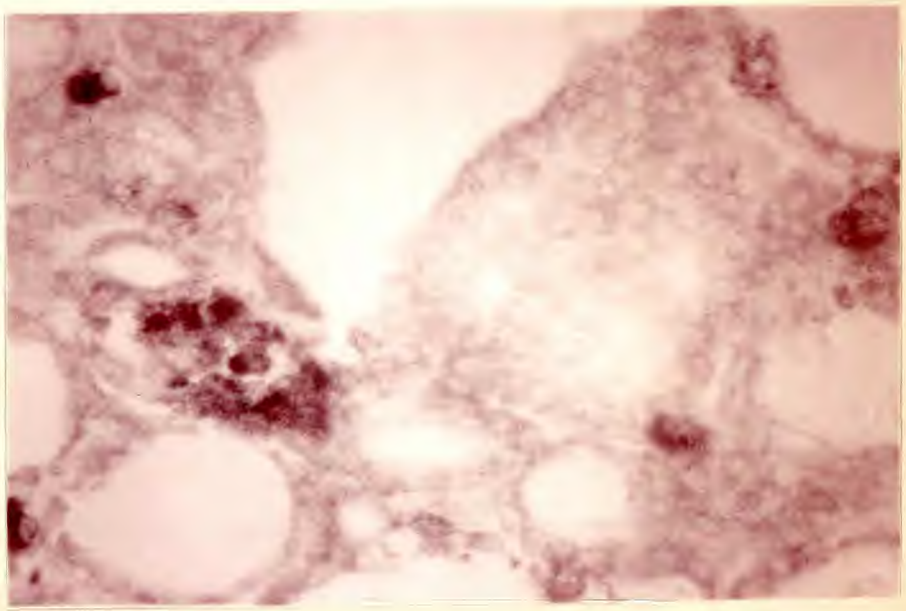


Figure IV - 2. Higher magnification (X 600) of the above showing a proportion of parafollicular cells to be "mitochondrion-poor".

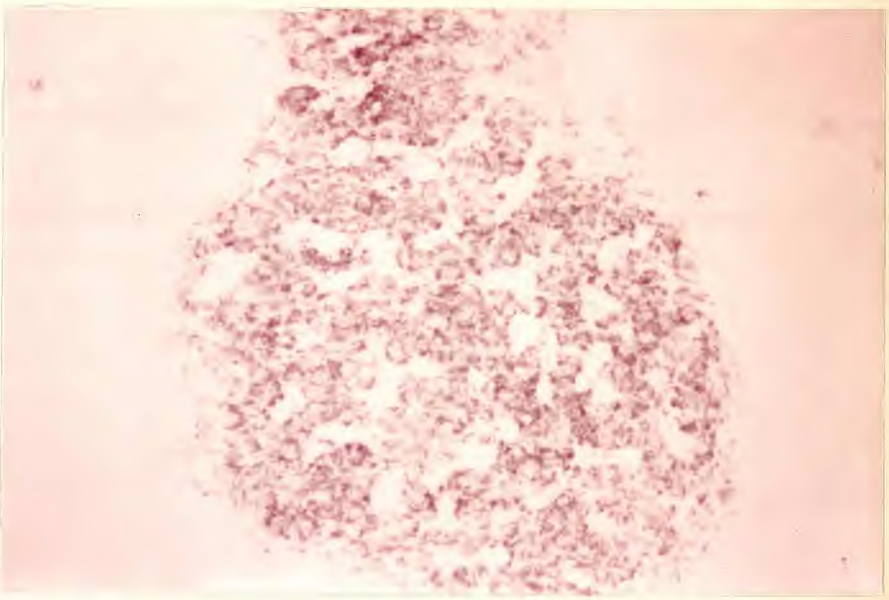


Figure IV - 3. 4 μ cryostat section of rat pancreas showing very high α-glycerophosphate dehydrogenase levels in the β cells of an islet of Langerhans (X 200).

Table IV - 1. Histochemical changes with thyroid perfusion.

Enzyme	High Ca	Low Ca
Acid Phosphatase	↑	0
∝-Glycerophosphatase	↓	0
Indol Esterase	↓	0
NADH - Diaphorase	0	0
Alkaline Phosphatase	0	0

histochemically or by electron microscopy. Striking histochemical changes were apparent in thyroid tissue indicating a change in enzymatic activity in response to variations in calcium concentrations. High calcium produced: (1) an increase in acid phosphatase, particularly in the parafollicular cells, (2) a decrease in mitochondrial ∝-glycerophosphate dehydrogenase and (3) a degrease in non-specific esterase. No change was observed in either NADH-diaphorase or alkaline phosphatase.

The change in acid phosphatase produced by high calcium perfusion is shown in Figures IV - 4 and IV - 5. Figure IV - 4 shows dog thyroid perfused with normocalcemic blood to illustrate the normal level of the enzyme. Figure IV - 5 is a section of the contralateral gland following a 3 hr. perfusion with hypercalcemic blood. The increase in pink stain reflects a marked increase in acid phosphatase. Changes in mitochondrial ∝-glycerophosphate dehydrogenase and non-specific esterase were less striking.

No difference in enzyme levels was noted between thyroid tissue perfused with hypocalcemic blood and controls. Haematoxylin and eosin staining, however, revealed an unusual sensitivity of the intra-follicular and parafollicular "mitochondrion-rich" cells to low calcium levels. Unperfused thyroid tissue in Figure IV - 6 is contrasted with perfused tissue in Figure IV - 7 which shows extensive vacuolation in the cells. The changes may or may not be artefactual.

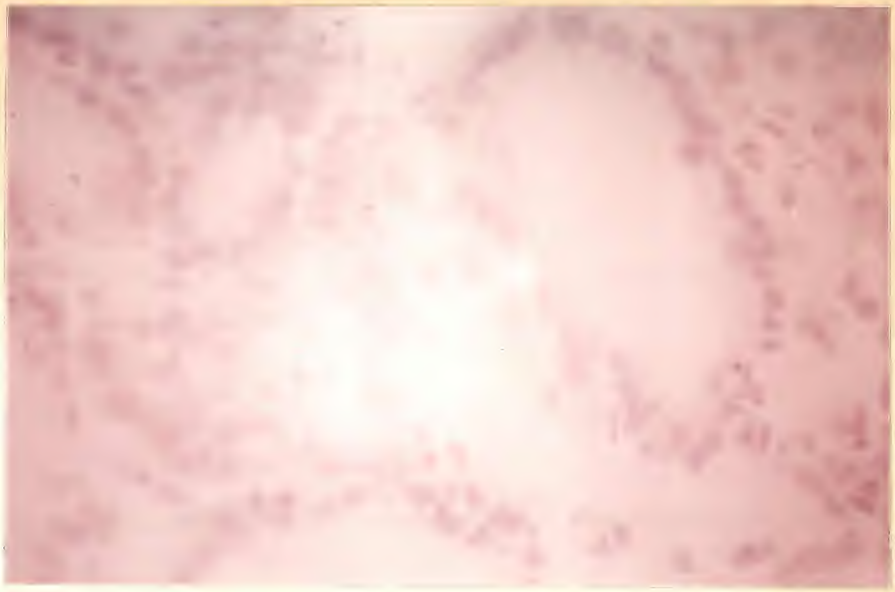


Figure IV - 4. 8 μ cryostat section of dog thyroid perfused with normocalcemic blood stained for acid phosphatase (X 600).

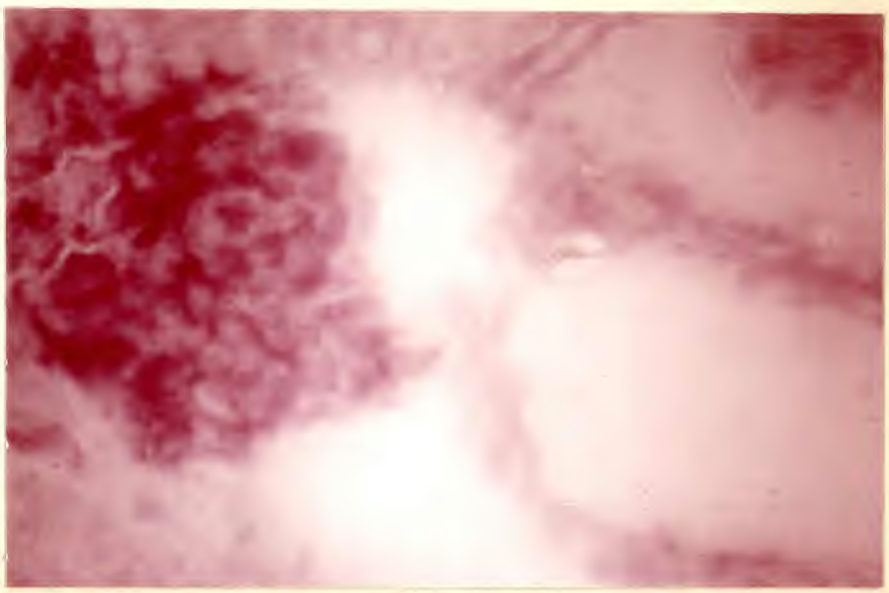


Figure IV - 5. Contralateral gland of the same animal as above following high calcium perfusion for 3 hr. Marked increase in acid phosphatase as shown by the red stain is apparent (X 600).

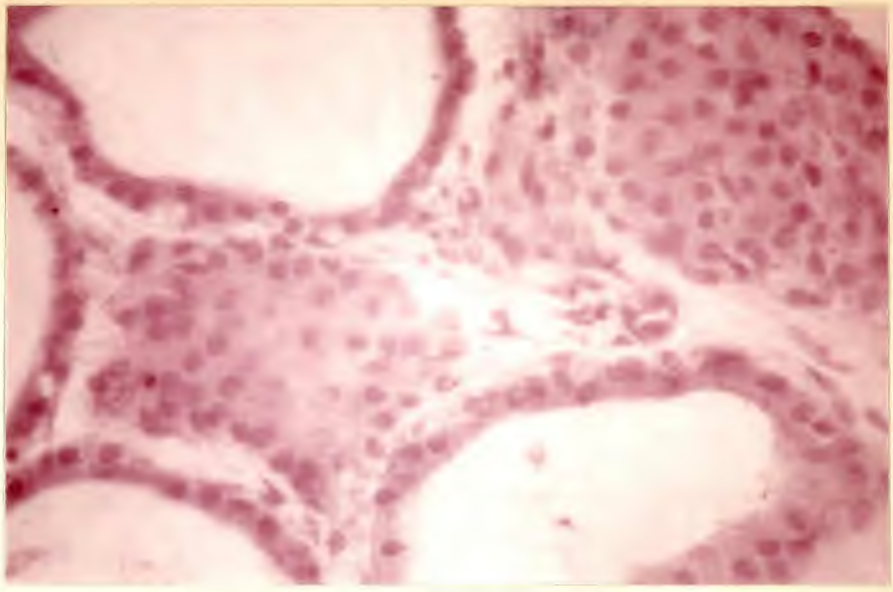


Figure IV - 6. 4 μ cryostat section of unperfused dog thyroid stained with haematoxylin and eosin (X 600).

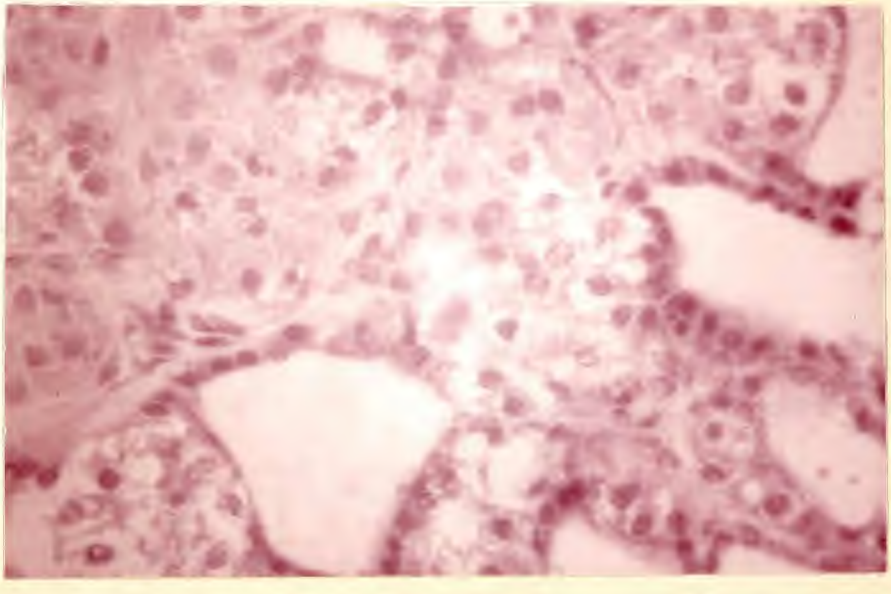


Figure IV - 7. 4 μ cryostat section of the contralateral thyroid of the same dog following low calcium perfusion (X 600). Note extensive vacuolation in inter- and parafollicular cells.

The results of efforts to demonstrate the adrenergic nerve supply to the thyroid cells are seen in Figure IV - 8. Adrenergic nerve fibres appear as faint fluorescent lines associated with inter-follicular blood vessels. It was presumed that these gave off branches to adjacent parafollicular cells, though this could not be demonstrated.

DISCUSSION

The results suggest that calcitonin is secreted by the "mitochondrion-rich" parafollicular cells of the thyroid. They further support the contention that the parathyroids are not involved. Recently, Capen, Koestner and Cole (1965) studied the ultrastructure and histochemistry of the parathyroids of pregnant and non-pregnant cows made hypercalcemic with vitamin D. No evidence of increased secretory activity of any cell type was found. Had such evidence been observed it would have been tempting to propose a common cell type in both glands. Rolleston (1936) has claimed that Hurthle cells, which closely resemble thyroid parafollicular cells, are actually parathyroid oxyphil cells included in the thyroid. In view of the present data such a hypothesis appears untenable.

As pointed out by Taylor (1960) lobules within the thyroid appear to vary considerably in their activity. The objection might be raised that the changes in thyroid cells subsequent to high calcium perfusion reflected the local variations in activity of a single cell type. This

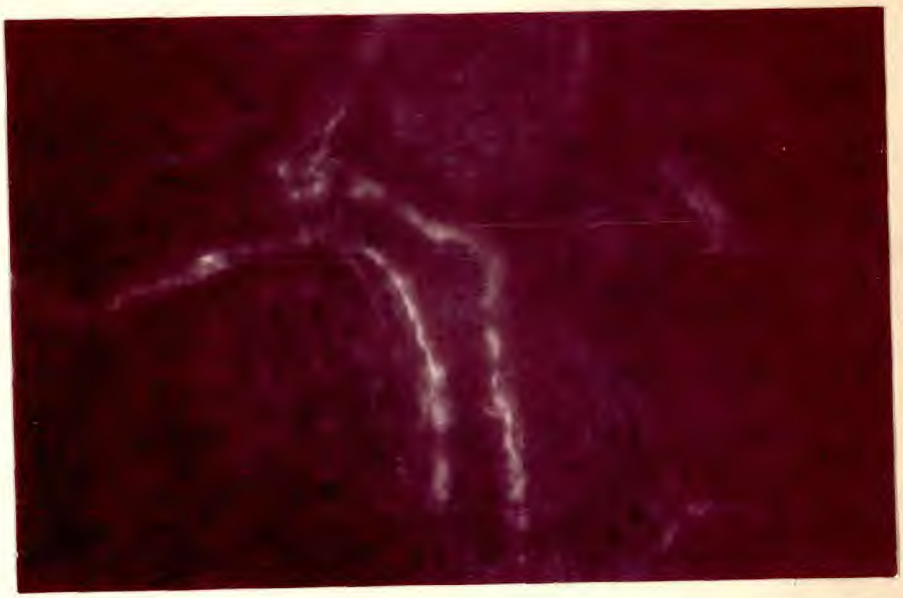
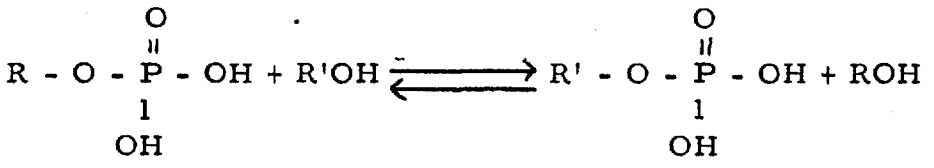


Figure IV - 8. 4 μ cryostat section of normal dog thyroid stained to demonstrate the distribution of adrenergic nerve fibres (X 600). The fluorescent stain shows them to be associated with the interfollicular blood vessels.

criticism cannot be accepted. Lindsay and Jenks (1961) have studied the histochemical profile of thyroids from rats prepared under varying conditions. In those animals treated with thyrotropin the changes observed were dissimilar to those reported in this chapter. They found increases in α-glycerophosphate dehydrogenase, indoxyl esterase and alkaline phosphate, and no change in acid phosphatase or NADH-diaphorase.

It is unrewarding at this time to speculate on the significance of the enzymatic changes brought about by high calcium perfusion. The marked increase in acid phosphatase activity observed suggest that this enzyme is probably liberated from lysosomal elements (de Duve, Pressman, Gianetto, Walliaux and Applemans, 1955). Such a possibility implies that high calcium levels may be deleterious to cellular integrity. However, similar high levels of acid phosphatase are found in other actively metabolizing cells in the anterior pituitary and pancreas (Pearse, unpublished).

Acid phosphatase is concerned with the transfer of phosphate from one alcohol to another



(Danielli, 1958; Dixon and Webb, 1958) and probably becomes active during the secretion of peptide hormones (Pearse, unpublished).

Recent studies by Tagnon and Steens-Lievens (1960a, b, 1961, 1962a, b, c) on prostatic homogenates show that calcium in this tissue inhibits acid phosphatase. This suggests that changes observed in thyroid tissue perfused with high calcium are specific and not a general effect of calcium on all cells.

The other enzymatic changes secondary to variations in calcium concentration reported in this chapter are difficult to explain. A speculative analysis of possible biochemical changes brought about by these external stimuli will have to await further investigation.

The dog thyroids reported in this study have recently been examined with the electron microscope (Pearse, in press). A new cell type has been identified which is distinctly different from other cells. The cell contains a large irregular nucleus, numerous mitochondria and secretion granules which appear to be released into adjacent capillaries. The cell is believed to be the same as the "mitochondrion-rich" cell described in this chapter. Pearse has proposed that they be called "C-cells" for calcitonin secreting cells to differentiate them from non-calcitonin secreting acinar cells or "A-cells".

In conclusion this study has shown that the thyroid in the dog is composed of two metabolically distinct cells: the principal and the "mitochondrion-rich" cells. Sensitivity to changes in plasma calcium suggest that calcitonin is secreted by the latter. Final confirmation will have to await the results of investigations using immunofluorescent techniques.

Chapter V

Bioassay Methods for Calcitonin

Summary

An intravenous infusion technique was developed for the estimation of calcitonin activity. Falls in plasma calcium were measured in rats infused with calcitonin extracts over a 1 hr. period. A linear dose response curve was obtained with a mean slope of - 0.536 mEq calcium l^{-1} (log µg protein N)$^{-1}$. The time course relations of plasma calcium falls produced by extracts given in a single injection subcutaneously, intraperitoneally, intravenously or by intravenous infusion were studied. Intravenous single injection proved almost as sensitive as intravenous infusion. A dose response curve was found linear for greater than a 100 fold dose range. The mean slope was 0.438 mEq calcium l^{-1} (log µg protein N)$^{-1}$. Because of greater simplicity the injection method is to be preferred. Preparation of a calcitonin standard for future assays is described and a unit of activity defined.

Before progress could be made on the extraction and purification of calcitonin a suitable bioassay was needed. Early thyroid tissue digests had been assayed in the dog and the goat. These animals proved disadvantageous because of: (1) cost, (2) unfeasibility to carry out large numbers of assays and (3) wastefulness, since relatively large quantities of extract had to be used. In short, these animal assays suffered the same defects of the Collip PTH dog assay.

To avoid these difficulties the rat was adopted as the assay animal, and the technique of MacIntyre (MacIntyre, Boss and Troughton, 1963)

for tail vein infusion was adapted for assessing plasma calcium-lowering activity. The statistical design and regression analyses of the bioassays were assayed by the method of Finney (Burn, Finney and Goodwin, 1950).

Experiment 1 - Intravenous infusion assay.

Animals: Healthy 135-185 g Hammersmith strain piebald rats, matched for sex, were used in all experiments. Animals were maintained on a normal diet. Food was withdrawn the evening prior to assay.

Technique: The animals were infused intravenously over a 1 hr. period with 3.0 ml containing 0.2 - 0.8 ml of calcitonin extract. The remainder was made up of a solution containing 44 g/l glucose, 19 mEq/l sodium and 0.1 g/l soldium heparin (Evans). Infusion solutions were delivered by means of a multichannel Technicon automated analyzer pump. The tubing used had an internal diameter of 0.1 mm and was fitted terminally with a 23 gauge needle.

The animals were put into separate cylindrical plastic containers (14 cms long and 5.5 cms in diameter). Their tails were prewarmed in a water bath at 48-52^{o} for approximately 1 min. before introduction into a caudal vein of the needle tipped end of the infusion tubing. The needle was held in place with cellotape. Individual rats were mounted on a frame as shown in Figures V - 1 with their hind legs restrained.

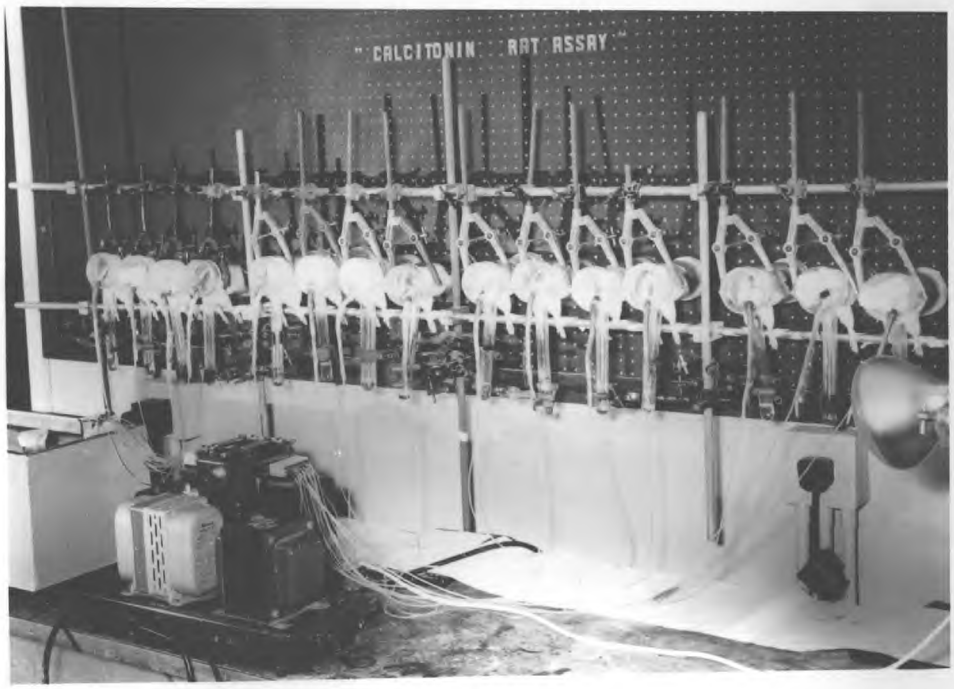


Figure V - 1. Intravenous infusion bioassay. Extracts are administered at a rate of 3 ml/hr. by means of a multiple channel constant infusion pump to caudal veins of individually restrained rats. Patency of the vessels is facilitated by externally heating the tails with infra-red lamps and by administering extracts prewarmed in a constant temperature bath.

In this way 15 rats could be accommodated. Prior to assay all animals were infused with the stock glucose-saline solution for a 3 hr. control period. The intake ends of the infusion tubes were then transferred to the extract solutions for assay. To prevent vaso-constriction in the rats' tails the infusion solutions were kept in a water bath at 40° and the tails were warmed externally with infra-red lamps. Animals in which infusions were suspected to be faulty were rejected.

Blood collection: At the conclusion of the 1 hr. extract perfusion period the rats were dismounted, anaesthetized with ether and their peritoneal cavities exposed with scissors. Using a 23 gauge needle on a heparinized syringe 3 - 7 ml of blood were removed from the abdominal aorta and the animals sacrificed. The blood collected was centrifuged in acid-washed tubes and the plasma collected.

Estimations: Dilutions were carried out for calcium within 30 min. of collection as described in Chapter I. In each assay run 4-5 animals were used as controls. These received 0.2 - 0.8 ml of the extract solvent made up to 3.0 ml with stock glucose-saline solution to provide suitable controls for each extract solution assayed.

Extract solutions: The extracts used in this and subsequent experiments in this chapter were prepared from a sodium chloride precipitated fraction of a 0.2$\underline{N}$-hydrochloric acid digest of acetone

dried pork thyroid. (Full details of this preparation will be given in the next chapter). The precipitate was taken up in 0.1 M-acetate buffer, pH 4.6, and seven dilutions made from the original solution over a hundred-fold dose range. In addition, a four-point bioassay was carried out using two different calcitonin extracts (TE 92A and 92B) at two dilutions.

Results.

The results obtained are expressed in Figure V - 2. The plasma calcium values are shown as the difference between the mean of control animals and individual plasma calcium levels. The dose response curve showed no evidence of non-linearity. The mean slope was -0.509 ± 0.034 mEq calcium l^{-1}, (log µg protein N)$^{-1}$. This was significantly different from zero ($p<0.01$). On the basis of this assay an arbitrary unit of activity was defined as

> 1/10 that amount of calcitonin which produced a fall in plasma calcium of 1 mg/100 ml (0.5 mEq/l) plasma when infused intravenously into a 150 g Hammersmith strain piebald rat over an hour period.

The results of the four-point assay showed parallelism (Figure V-3a). The potency ratio of the two preparations was 0.771 with 95 percent confidence limits of 1.131 and 0.457.

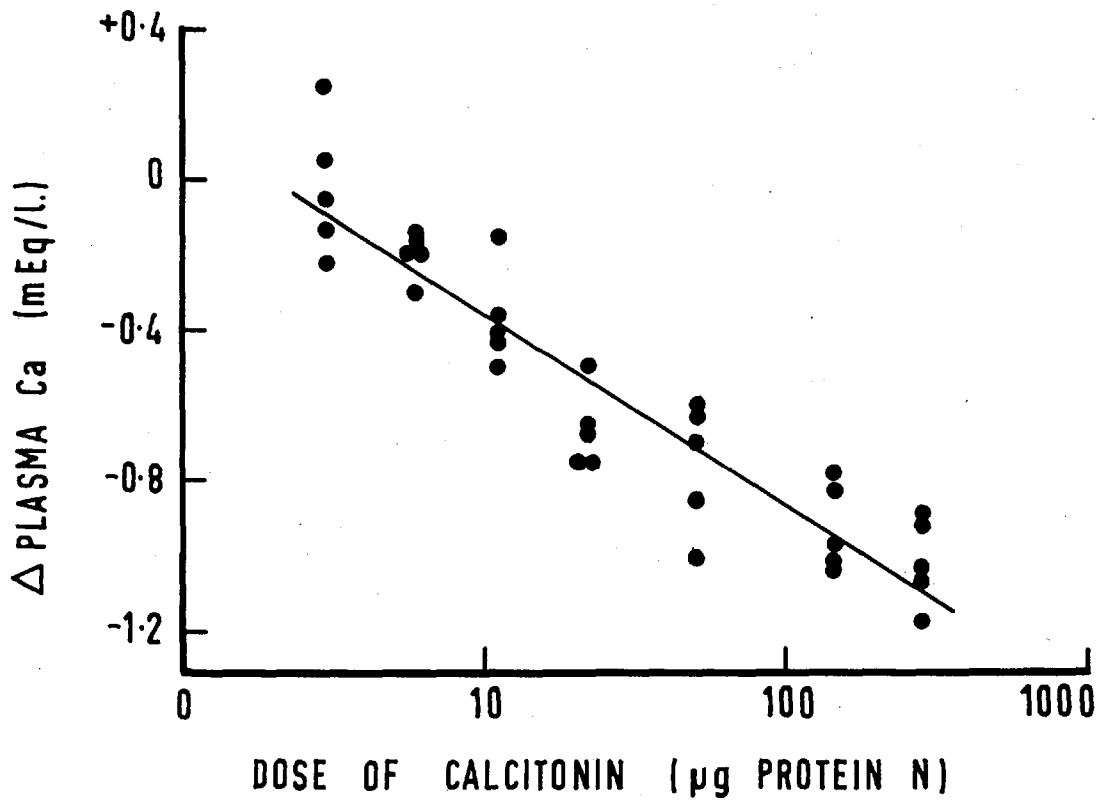


Figure V - 2. Dose response curve obtained by intravenous infusion of salt precipitated extract over a 1 hr. period in 150 g Hammersmith piebald rats. The calculated slope is -0.509 ± 0.034 mEq calcium l^{-1} (log µg protein N)$^{-1}$.

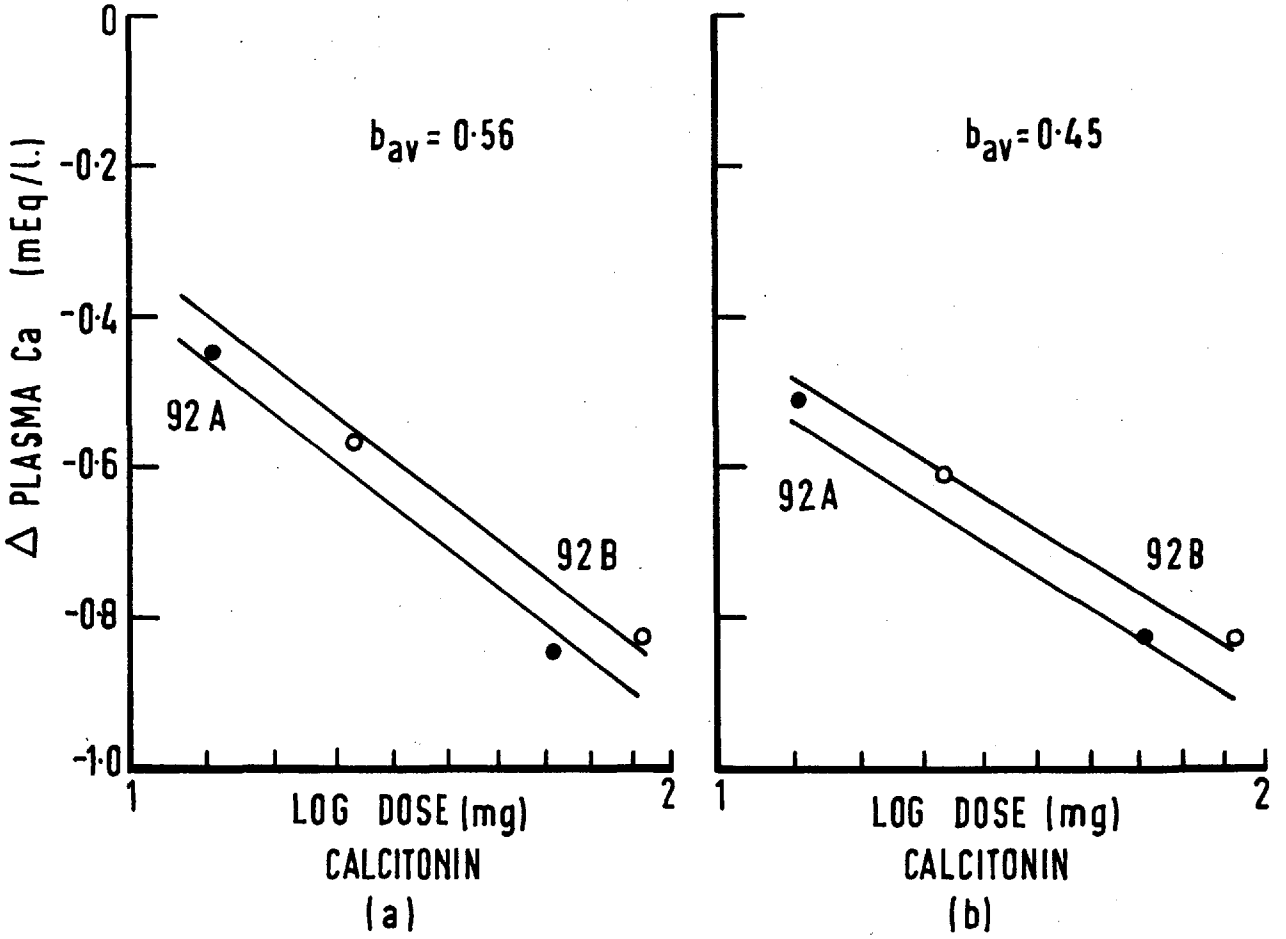


Figure V - 3. Four point biological assay of partially purified calcitonin (salt precipitated TE 92A and 92B) by (a) intravenous infusion and (b) single injection technique. Each point represents the mean value obtained in six assay rats. Statistical analyses of data obtained by each method indicated no significant non-parallelism. A common slope for each technique was calculated and the regression lines were drawn parallel.

Experiment 2 - Investigation of other possible routes of administration for assay.

Preliminary studies (Foster, Baghdiantz, Soliman and MacIntyre, 1965) on the time course relationship of a single extract (TE 29) given subcutaneously, intraperitoneally. and by intravenous infusion, suggested intravenous infusion as the route of choice (Figure V - 4, Table V - 1). This route had produced maximum fall in plasma calcium in 60 min. Since the calcium-lowering effect had not been studied for longer than 60 min. and a single intravenous dose assay had not been evaluated, a more detailed investigation of various routes of administration was undertaken.

Methods.

The intravenous assay was similar to that described in the preceding experiment. It differed only in that groups of four rats each received 0.2 ml of TE 92A over 30, 60, 120, 150 and 180 min. Four control animals received 0.2 ml of acetate buffer, pH 4.6, over each time period.

Subcutaneous and intraperitoneal routes of administration were studied as follows. Injections of 0.2 ml of TE 92A were given to 24 rats and plasma calcium measured in groups of 4 animals, every 30 min. for 3 hr. The mean base value from six untreated rats served as controls.

0.2 ml of the same extract was given intravenously as a single

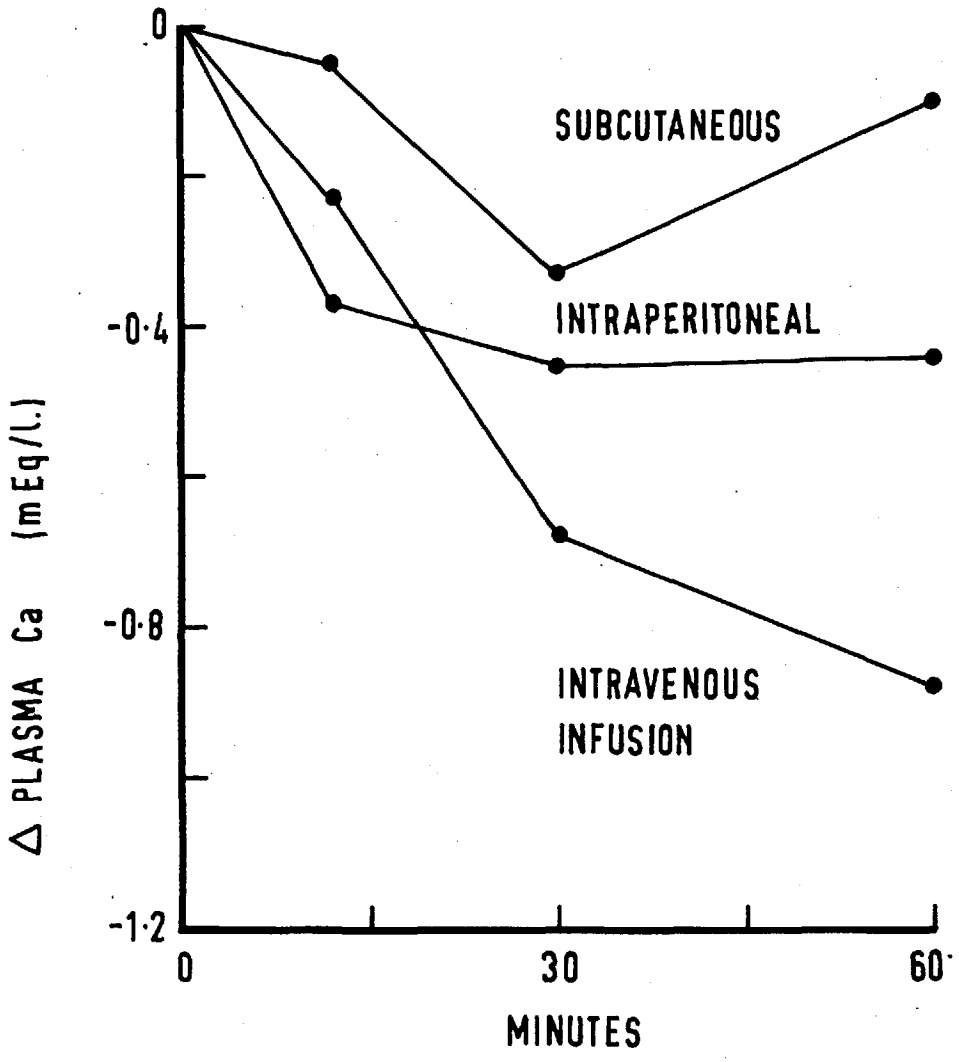


Figure V - 4. Preliminary study to investigate rat assay systems, using varying routes of administration. 0.4 ml of crude extract (TE 29) was injected into each animal. The average of plasma values obtained in three animals is plotted as a single point.

Table V - 1. Pilot study to investigate assay systems using various routes of administration. 0.4 ml of crude extract (TE 29) dialyzed against 0.1 M-acetate buffer, pH 4.6, was used. Each value represents the average of plasma values obtained in three rats.

Route of Administration	Time	Ca mEq/l	P_i mEq/l	Protein g%
Subcutaneous	0*	5.20	3.64	6.73
	15	5.15	3.82	6.59
	30	4.87	3.75	6.52
	60	5.10	3.32	6.48
Intraperitoneal	0*	5.20	3.64	6.73
	15	4.83	3.48	6.56
	30	4.75	3.69	6.77
	60	4.76	3.51	6.74
Intravenous	0	5.01	3.20	6.45
	15	4.78	3.34	6.39
	30	4.33	2.82	6.16
	60	4.13	2.32	6.13

* No injection given

injection into the tail vein of 36 rats. Plasma calcium was measured after 10, 20, 30, 60, 90, 120, 150 and 180 min. Again 6 untreated rats served as controls.

Collection of blood specimens and estimation of plasma calcium were as described in Experiment 1.

Results.

Figures V - 5 and V - 6 show the varying responses. Intravenous infusion proved the most sensitive assay route. A maximum fall was obtained between 60 and 90 mins. Almost as great a fall was observed by intravenous injection. The fall by this route was maximum between 30 and 60 min. As in the pilot experiment (Figure V - 4) relatively small falls were obtained with subcutaneous injection.

Experiment 3 - Intravenous injection assay.

The intravenous infusion method had two disadvantages. It required the use of an automated pump and the number of assay animals was limited to 15 at a time. For these reasons a simplified alternative method was devised.

Methods.

The same methods, protocols and controls were used as in Experiment 1, excepting dilutions of extract (TE 92A) were injected intravenously over a greater than 100 fold dose range. A four-point assay was carried out identically as before.

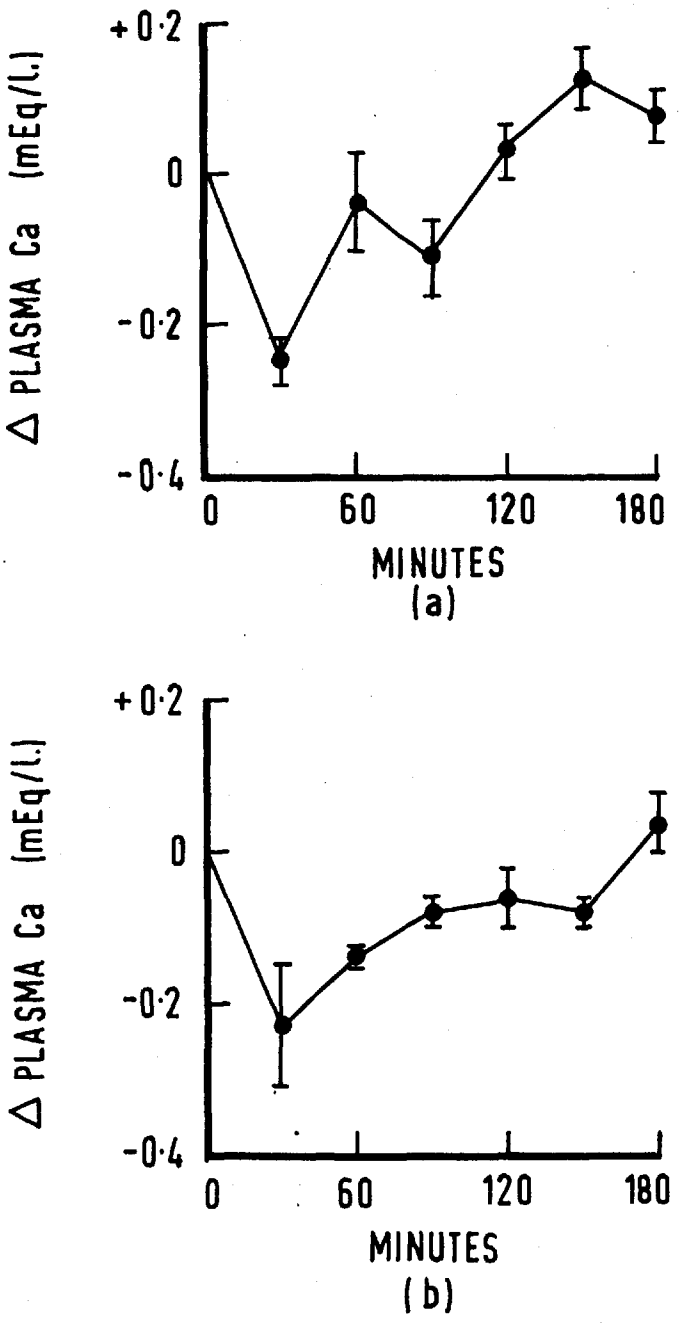


Figure V - 5. Time course relationship of hypocalcemia produced by a salt precipitated extract of calcitonin when given (a) subcutaneously and (b) intraperitoneally. The mean average falls (controls - test) for six animals is represented by each point and the standard errors of the mean by the vertical bars.

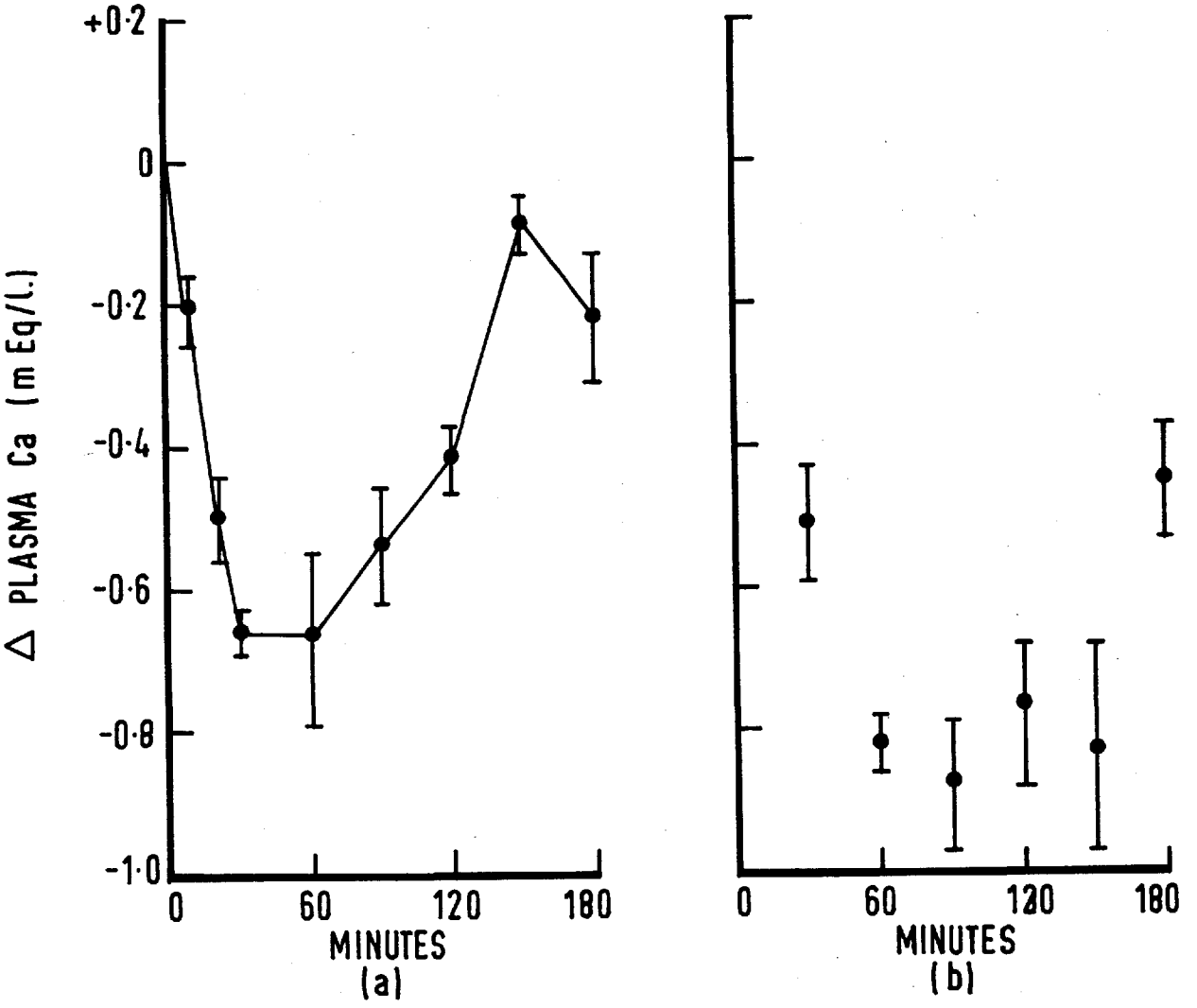


Figure V - 6. Time course relationship of hypocalcemia produced by a salt precipitated extract of calcitonin when given as (a) a single intravenous injection and (b) an intravenous infusion over a 1 hr. period. The mean average falls (control - test) for six animals is represented by each point and the standard errors of the mean by the vertical bars.

Results.

The results depicted in Figure V - 7 show a linear dose response curve was obtained. The slope was 0.460 ± 0.34 mEq calcium l^{-1} (log ug protein N)$^{-1}$. Analysis of variance showed the mean slope was significantly different from zero ($p < 0.01$) and that the slopes for the two preparations individually were not significantly different from each other (Figure V - 3b). The potency ratio of the two preparations was 0.805 (TE 92A and TE 92B) with 95 percent confidence limits of 2.847 and 0.311.

Experiment 4 - Preparation of a calcitonin standard for future assays.

A standard calcitonin preparation was necessary to enable all subsequent intra- and extra-mural assays to be related to a known standard of activity (Baghdiantz, Foster, Edwards, Kumar, Slack, Soliman and MacIntyre, 1964; Foster, Baghdiantz, Soliman and MacIntyre, 1965; MacIntyre, Foster and Kumar, 1965). Both the Hammersmith and Harvard investigators (Hirsch, Gauthier and Munson, 1963) had observed that relatively crude preparations of calcitonin were stable when stored in the frozen state for prolonged periods.

The salt precipitated fraction (Chapter VII) from TE 90A and TE 90B were pooled, taken up in 0.1M-acetic acid and lyophilized in multiple 2 ml ampoules. These were sealed and stored at - 20°.

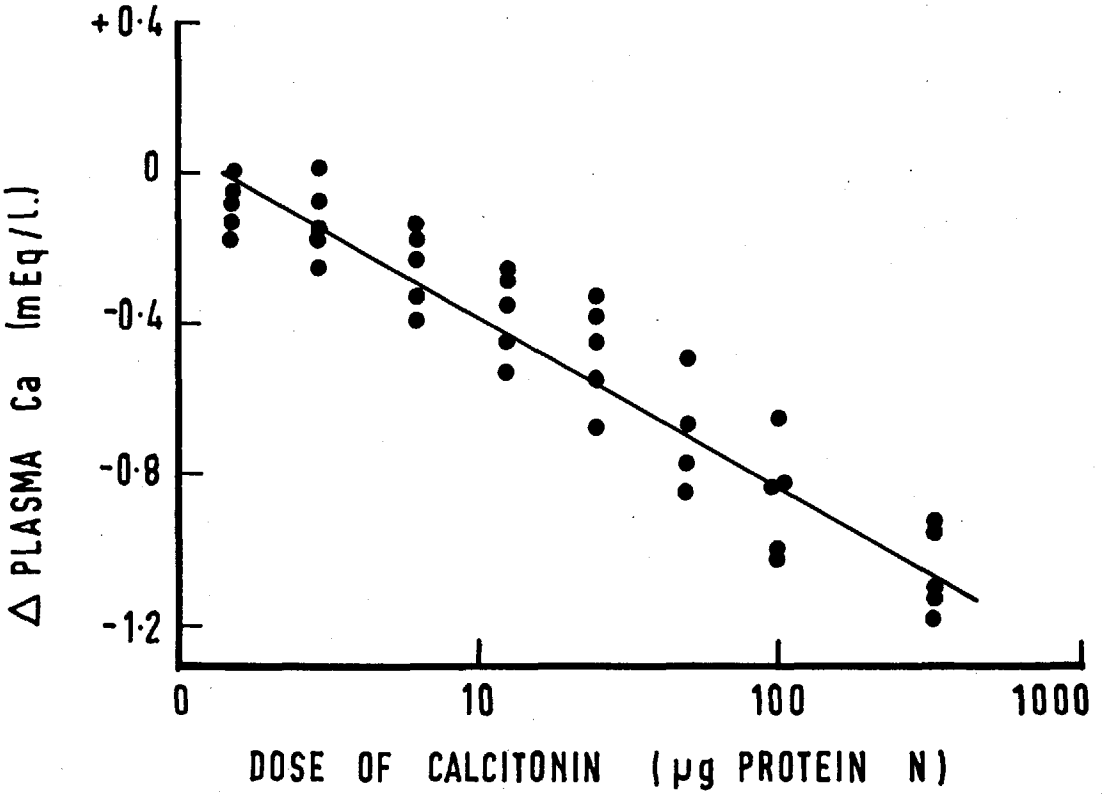


Figure V - 7. Dose response curve obtained by single intravenous injection of salt precipitated extract over a 1 hr. period in 150 g Hammersmith piebald rats. The calculated slope is -0.460 ± 0.034 mEq calcium l^{-1} (log μg protein N)$^{-1}$.

The extract contained 75 μg protein nitrogen per mg. A unit of calcitonin was redefined as that amount contained in 13.3 μg of this powder. Intravenous injection of 10 units produced approximately a 10 percent fall in plasma calcium after 50 min. in 150 g Hammersmith assay rats.

DISCUSSION

The potency ratio estimates by each of the intravenous assay methods described in this chapter were similar: the infusion method is more precise. The confidence limits and index of precision ($\lambda = \frac{S}{b}$ where S^2 = error mean square and b = slope; Bliss, 1952) were 0.26 for the infusion and 0.42 for the injection method. Two previous injection assays (not reported) gave an index of precision of 0.51; a previous infusion assay value of 0.29.

The bioassay systems compare favourably with the method developed by Hirsch, Voelkel and Munson (1964). These investigators used 150 - 180 g male Holtzman rats maintained on a purified low calcium diet for 4 days prior to assay. Standard and unknown extracts were administered subcutaneously to parallel groups of rats and blood samples drawn by cardiac puncture after 1 hr. The dose response curve obtained under these conditions was linear over only an eight fold dose range. Their method has the additional disadvantage in that the purity of extracts assayed affects the speed

with which they are absorbed from their injection sites. This objection is precluded by intravenous administration.

The intravenous infusion technique (Experiment 1) was used exclusively in the studies described in the following chapters since the injection method had not been developed at that time. The relatively greater simplicity of the single injection makes it the method of choice for future work. Pending further purification of calcitonin, all animal bioassay systems may someday be replaced by a simpler immunological procedure.

Since these studies were completed a standard preparation of calcitonin has been prepared by the Medical Research Council Department of Biological Standards to replace those originally proposed. It consists of material purified to the stage of salt fractionation (Chapter VI), distributed into ampoules and freeze-dried. Each ampoule (containing approximately 250 μg protein/mg) has been assigned a potency of 0.25 units. This unit is about 1000 times larger than the unit described in this chapter. Intravenous injection of 10 m-u into 150 g rats produces a fall of approximately 10% in plasma calcium after 50 min.

Hirsch and Munson (1964) have also defined a unit based on the depression of serum calcium values in the rat. Their unit appears to be about 100 times smaller than the MRC unit but no exact comparison has yet been made.

MRC Calcitonin Standard A is available from the Department of Biological Standards, National Institute for Medical Research, Mill Hill, London N.W.7.

Chapter VI

Extraction and Partial Purification of Calcitonin

Summary

Calcitonin activity was extracted from acetone dried pork thyroid with 0.2N-hydrochloric acid. The crude extract was relatively stable to heat and ageing, but was inactivated by boiling in the presence of excess acid or alkali. When dialyzed at 4° for up to 48 hr. against 0.1M-acetate buffer at pH 4.6 or 0.1N-hydrochloric acid in 24/32 "Visking" tubing all the activity remained with the dialysis residue. Extract dialyzed against 0.2N-hydrochloric acid was dried from the frozen state and taken up in 0.1M buffers at pH 1.2-9. No activity was lost below pH 4.6 after 2 weeks at 4°. Above pH 7.2 a precipitate formed in the crude extract and some activity was lost with the precipitate.

Preliminary fractionation studies of undialyzed crude extract were carried out using ammonium sulphate. At 0.2 saturation ammonium sulphate precipitated most of the inactive protein. Since ammonium sulphate was found to be toxic to assay animals sodium chloride was tried. Crude extract was dialyzed first against 0.1M-sodium acetate buffer. With 1M-sodium chloride most of the inactive protein was precipitated leaving 48% of the calcium-lowering activity in the supernatant. With 2M-sodium chloride all activity was precipitated. Utilizing a cut between 1M- and 2M-sodium chloride a six fold purification of the crude extract was achieved.

Preliminary extraction studies demonstrated that calcitonin activity was readily extractable with hydrochloric acid, acid alcohol, saline alcohol and alkali (Table VI - 1). Pig thyroid was found to be a richer source of activity than beef glands. For this reason porcine

Table VI - 1. Preliminary study on the effect of thyroid extracts on blood plasma constituents. Minced fresh frozen pork thyroid was extracted in a ratio of 1 g wet weight to 10 ml of solvent. Digestion was carried out at 70-90° for 5 minutes and the mixture allowed to stand for 1 hour at room temperature. The residue was washed with an equal volume of water before centrifugation. Each dog received 10 ml of extract intravenously and each rat 0.2 - 0.3 ml by intravenous infusion over a 1 hour period. Values given for rats represent mean deviation of 4 - 5 animals from 4 - 5 control animals not receiving extract.

	DOGS								RATS			
Solvents	Animal No.	B.P. mmHg	Δ Ca mEq/l	Δ Mg mEq/l	Δ P_i mEq/l	Δ Na N/100 ml	Δ K N/100 ml	Δ Protein g/100ml	Δ Ca mEq/l	Δ Mg mEq/l	Δ P_i mEq/l	Δ Protein g/100ml
50% ethanol 0.2N-HCl	115	-10	-0.35	-0.23	-0.35	.0	0	0	-0.51	-	-	-0.39
0.2N-HCl	116	-10	-0.24	0	0	0	0	0	-0.52	-	-	+0.19
4.5% saline 50% ethanol	120	-10	-0.41	-0.27	0	0	0	0	-	-	-	-
0.2N-Na_2CO_3	128	-10	-0.35	-0.16	-0.67	0	0	0	-0.62	-	-0.54	-0.59

Compare with Table II - 8.

thyroid was used in subsequent experiments. It had three advantages over thyroid tissue from other species. First, it contained no parathyroid tissue (Bourdelle, 1920; Care, personal communication). Second, it was far less fatty than beef. Third, it was readily available from commercial sources. Since early investigations had shown it could be defatted with acetone without any appreciable loss of activity, extracts were prepared from acetone dried material.

Methods.

Preparation of acetone dried pork thyroid: The protocol used for the preparation of a fat-free gland powder was that of L'Heureux, Tepperman and Wilhelmi (1947) with minor modifications. Fresh frozen pig thyroid was ground together with crushed dry ice through a mechanical mincer with 9/16" cutters. The material was collected in additional dry ice and the procedure repeated two more times. Four volumes of prechilled reagent acetone for each volume of ground glands was added to the mince. The mixture was stirred mechanically for 15 min. and allowed to settle at 4°. The acetone was syphoned off. Acetone extraction was repeated twice again. When processing more than 1 kg quantities of fresh glands it was found necessary to extract with acetone an additional time. Following the last syphoning the tissue residue was transferred to a Buchner funnel with 541 paper and sucked dry. The final residue was transferred to a cold vacuum oven

and vacuum dried for 36 hr. The product was approximately 25 percent of the original frozen weight and had a light pink colour. If defatting was incomplete the material had a brownish tint and was difficult to work with.

Hydrochloric acid extraction: The acetone dried thyroid powder was stirred with 0.2N-hydrochloric acid at 60° - 80° for 5 min. in a ratio of 1 g of gland powder to 10 ml of acid. The mixture was allowed to stand at room temperature for 1 hr. It was filtered through three folds of cheese cloth and the residue washed with an equal quantity of distilled water. Washings were added to the filtrate. Together they were centrifuged at 1.6×10^3 g_{av} for 30 min. to remove particulate matter. The supernatant was decanted and the residue discarded. The crude extract had a potency of approximately 180 units calcitonin/µg protein nitrogen.

Bioassays: Calcium-lowering activity was assayed by measuring the fall in plasma calcium in a 150 g rat following intravenous infusion over a 1 hr. period (see Chapter V). Protein nitrogen determinations were done by the method of Lowry (Bailey, 1962).

Study on the effect of boiling: A 10 ml aliquot of crude extract (TE 49 dialyzed overnight against 0.1N-hydrochloric acid at 4°) was boiled for 30 min. in a reflux condenser. The boiled extract, unboiled extract and solvent were then immediately assayed.

Study on the effect of dialysis: 10 ml of crude extract (TE 52) was put in three individual 24/32 Visking dialysis bags. These were dialyzed against 3 l of 0.1$\underline{\underline{N}}$-hydrochloric acid at 4° for 3, 24 and 48 hr. respectively. The dialysis residues were assayed for calcitonin activity and protein and their volumes recorded. The results were compared with an undialyzed sample.

Study on the effect of pH: Multiple 4 ml aliquots of crude extract (TE 55) were dried from the frozen state and restored to their original volumes in 0.1$\underline{\underline{M}}$ buffers ranging from pH 1.2-9 (see Table VI - 2). After storage at 4° for 2 weeks they were centrifuged and assayed. For each sample a parallel assay was run using the buffer alone and the same lyophilized extract freshly made up with the buffer. Activity was recorded as the difference between plasma calcium-lowering activity of the extract and the buffer.

Ammonium sulphate fractionation study: Precipitations were carried out at room temperature with additions of a saturated solution of analar ammonium sulphate to a 25 ml system of crude extract (TE 53). An amount of ammonium sulphate sufficient to bring the solution to 0.1 saturation was added slowly with constant stirring. Stirring was continued for an additional 20 min. after completion of the addition. The precipitate which formed was collected by centrifugation at 1.6×10^3 g_{av} for 30 min. Precipitation with ammonium sulphate was

Table VI - 2. Composition of buffer solutions used in studying the effect of pH on crude calcitonin extracts.

Buffer	pH	Stock Solutions		Composition (made up to 100 ml)
		A	B	
Clark & Lub's potassium chloride-hydrochloric acid (Clark & Lubs, 1917)	1.2 2.0	0.2M K Cl (14.9g/l)	0.2N HCl	25 ml A + 48.50 ml B.
Walpole's acetate (Walpole, 1914, Gomori, 1955)	3.6	0.2M acetic acid (12.0g/l)	0.2M sodium acetate (16.4g anhydrous or 27.2g hydrous/litre)	46.3ml A + 3.7 ml B.
	4.6			25.5ml A + 24.5 ml B.
	5.6			4.8 ml A + 45.2 ml B.
Gomori's tris (Gomori, 1948, 1955)	7.2	0.2M tris (24.2g tris(hydroxymethyl) aminomethane/l)	0.2N HCl	25 ml A + 22.1 ml B.
	8.0			25 ml A + 13.4 ml B.
	9.0			25 ml A + 2.5 ml B.

continued stepwise, collecting precipitates at 0.2, 0.3, 0.4 and 0.6 saturation. The precipitates harvested were taken up in 4.0 ml volumes of 0.1N-hydrochloric acid and assayed for protein. Each fraction was further diluted with 0.1N-hydrochloric acid so that each contained 2.0 µg protein nitrogen per ml. Bioassays were carried out for each extract and for 0.1N-hydrochloric acid as control.

Sodium chloride fractionation study: 5 ml aliquots of crude extract (TE 53) were dried in vacuo from the frozen state in vials and stored at 4° until used. Each residue was taken up in 2.5 ml of 0.2M-acetate buffer pH 4.6 and stirred until dissolved. Equal volumes of sodium chloride solution of such molarity as to give final concentrations of 0.1, 0.2, 0.5, 0.7, 1.0, 1.2, 1.5 and 2.0 M were added. The extracts were allowed to stand overnight at 4°. After centrifugation at 1.6×10^3 g_{av} for 30 min., the resultant supernatants were assayed for calcitonin activity. Controls of 0.1, 0.2, 0.5 and 1.0 M-sodium chloride in 0.1M-acetate buffer were run simultaneously.

Starch gel electrophoresis: Starch gel was prepared using a buffer with a pH of 2.9 containing 0.065M-formic acid, 0.001M-sodium hydroxide and 0.005M-ethylenediaminetatraacetate. Approximately 1 mg of lypholized crude extract and 1 mg of material harvested between a 1 and 2 M-sodium chloride fractionation of dialyzed extract were applied and the system developed under a potential gradient of 12 volts for 12 hr. at room temperature.

Results.

Effect of boiling: Crude extract boiled for 30 min. lowered plasma calcium in assay animals only 0.18 mEq/l less than unboiled extract (Figure VI - 1a and Table VI - 3). The data showed the crude extract to be relatively stable to heat and suggested that it is either protected by associated proteins or is moderately resistant to acid hydrolysis.

Effect of dialysis: As shown in Figure VI - 1b and Table VI - 4, dialysis in the cold produced no change in activity. Since the membrane used was porous to molecules with molecular weights less than 20,000 the results suggested that the activity was associated with a molecule larger than this. The study showed that dialysis could effectively be used to:-

(1) remove small inactive material, and

(2) exchange the acid solvent for a buffer closer to a physiological pH.

Effect of pH: The results of changes in activity in extracts stored at 4° for 2 weeks are shown in Figures VI - 2a and b and Table VI - 5. Calcium-lowering activity remained relatively constant at pH 1.2 - 5.6. Above pH 7.2 a precipitate formed and activity was lost in the supernatant. Precipitate which formed at pH 9.0 was redissolved in 0.1M-acetate buffer pH 3.6 and activity was recovered.

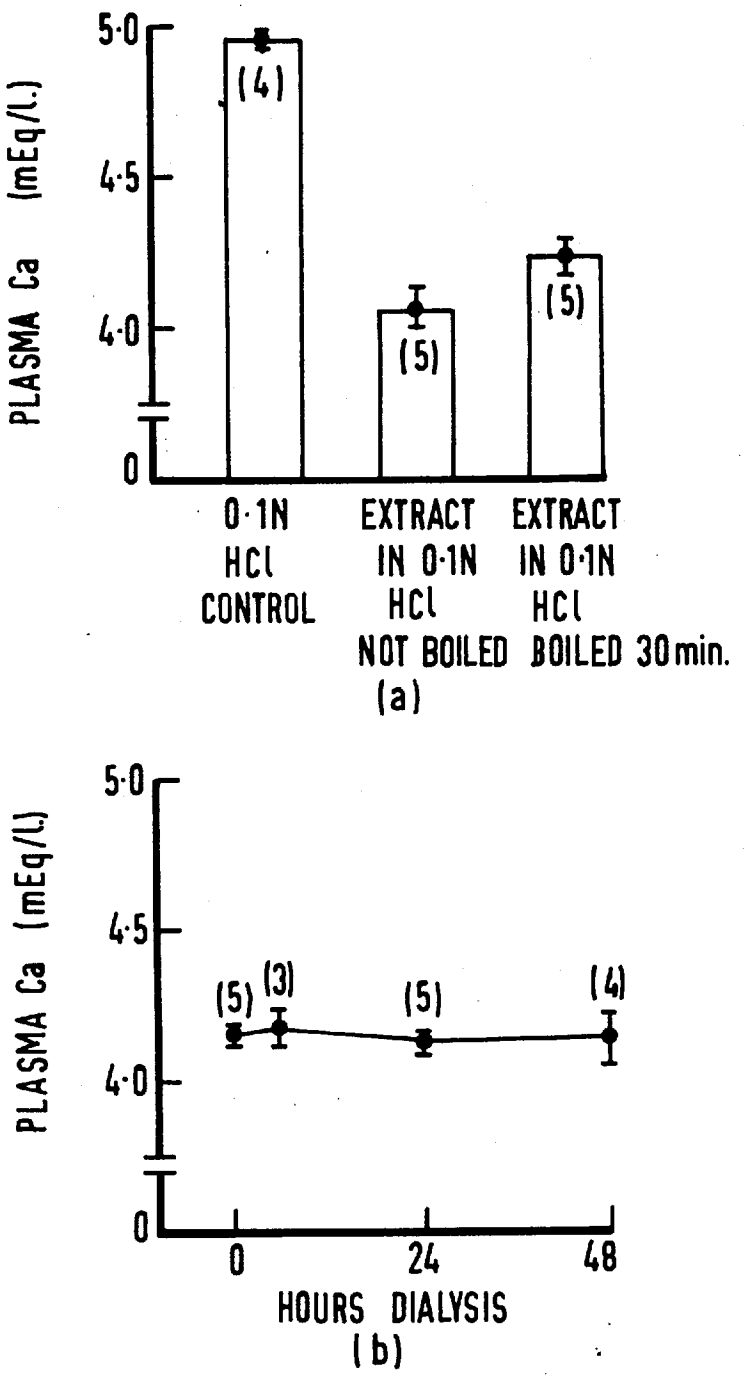


Figure VI - 1. Studies on crude extract: (a) effect of boiling crude extract in the presence of 0.1N-hydrochloric acid, (b) effect of dialyzing crude extract against 0.1M-sodium acetate, pH 4.6 at 4°C. Plasma calcium values refer to assay rats which received 0.4 ml of extract by intravenous infusion over a 1 hr. period. Points indicate mean values, vertical bars the standard errors of the mean and parentheses the number of assay animals used for each determination.

Table VI - 3. Effect of boiling crude calcitonin extract (TE 49) in the presence of 0.1N-HCl.

Preparation	No. rats	Plasma Ca mean ± S.E.	mEq/l range	Δ Ca
Extract in 0.1N-HCl not boiled	5	4.06±0.04	(3.95-4.21)	-0.90
Extract in 0.1N-HCl boiled 30 min.	5	4.24±0.07	(4.10-4.50)	-0.72
Controls using 0.1N-HCl	4	4.96±0.02	(4.95-5.00)	-

Table VI - 4. Study on the effect of dialysis on the activity of crude extract (TE 52).

Duration of dialysis (hr.)	Protein mg N %	No. rats	Plasma Ca mean ± S.E.	mEq/l range	ΔCa
0	67.3	5	4.15±0.03	(4.10-4.25)	-0.95
3	64.8	3	4.17±0.06	(4.10-4.30)	-0.93
24	63.0	5	4.13±0.04	(4.00-4.20)	-0.97
48	63.8	4	4.14±0.08	(3.95-4.30)	-0.96

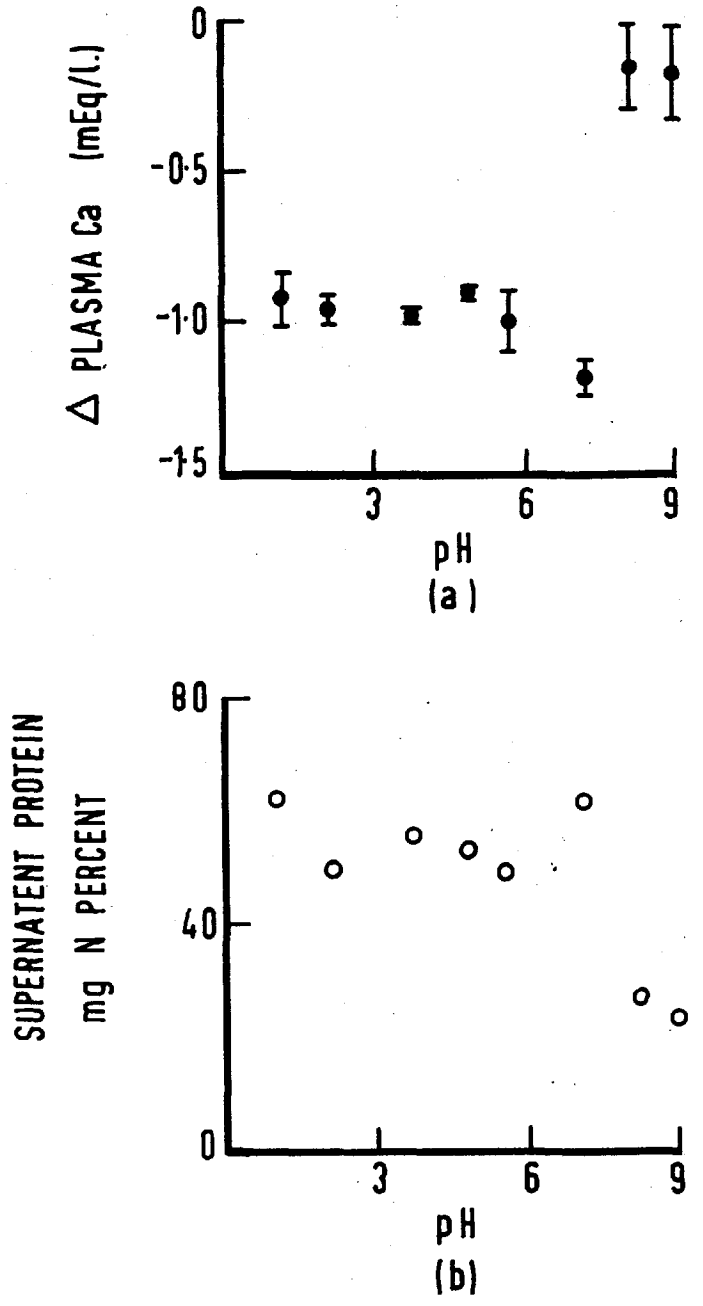


Figure VI - 2. Effect of storage of salt precipitated extract (TE 55) at varying pH for 14 days at 4°. Diagram (a) indicates residual calcium-lowering activity in extracts made up in buffers of different pH. Activity is expressed as the difference between plasma calcium in rats receiving control solutions and those receiving extracts. Points represent mean values, bars the standard error of the means and the number in parentheses the number of animals tested. Diagram (b) shows the protein concentration in each extract. 0.4 ml of extract was administered by intravenous infusion to each assay animal.

Table VI - 5. pH stability studies on crude extract (TE 55).

The effect on calcium-lowering activity of each buffer, extract freshly prepared with each buffer and extract made with each buffer and stored for 2 weeks at 4° were studied. Assays were carried out with 120-190 g male rats. 0.4 ml buffer or extract in buffer was administered by intravenous infusion and plasma calcium measured after 60 min.

	pH	Protein mg N %	No. rats	Plasma calcium mEq/l mean	range	Δ Ca	Calcitonin units/mg	Percent Change
Buffer	1.2		3	5.07	(5.00 - 5.15)			
Fresh extract	1.2	68.44	4	4.21	(4.00 - 4.40)	-0.86	193	
Stored extract	1.2	63.47	4	4.21	(3.95 - 4.30)	-0.86	205	0
Buffer	2.0		3	5.12	(4.95 - 5.25)			
Fresh extract	2.0	57.77	4	4.25	(4.10 - 4.35)	-0.87	237	
Stored extract	2.0	48.88	4	4.21	(4.10 - 4.25)	-0.91	322	+ 57
Buffer	3.6		4	5.23	(5.25 - 5.35)			
Fresh extract	3.6	68.44	4	4.24	(4.10 - 4.30)	-0.99	336	
Stored extract	3.6	56.53	4	4.26	(4.25 - 4.30)	-0.97	371	+ 81
Buffer	4.6		4	5.01	(4.95 - 5.10)			
Fresh extract	4.6	64.88	4	4.23	(4.00 - 4.35)	-0.78	139	
Stored extract	4.6	53.69	4	4.20	(4.10 - 4.25)	-0.81	191	- 7
Buffer	5.6		3	5.37	(5.30 - 5.45)			
Fresh extract	5.6	52.08	4	4.15	(4.10 - 4.21)	-1.22	128	
Stored extract	5.6	48.41	4	4.36	(4.10 - 4.50)	-1.01	547	+ 97
Buffer	7.2		4	5.42	(5.28 - 5.60)			
Fresh extract	7.2	66.06	4	4.29	(4.10 - 4.60)	-1.13	680	
Stored extract	7.2	63.00	4	4.25	(4.15 - 4.30)	-1.17	833	+ 209
Buffer	8.0		4	4.89	(4.65 - 5.10)			
Fresh extract	8.0	33.19	4	4.06	(3.95 - 4.15)	-0.83	331	
Stored extract	8.0	27.00	4	4.70	(4.20 - 5.10)	-0.29	36	- 82
Buffer	9.0		4	5.05	(4.90 - 5.20)			
Fresh extract	9.0	25.65	4	4.30	(4.20 - 4.40)	-0.75	302	
Stored extract	9.0	23.40	4	4.70	(4.20 - 5.10)	-0.35	53	- 75
Buffer	3.6		4	5.23	(5.15 - 5.36)			
pH 9.0 precipitate			2	4.28	(4.15 - 4.40)	-0.98		

Ammonium sulphate fractionation: The data is shown in Figure VI - 3a and b and Table VI - 6. The Cohn fractionation method (Cohn, 1940) was not used in this study since the effect of dialysis had not at the time been determined. 0.2 saturation ammonium sulphate precipitated most of the inactive protein while not affecting calcium-lowering activity in the supernatant. At 0.6 saturation all activity was precipitated.

This method appeared to give satisfactory fractionation of crude extract. It was not developed further because ammonium sulphate was found to be toxic to assay animals and to interfere with protein nitrogen determinations (Keller and Block, 1960). It was abandoned in favour of a method using sodium chloride.

Sodium chloride fractionation: As can be seen from the results shown in Figure VI - 4 and Table VI - 7 sodium chloride provided an alternative method of fractionation. At 0.1M-sodium chloride 11 percent of the activity was lost from the supernatant. At 0.5M 17 percent, 1.0M 52 percent, 1.5M 87 percent and 2.0M all activity was removed. Concomitantly, 21 percent of the protein was precipitated at 0.1M, 43 percent at 0.5M, 67 percent at 1.0M and 82 percent at 2M-sodium chloride.

Starch gel electrophoresis: The results are shown in Figure VI - 5. Fourteen bands could be demonstrated in crude extract and eleven after salt precipitation.

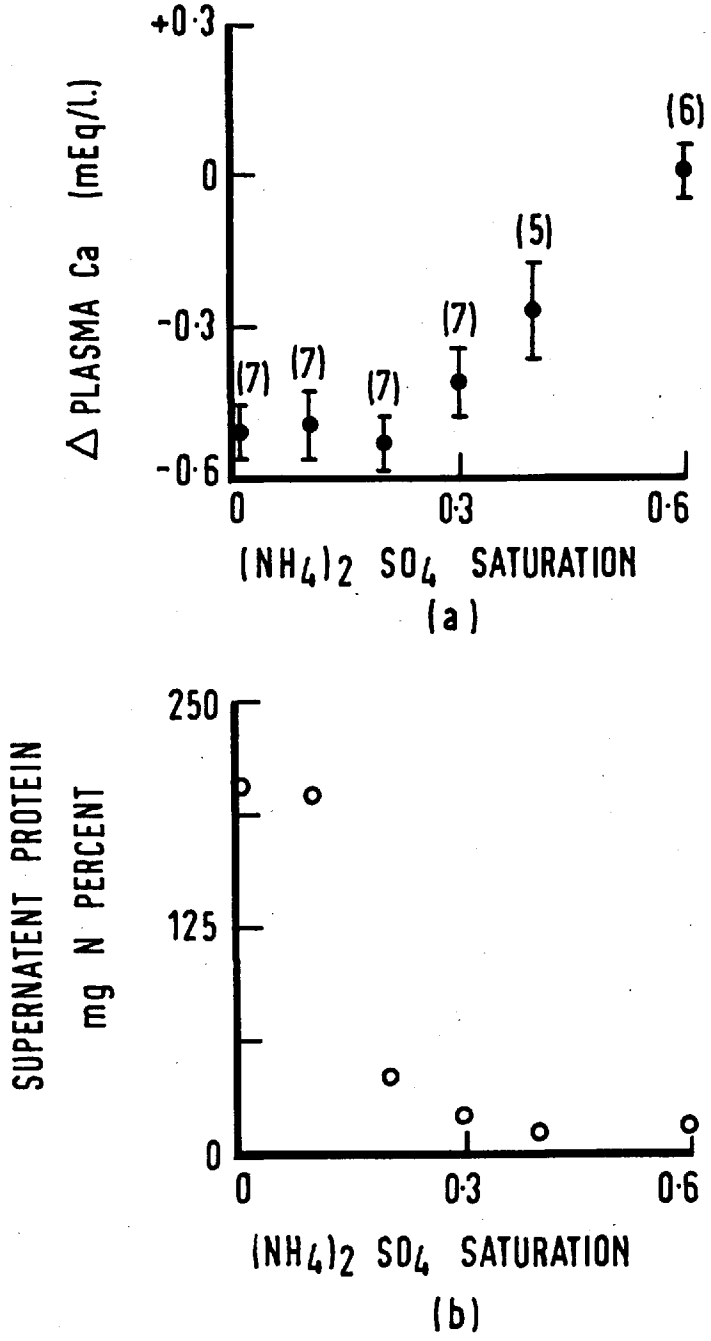


Figure VI - 3. Effect of ammonium sulphate precipitation on calcium-lowering activity of undialyzed crude extract (TE 53). Diagram (a) indicates the relative residual activity in supernatants following serial precipitation as expressed by the difference between plasma calcium in rats receiving control solutions and those receiving extracts. Points represent mean values, bars the standard error of the means and numbers in parentheses the number of animals tested. Diagram (b) shows protein concentration in each fraction used in the diagram above. 0.4 ml of extract was administered by intravenous infusion to each assay animal.

Table VI - 6. Ammonium sulphate fractionation study on crude extract (TE 49).

Crude extract and each precipitate were taken up in 5 ml 0.1N-hydrochloric acid and dialyzed against 3 litre 0.1N-hydrochloric acid at 4° overnight. Dilution of the dialysis residue was carried out so that a 0.08 mg N/0.4 ml dose was given to each assay animal.

Preparation	$(NH_4)_2SO_4$ saturation	Protein mg N %	No. rats	Plasma calcium mEq/l mean ± S.E. range	Δ Ca	Units Calcitonin	Percent Activity
0.1N-HCl	-	-	6	4.94±0.06 (4.90 - 5.00)			
Fraction 1	0 - 0.1	216.0	7	4.44±0.06 (4.05 - 4.71)	-0.50	10.0	100
Fraction 2	0.1 - 0.2	38.0	7	4.40±0.07 (4.15 - 4.65)	-0.54	10.2	100
Fraction 3	0.2 - 0.3	20.0	7	4.53±0.07 (4.15 - 4.75)	-0.41	6.6	65
Fraction 4	0.3 - 0.4	8.9	5	4.67±0.10 (4.40 - 4.95)	-0.27	3.5	35
Fraction 5	0.4 - 0.6	16.0	6	4.95±0.06 (4.75 - 5.00)	0	0	0
Crude extract dialyzed		222.0	7	4.42±0.06 (4.20 - 4.60)	-0.52	10.1	100

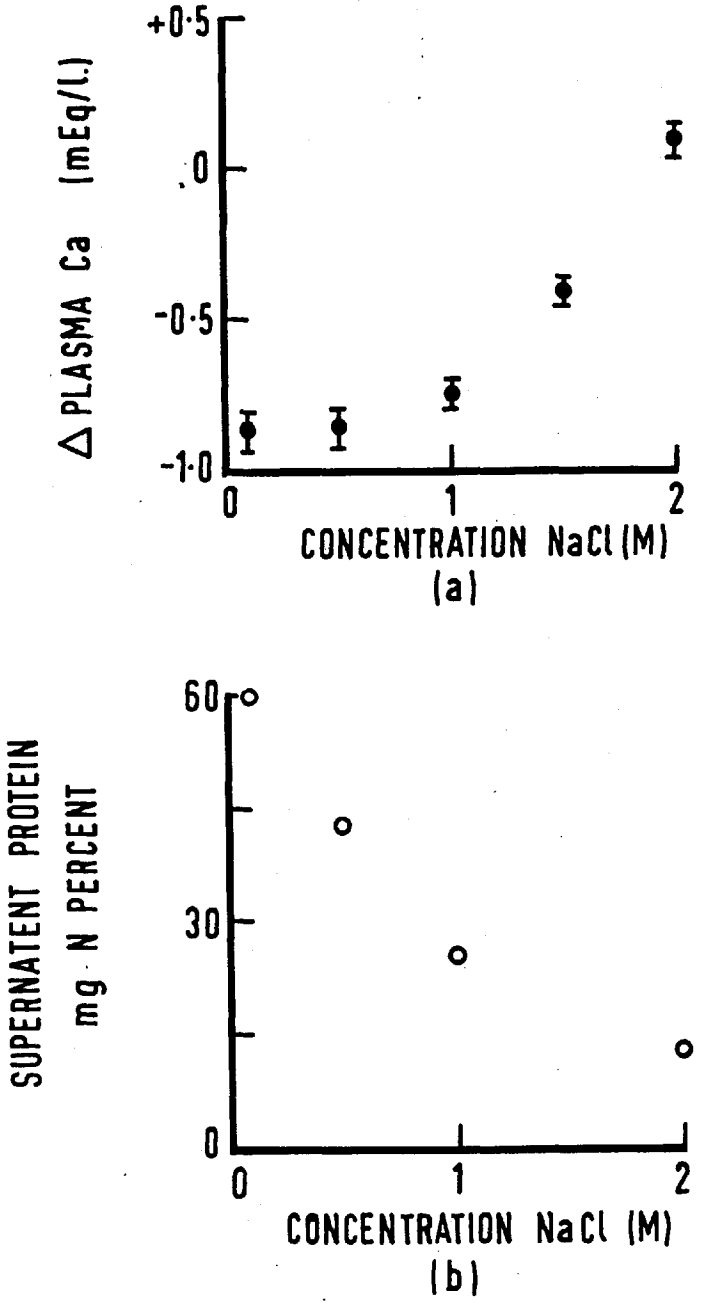
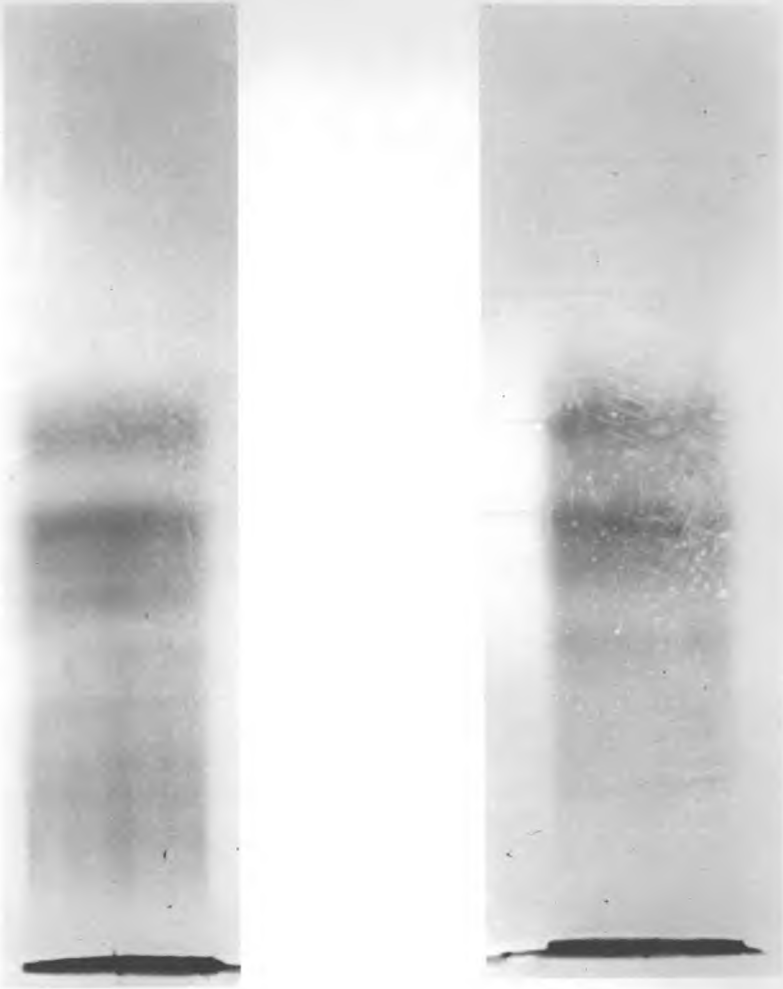


Figure VI - 4. Effect of sodium chloride precipitation on calcium-lowering activity of crude extract (TE 53) dialyzed against 0.1M-sodium acetate, pH 4.6. Diagram (a) indicates the relative activity in supernatants following precipitation as expressed by the difference between plasma calcium in rats receiving control solutions and those receiving extracts. Points represent mean values, bars the standard error of the means and the numbers in parentheses the number of animals tested. Diagram (b) shows protein concentration used in each fraction in the diagram above. 0.4 ml of extract was administered by intravenous infusion to each assay animal.

Table VI - 7. Salting-out study on crude extract (TE 53) using sodium chloride. Values reported below refer to residual supernatant.

Preparation	Protein mg N %	No. rats	Plasma calcium mEq/l mean	range	△ Ca	Units calcitonin	Percent loss of activity	Percent loss of protein
0.1M Supernat.	59.80	4	4.05	(3.90 - 4.20)	-0.88	56	-11	-21
0.1M Control		3	4.93	(4.85 - 5.00)				
0.5M Supernat.	42.74	4	4.01	(3.90 - 4.15)	-0.86	52	-17	-43
0.5M Control		4	4.87	(4.80 - 4.90)				
1.0M Supernat.	25.85	4	4.13	(4.00 - 4.20)	-0.74	30	-52	-67
1.0M Control		4	4.87	(4.80 - 4.95)				
1.5M Supernat.		4	4.40	(4.30 - 4.50)	-0.47	8	-87	
2.0M Supernat.	13.36	4	4.90	(4.80 - 5.10)	+0.10	0	-100	-82
4.0M Supernat.		4	4.96	(4.70 - 5.05)	+0.09	0	-100	
Extract in buffer	75.59	5	4.00	(3.85 - 4.20)	-0.91	63	0	0
Buffer, pH 4.6		6	4.91	(4.85 - 4.95)	-	0		



(A) (B)

Figure VI - 5. Electrophoretic patterns on starch gel of crude extract (A) and calcitonin partially purified by salt fractionation (B).

DISCUSSION

Use was made of the preceding observations to partially purify the crude extracts as follows: 30-50 ml of crude extract was dialyzed in 24/32 Visking tubing against 3 l of 0.1M-acetate buffer, pH 4.6, overnight at 4°. The dialysis residue was removed and its volume recorded. An equal volume of 2M-sodium chloride was added slowly with stirring to bring the final concentration to 1M. The solution was stored at 4° for 12 hr. The resulting precipitate was separated by centrifugation for 20 min. at 1.1×10^3 g_{av} and discarded. An equal volume of 3M-sodium chloride was added to give a concentration of 2M and allowed to stand at 12 hr. at 4°. The active fraction was harvested by centrifugation at 1.1×10^3 g_{av} for 20 min. and the supernatant discarded. In subsequent experiments the final precipitate was taken up in one-tenth the original volume of the crude extract in either 0.1N-hydrochloric acid or 0.1M-acetate buffer, pH 4.6 This solution had an average potency of approximately 1,200 units/μg protein nitrogen and represented a greater than 6-fold purification of the crude extract.

This method has four major advantages. First, it is simple, inexpensive and requires a minimum of laboratory equipment. Second, it is useful in preparing large quantities of material. Third, the salt precipitated material is stable. Fourth, since the suspending

buffer is non-toxic, relatively large doses of activity can be given to laboratory animals.

Some chemical studies carried out on this preparation are presented in the following chapter.

Chapter VII

Some Properties of Partially Purified Calcitonin

Summary

Sodium chloride fractionated calcitonin was found to be relatively stable to boiling at pH 4.6. 46 percent activity was lost in 30 min. and 73 percent in 2 hrs. All activity was lost if the extract was boiled in 1N-sodium hydroxide or 1N-hydrochloric acid. The peptide nature of the hormone was shown by its inactivation by enzymatic digestion. Extracts incubated at 37° in the presence of trypsin or pepsin lost all activity within 6 hr. Oxidation carried out for 2 hr. at pH 4.6 with 1M-hydrogen peroxide at room temperature did not affect activity. All activity was lost after $2\frac{1}{2}$ hr. of oxidization at pH 4.6 with performic acid at -10°. Activity was unaffected by reduction with cysteine for 5 hr. or by incubation with 8M-urea for 24 hr. The data suggest that calcitonin is a relatively stable polypeptide.

Experiments reported in this chapter were all carried out with calcitonin prepared as previously described in Chapter VI. The precipitate harvested between 1M- and 2M-sodium chloride was dissolved in 0.1M-acetate buffer, pH 4.6, and made up to one-tenth the volume of the original crude extract unless otherwise stated. The studies were done to assess the stability of the hormone under a variety of conditions.

Methods.

Boiling at pH 4.6: 20 ml of salt precipitated extract (TE 60) were diluted with an equal volume of buffer to obtain a total volume one-fifth that of the crude extract. 10 ml was saved for control studies. The remaining 30 ml were boiled under a reflux condenser. After 15, 30 and 120 min., 5 ml samples were taken and kept at 4^{o} until assayed.

Boiling in excess acid or base: Two 25 ml samples of supernatant remaining after 1 M-sodium chloride precipitation of the crude extract (TE 60) were precipitated separately by the addition of 25 ml of 3 M-sodium chloride. After centrifugation one pellet was taken up in 5 ml of 1.0 N-hydrochloric acid and the other in 1.0 M-sodium hydroxide. Both were boiled under reflux conditions for 1 hr., adjusted to pH 4.6 by the addition of a molar solution of the corresponding reagent and made up to a volume of 25 ml with distilled water. Controls were prepared by treating a pair of 5 ml systems of distilled water in the same way without boiling. Each extract was assayed and compared with its corresponding control.

Pepsin digestion: To a 4 ml aliquot of salt precipitated extract (TE 57 made up in 0.1 N-hydrochloric acid to one-sixth the volume of the original crude extract) was added 1 ml of a pepsin solution containing 10 mg pepsin (British Drug Houses Ltd., 1:2500) per ml of

0.1N-hydrochloric acid. The system was incubated at pH 1 - 2 at 37° in a water bath. After 6 hr. 2.5 ml were removed and neutralized to pH 7.0 with 0.1N-sodium hydroxide. The remaining solution was incubated for a total of 24 hr. and similarly neutralized. Control systems of (1) 4.0 ml of 0.1N-hydrochloric acid plus 1 ml of pepsin solution, and (2) 4.0 ml of extract with 1.0 ml of 0.1N-hydrochloric acid were concurrently incubated and sampled. Specimens were stored at 4° and adjusted to 4.5 ml with 0.1M-hydrochloric acid prior to assay (final pH 1.2).

Trypsin digestion: A 4 ml aliquot of the same extract in 0.1N-hydrochloric acid (TE 57) was neutralized to pH 7.0 with 0.5 ml of 0.5N-sodium hydroxide. An equal volume of tris (tris-hydroxymethyl aminomethane) buffer, pH 7.2, was added. 4 ml was incubated with 1 ml of a trypsin (British Drug Houses Ltd., 2500 - 2700 units/mg) solution containing 10 mg per ml. Controls were run similar to the experiment using pepsin. One contained 4 ml extract plus 1 ml trypsin; the other 4 ml buffer plus 1 ml of the enzyme. Sampling was carried out again at 6 and 24 hr. Prior to assay the samples were acidified to pH 1.2.

Note on method used to determine activity of proteolytic enzymes: Varying concentrations of casein in suspension in normal saline were treated with fixed amounts of pepsin or trypsin at pH 2 and 7 respectively.

Incubation was carried out at 37° for 6 and 24 hr. The degree of hydrolysis was estimated (1) spectrophotometrically at 230 μm and (2) by formol titration. Protein nitrogen of the casein solutions and the calcitonin extract were determined. The amount of proteolytic enzyme used in the experiments with calcitonin was calculated on the basis of the amount necessary to hydrolyse a similar quantity of casein.

Hydrogen peroxide oxidation: The oxidation method of Rasmussen (1959c) was used. To 5 ml of salt precipitate fraction (TE 91, containing 74 μg protein nitrogen per 100 ml in 0.1M-acetate buffer, pH 4.6) was added sufficient 30 percent hydrogen peroxide to make a 1M solution. The reaction was carried out at room temperature for 2 hr. and terminated by the addition of 10 μg of catalase. The reaction mixture was immediately assayed. Parallel controls containing 5 ml of buffer plus all reagents were concurrently run.

Performic acid oxidation: Performic acid oxidation was carried out using the method described by Hirs (1956) for ribonuclease. Two 50 ml flasks connected by a removable right angle joint were used. Performic acid was made in one flask by the addition of 0.2 ml of 30 percent hydrogen peroxide and 1.8 ml of 90 percent analar formic acid. The mixture was allowed to stand for 2 hr. at room temperature. In the second flask 1 ml of 90 percent analar formic acid was added to 3.7 μg protein nitrogen of salt precipitated fraction (TE 94, lyophilized).

After solvation 0.2 ml of anhydrous methanol was added to prevent freezing of the solution. Both vessels were cooled for 30 min. in an ice bath at -10° (dry ice in 30 percent w/v glycerol) with the side arm in place to prevent chloride interference. The solutions were mixed by tipping and the reaction carried out at -10° for 2½ hr. At the conclusion 10 ml of iced distilled water was added to the reactants and the mixture poured into 70 ml of distilled water at 0°. The aqueous system was frozen and lyophilized. The residue was taken up in an additional 70 ml of iced distilled water, frozen and lyophilized a second time to remove traces of formic acid. The final residue was stored at -20° until assayed.

Cysteine reduction: 10 mg cysteine hydrochloride was added to 5 ml of salt precipitated fraction (TE 91, containing 74 µg protein nitrogen/100 ml in 0.1M-acetate buffer, pH 4.6). The incubation was carried out for 5 hr. at 22° (Rasmussen, 1959c). Assays were done concurrently with controls containing buffer and reagents.

Incubation with 8M-urea: 0.5 ml of 8M-urea (analar) was added to 3.7 µg protein nitrogen of salt precipitated material (TE 94, lyophilized). The solution was allowed to stand for 12 hr. 3.5 ml of 0.1M-acetate buffer, pH 4.6, was added immediately prior to assay. Controls run simultaneously consisted of a similar amount of lyophilized extract made up to 4.0 ml with buffer 12 hr. prior to assay and 0.5 ml of

8M-urea together with 3.5 ml buffer made up just prior to assay. Each assay animal received 12 µg urea.

Results.

Effect of boiling: Boiling of partially purified calcitonin extract resulted in 12 percent loss of activity in 15 min., 46 percent in 30 min. and 73 percent in 2 hr. (Figure VII - 1 (a) and Table VII - 1). The loss of activity after 30 min. of boiling was somewhat less than that observed when the crude extract was boiled (Figure VI - 1a and Table VI - 3). The difference may have been due to the greater stability at pH 4.6 than at pH 1, ~~or the protective effect of a higher concentration of foreign proteins~~.

Effect of boiling in excess acid or base: All activity was lost after boiling for 1 hr. in either 1N-sodium hydroxide or 1N-hydrochloric acid (Figure VII - 1b and Table VII - 2).

Effect of proteolytic enzymes: All activity was lost within a 6 hr. incubation period with either pepsin (Figure VII - 2 and Table VII - 3) or trypsin (Figure VII - 3 and Table VII - 4). Trypsin attacks bonds linking the carboxyl group of a basic amino acid to the amino group of another amino acid or to the hydroxyl group of an alcohol: pepsin attacks only peptide bonds. Inactivation of calcitonin by these enzymes clearly demonstrated the peptide nature of the hormone.

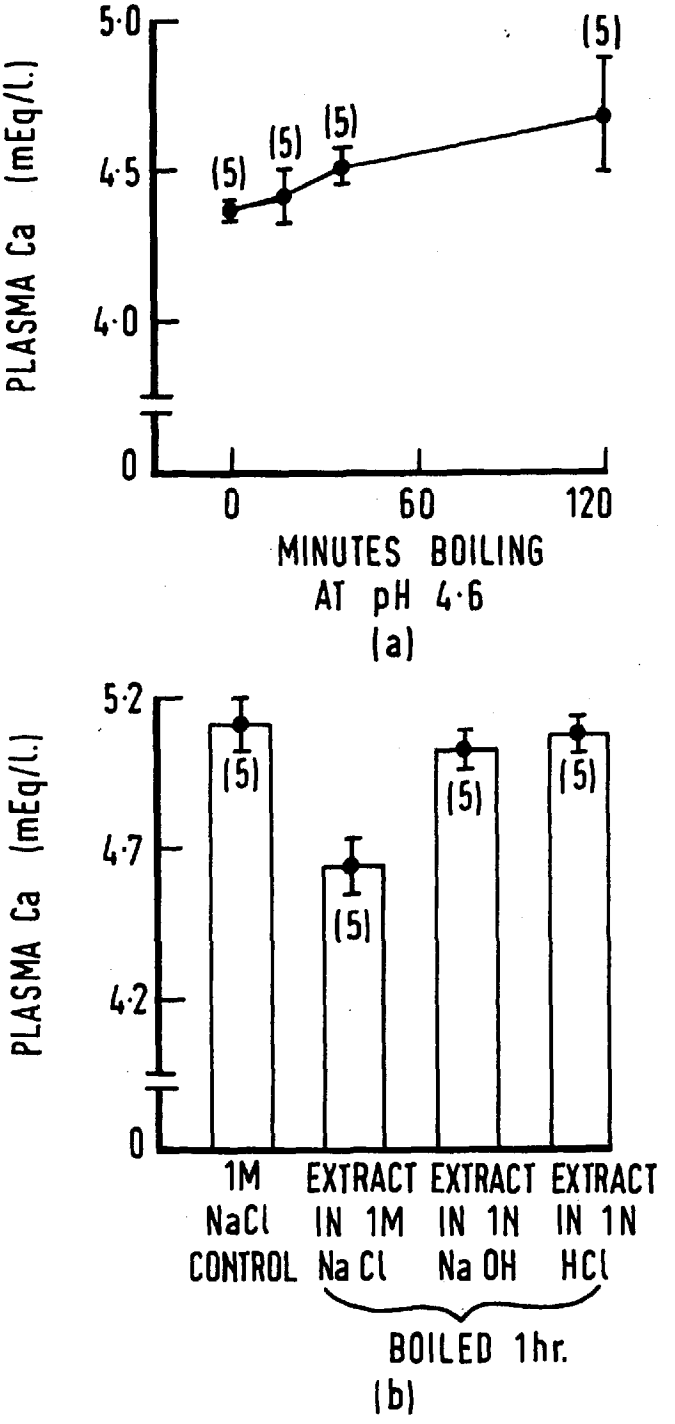


Figure VII - 1. (a) Effect of boiling on calcitonin activity of salt precipitated extract (TE 60) in 0.1M-acetate buffer, pH 4.6. (b) Effect of boiling for 1 hr. on calcitonin activity of extract in the presence of 1M-NaCl, 1N-NaOH and 1N-HCl. pH of extracts was adjusted prior to intravenous assay. Points indicate actual plasma calcium values in animals receiving 0.4 ml aliquots of assay solutions.

Table VII - 1. Effect of boiling on calcitonin activity of salt precipitated extract (TE 60) in 0.1M-acetate buffer, pH 4.6.

Preparation	No. rats	Plasma Calcium mEq/l mean ± S.E.	range	Δ Ca	Units Calcitonin	Percent Change
Extract, not treated	5	4.38 ± 0.04	(4.29-4.50)	-0.71	26	0
Extract boiled 15 minutes	5	4.41 ± 0.09	(4.25-4.50)	-0.68	23	-12
Extract boiled 30 minutes	5	4.51 ± 0.06	(4.40-4.67)	-0.58	14	-46
Extract boiled 120 minutes	5	4.69 ± 0.19	(4.35-5.40)	-0.42	6.8	-73
Buffer, alone	5	5.09 ± 0.07	(4.95-5.29)	-	0	-

Table VII - 2. Effect on calcitonin activity of boiling salt precipitated extract (TE 60) in presence of 1N-HCl and 1N-NaOH for 1 hr.

Preparation	No. rats	Plasma Calcium mEq/l mean ± S.E.	range	Δ Ca	Units Calcitonin	Percent Change
Buffer + 1M-NaCl (not boiled)	5	5.11 ± 0.09	(4.90-5.43)	-	-	-
Extract + 1M-NaCl (boiled)	5	4.63 ± 0.09	(4.45-4.95)	-0.48	18	0
Extract + 1N-NaOH (boiled)	5	5.02 ± 0.06	(4.80-5.15)	-0.09	3	-88
Extract + 1N-HCl (boiled)	5	5.08 ± 0.06	(4.86-5.21)	-0.03	0	-100

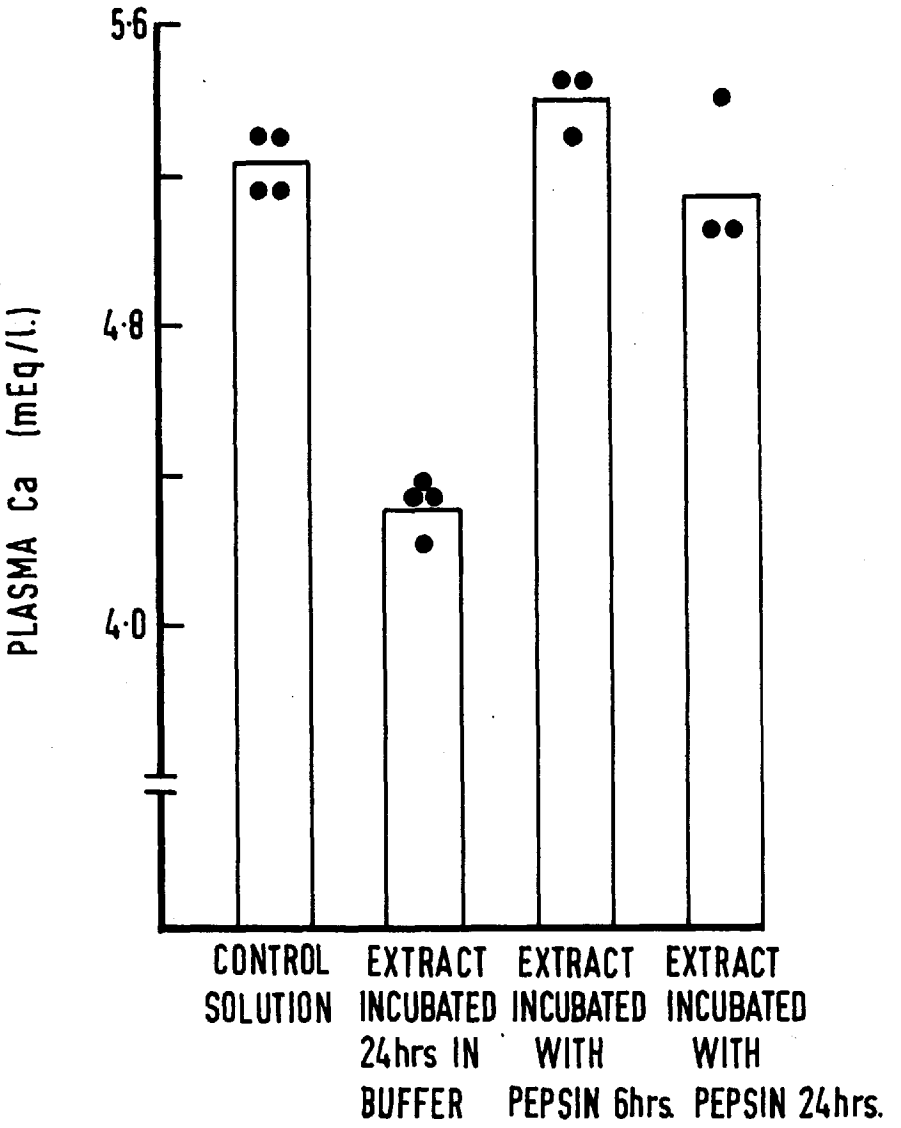


Figure VII - 2. Effect of pepsin incubation on the calcitonin activity of salt precipitated extract (TE 57) in 0.1M-HCl. 4 ml of extract was incubated in the presence of 10 mg of pepsin at 37°. Samples were removed at 6 and 24 hr., neutralized to pH 7.0 with 0.1N-NaOH and 0.4 ml aliquots assayed by intravenous infusion. Points indicate actual plasma calcium values in animals receiving 0.4 ml of assay solution.

Table VII - 3. Effect of pepsin incubation on calcitonin activity of salt precipitated extract (TE 57).

Preparation	No. rats	Plasma Calcium mEq/l mean	range	△ Ca	Units Calcitonin	Percent Change
Untreated extract	4	4.31	(4.21-4.39)	-0.91	62	0
Extract, incubated with pepsin 6 hrs.	3	5.40	(5.30-5.45)	+0.17	0	-100
Extract, incubated with pepsin 24 hrs.	4	5.15	(5.05-5.40)	-0.08	0	-100
Controls, 0.1N-HCl and pepsin	4	5.23	(5.15-5.30)	-	0	-

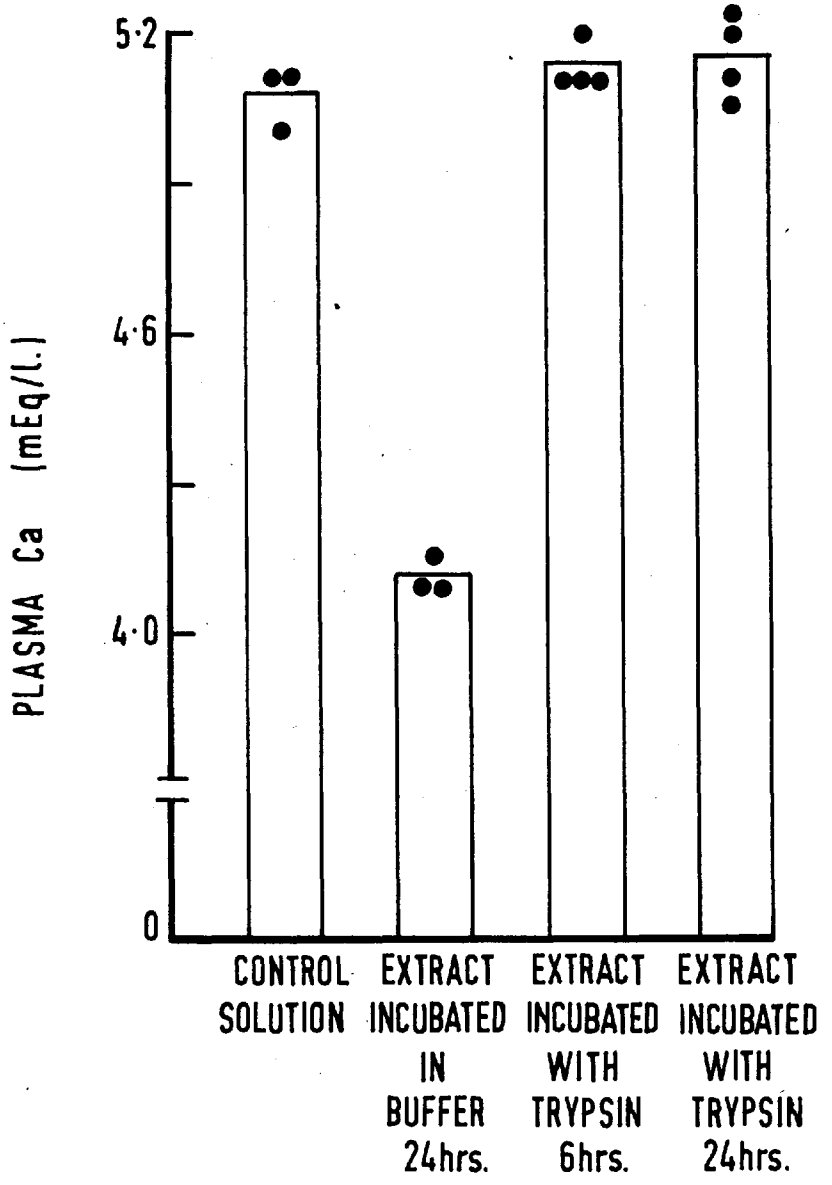


Figure VII - 3. Effect of trypsin incubation on calcitonin activity of salt precipitated crude extract (TE 57). See text. Incubation was carried out at neutral pH for 6 and 24 hr. at 37°. Prior to intravenous infusion assay samples were acidified to pH 1.2. Points indicate actual plasma calcium values in animals receiving 0.4 ml of assay solutions.

Table VII - 4. Effect of trypsin incubation on calcitonin activity of salt precipitated extract (TE 57).

Preparation	No. rats	Plasma Calcium mEq/l mean	range	$\triangle$Ca	Units Calcitonin	Percent Change
Untreated extract in buffer 24 hrs.	3	4.12	(4.10-4.15)	-0.95	78	0
Extract, incubated with trypsin 6 hrs.	4	5.13	(5.10-5.20)	+0.06	0	100
Extract, incubated with trypsin 24 hrs.	4	5.15	(5.05-5.25)	+0.08	0	100
Controls, buffer 0.1N-HCl with trypsin	3	5.07	(5.00-5.10)	-	0	-

Effect of hydrogen peroxide oxidation: As seen in Figure VII - 4 and Table VII - 5, no appreciable activity was lost following a 2 hr. incubation with hydrogen peroxide at pH 4.6. Inability to alter potency suggests that thiol groups are not necessary for activity. For verification oxidation would have had to have been tried over a wide range of pH since all reactive groups might not be exposed at any one given pH (Hamilton, 1960).

Effect of cysteine reduction: The results are shown in Figure VII - 5 and Table VII - 6. No change was induced by mild reduction. Reduction with stronger reducing agents such as sodium borohydrate would have been inappropriate since they affect peptide bonds.

This experiment was undertaken to investigate if cysteine might restore activity lost by possible oxidation. Oxidation appeared unlikely since calcitonin was not affected by hydrogen peroxide. A number of polypeptide hormones including PTH, however, are known to be inactivated by oxidation to air alone. Rasmussen (1959c) has shown that activity lost by PTH can be partially restored by reduction with cysteine.

Failure to increase the calcium-lowering activity of calcitonin with cysteine treatment is further supportive evidence that it is not readily affected under mild oxidizing conditions.

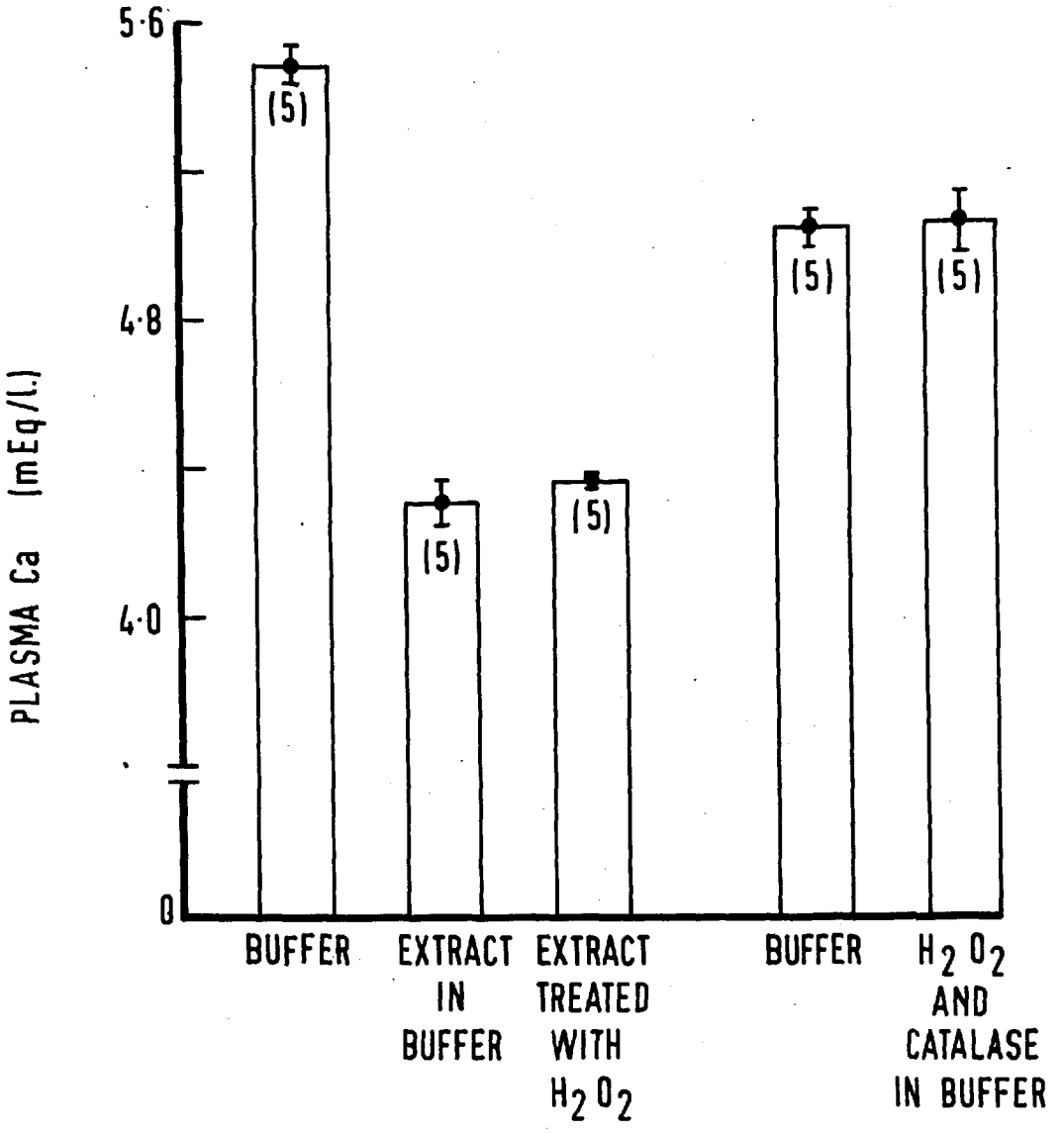


Figure VII - 4. Effect of H_2O_2 oxidation on calcitonin activity of salt precipitated extract (TE 91). Buffer used was 0.1M-sodium acetate, pH 4.6. Oxidation was carried out with 1M-H_2O_2 at room temperature for 2 hr. and terminated by the addition of 10 µg of catalase. Reaction mixture and control solutions were assayed by intravenous infusion. The H_2O_2 and catalase in buffer assay was performed on a separate day and is recorded with its own buffer control. Points indicate actual mean assay values, vertical bars the standard error of the mean and numbers in parenthesis the number of assay animals tested.

Table VII - 5. Effect of peroxide oxidation on calcitonin activity of salt precipitated extract (TE 91).

Preparation	Protein mg N %	No. rats	Plasma Calcium mEq/l mean ± S.E.	range	Δ Ca	Units Calcitonin	Percent Change
Extract untreated	18	5	4.31 ± 0.06	(4.10-4.40)	-1.18	220	0
Extract incubated with H_2O_2	18	5	4.37 ± 0.02	(4.30-4.40)	-1.12	170	-23
Buffer		5	5.49 ± 0.05	(5.35-5.65)	-	0	-
Buffer H_2O_2 and Catalase		5	5.06 ± 0.08	(4.80-5.30)	+0.01	0	0
Buffer		5	5.05 ± 0.05	(4.95-5.25)	-	0	-

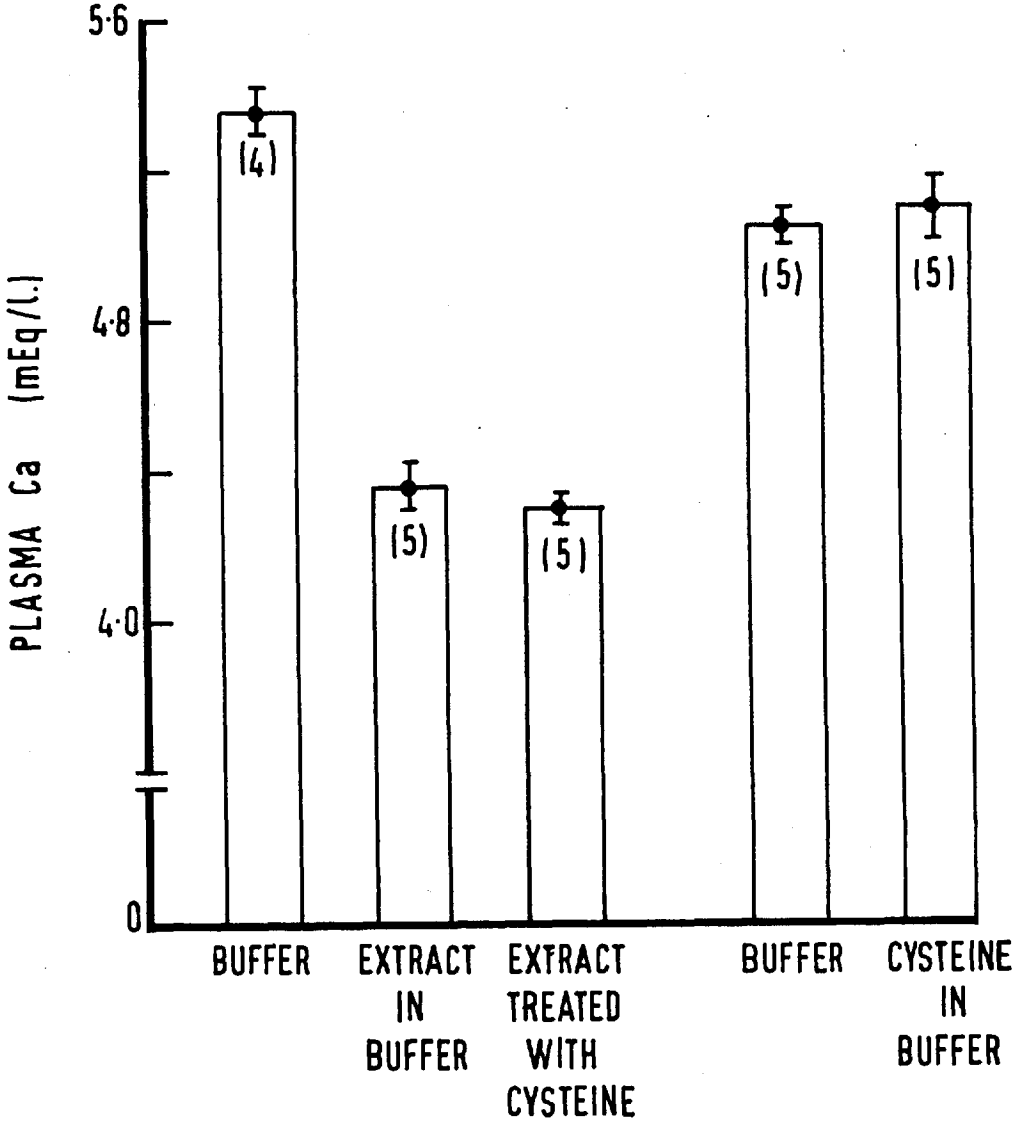


Figure VII - 5. Effect of cysteine reduction on calcitonin activity of salt precipitated extract (TE 91). 10 mg cysteine hydrochloride was added to 5 ml of extract containing 74 µg protein N/100 ml in 0.1M-acetate buffer, pH 4.6. Incubation was carried out for five hours at room temperature. Reaction mixture and control solutions were assayed by intravenous infusion. The cysteine in buffer assay was performed on a separate day and is recorded with its own buffer control. Points indicate actual mean assay values, vertical bars the standard errors of the means and numbers in parenthesis the number of assay animals tested.

Table VII - 6. Effect of cysteine reduction on calcitonin activity of salt precipitated extract (TE 91).

Preparation	Protein mg N %	No. rats	Plasma Calcium mEq/l mean ± S.E.	range	Δ Ca	Units Calcitonin	Percent Change
Extract, untreated	18	5	4.35 ± 0.07	(4.25-4.50)	-1.01	100	0
Extract incubated with cysteine	18	5	4.30 ± 0.04	(3.90-4.60)	-1.06	125	+25
Buffer alone		4	5.36 ± 0.07	(5.20-5.50)	-	0	-
Buffer and cysteine		5	5.10 ± 0.09	(4.90-5.40)	+0.05	0	+1
Buffer		5	5.05 ± 0.05	(4.95-5.25)	-	0	-

Effect of performic acid oxidation: Performic oxidation resulted in complete loss of activity (Figure VII - 6 and Table VII - 7). Oxidation by performic acid destroys disulphide bonds with the transformation of the residues into their corresponding sulphonic acids.

$$RSSR' + 6HCOOH \rightleftharpoons RSO_3H + R'SO_3H + 6HCOOH$$

The reaction is not limited to cystine residues but can affect tryptophan, methionine and tyrosine. Since oxidation by hydrogen peroxide failed to inactivate calcitonin it is more likely that performic acid altered one of the latter amino acids.

Effect of 8M-urea: Incubation for 12 hr. with 8M-urea had no effect on calcitonin activity (Table VII - 8). Any configurational changes brought about were either not important to the activity of the molecule or were readily reversed when diluted prior to assay.

Stork, Steine and Moore (1960) have reported that 8M-urea contains approximately 0.02M-cyanate. Since cyanate reacts with sulphydryl groups this further suggests that disulphide bonds are not necessary for activity.

DISCUSSION

The investigations described in this chapter were designed to assess the stability of partially purified calcitonin. The results suggest that calcitonin is a polypeptide that is relatively stable to heat and,

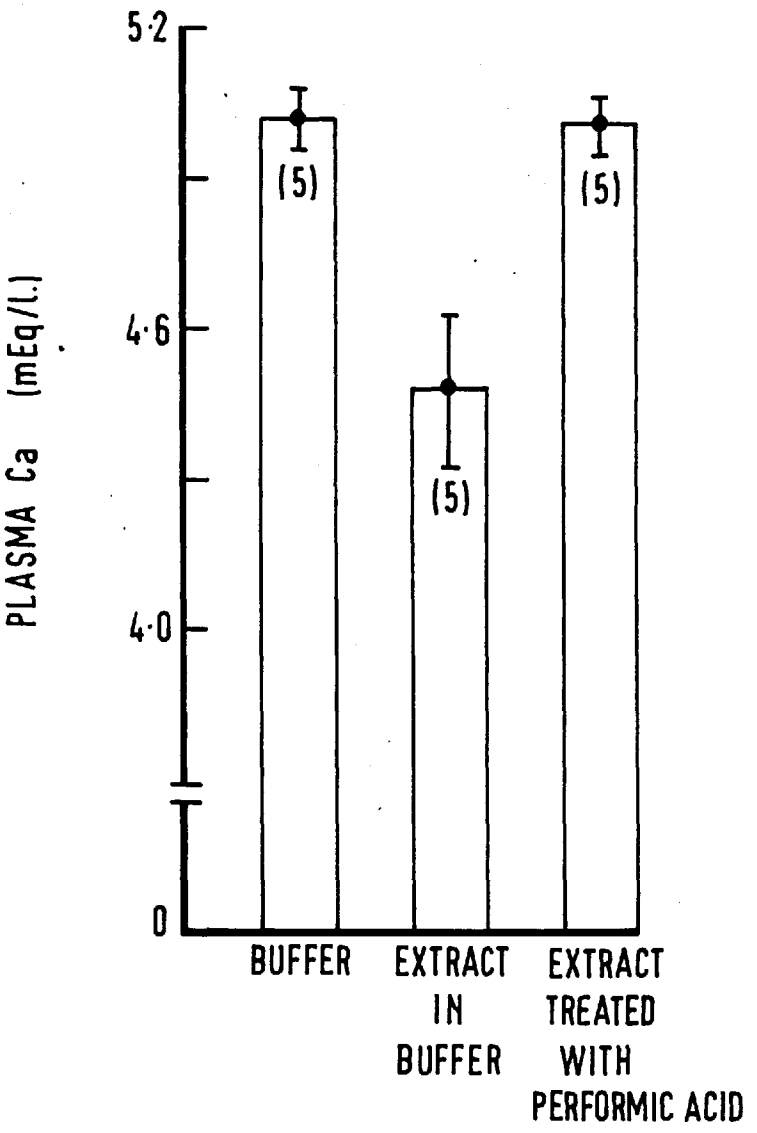


Figure VII - 6. Effect of performic acid oxidation on calcitonin activity of salt precipitated extract (TE 94). See text. Oxidation reaction was carried out for $2\frac{1}{2}$ hr. at -10°. The resulting solutions lyophilized twice and taken up in 0.1M-sodium acetate buffer, pH 4.6. Extracts and control solutions were assayed by intravenous infusion. Points indicate actual mean assay values, vertical bars the standard errors of the means and number in parenthesis the number of assay animals tested.

Table VII - 7. Effect of performic acid oxidation on calcitonin activity of salt precipitated activity (TE 94).

Preparation	Protein mg N	No. rats	Plasma Calcium mEq/l mean ± S.E.	range	Δ Ca	Units Calcitonin	Percent Change
Extract untreated	0.094	5	4.47 ± 0.16	(4.20-5.05)	-0.57	14	0
Extract treated with performic acid	0.094	5	5.03 ± 0.05	(4.85-5.10)	-0.01	0	-100
Buffer, alone		5	5.04 ± 0.05	(4.90-5.20)	-	0	-

Table VII - 8. Effect of 8M-urea on calcitonin activity of salt precipitated extract (TE 94).

Preparation	Protein mg N	No. rats	Plasma Calcium mEq/l mean ± S.E.	range	Δ Ca	Units Calcitonin	Percent Change
Extract untreated	0.188	5	4.43 ± 0.06	(4.25-4.55)	-1.02	105	0
Extract incubated with 8M-urea	0.188	5	4.39 ± 0.08	(4.15-4.55)	-1.06	125	+19
Buffer, alone		5	5.45 ± 0.06	(5.25-5.55)	-	0	-

under mild conditions, to oxidation and reduction.

As might be expected less loss of activity was noted on boiling at pH 4.6 than at pH 1 as previously reported (Chapter VI). At the lower pH hydrolysis of aspartyl peptide bonds could account for inactivation. Inactivation in the presence of higher concentrations of acid or base are explicable by less specific hydrolysis.

Susceptibility to changes in activity due to oxidation has now been found in several polypeptide hormones. Rasmussen (1959) has shown PTH loses potency with oxidation. Similar findings have been reported for ACTH (Dixon, 1956; Dedman, Farmer and Morris, 1957) and melanocyte stimulating hormone (Dixon, 1956). Loss of activity has been noted with these hormones under such mild conditions as exposure to air. Preparations of calcitonin, however, have appeared relatively stable to air and have even resisted inactivation by hydrogen peroxide as shown in this chapter.

Loss of calcitonin activity induced by performic acid can be accounted for by explanations other than oxidation of cystine. Tryptophan is converted to kynurenine and other degradation products, methionine to the sulphone (Bailey, 1962) and tyrosine to probably a modified chloride derivative in the presence of traces of chloride ion (Thompson, 1954). Studies by Hirs (1956) working with ribonuclease found the latter reaction could be minimized if carried out at -10° C.

This was the method followed in the present study. An alternative method could have been devised by ridding the system of unwanted chloride ions by passing the reactants first through an ion exchange column before mixing. Since precautions were taken it is unlikely that inactivation was due to alteration of tyrosine. If subsequent analysis studies of purified hormone are able to exclude the presence of the sulphone, tryptophan will be incriminated by exclusion.

Finally, it should be noted that most of the studies reported in this chapter were (1) carried out under acid conditions and (2) impure calcitonin was used. The stability studies in Chapter VI on the effect of pH suggest that the isoelectric point may be above pH 7.0 since activity was precipitated at pH's near or above neutrality. Conceivably the molecule under acid conditions may assume a more stable configuration when highly charged. Studies of other polypeptide hormones susceptible to oxidation, however, have shown loss of considerable activity even when oxidation was carried out at extreme pH's (Rasmussen, 1959c).

The salt precipitated preparations of calcitonin unquestionably contained amino acids, peptides and proteins. These may have contributed to stability by preventing oxidation of an active site of the hormone molecule.

The oxidation-reduction experiments reported here were not

designed as definitive studies but rather as guides to assess if the conditions under which purification was carried out were optimal for the recovery of activity.

Chapter VIII

Further Purification of Calcitonin with a Preliminary Estimate of Molecular Weight Range

Summary

A 20 fold purification of the salt precipitated extract was obtained on Sephadex G-100 using 0.1M-acetate buffer, pH 4.6, containing 0.2M-sodium chloride as eluant. The most active fraction which had 23,000 units/mg protein nitrogen, was collected from the descending shoulder of the major peak. The activity readily penetrated 24/32 Visking membrane which previously contained the activity of crude extracts. The data suggests that the active moiety had been dissociated from larger carrier-molecules. The molecular weight of calcitonin is less than 10,000 as judged by comparison of the elution volume on a Sephadex column with other peptides of known molecular weight.

The salt precipitated extract described in the last chapter was further purified by gel filtration. Its elution characteristics relative to other proteins and polypeptides were used to estimate the molecular weight.

Gel filtration (Flodin, 1962; Gelotte, 1964) is a chromatographic technique utilizing the molecular sieving properties of dextran polysaccharide. The particles of this gel form a three dimensional plexus with pore sizes which permit the entry of small molecules. Large

molecules readily pass through the interstitial spaces. The small molecules distribute themselves inside and outside the gel particles and pass through the gel bed at a rate relative to their size.

The commercially available products are made from dextran micro-biologically synthesized and cross-linked by glyceryl bridges with epichlorohydrine (see Figure VIII - 1). This linkage allows dextran to maintain its hydrophilic character while becoming insoluble. The degree of linkage determines the exclusion properties of the gel particles.

The operational parameters of a given column are described by the following basic equation (Gelotte, 1960):

$$V_e = V_o + K_D V_i \qquad (1)$$

The effluent peak volume (V_e) is related to the void volume (V_o), or volume of solvent external to the gel particles, and to the volume of solvent imbibed by the particles themselves (V_i). The "distribution coefficient" (K_D) characterizes the interaction or penetration of the solute with the gel and can be readily calculated.

Assuming the density of water as unity, V_i can be determined from the water regain (W_r) expressed in grams of solvent imbibed per gram of dry gel and the weight of the gel (a).

$$V_i = a \cdot W_r \qquad (2)$$

Substituting in equation (1)

$$K_D = \frac{V_e - V_o}{a \cdot W_r} \qquad (3)$$

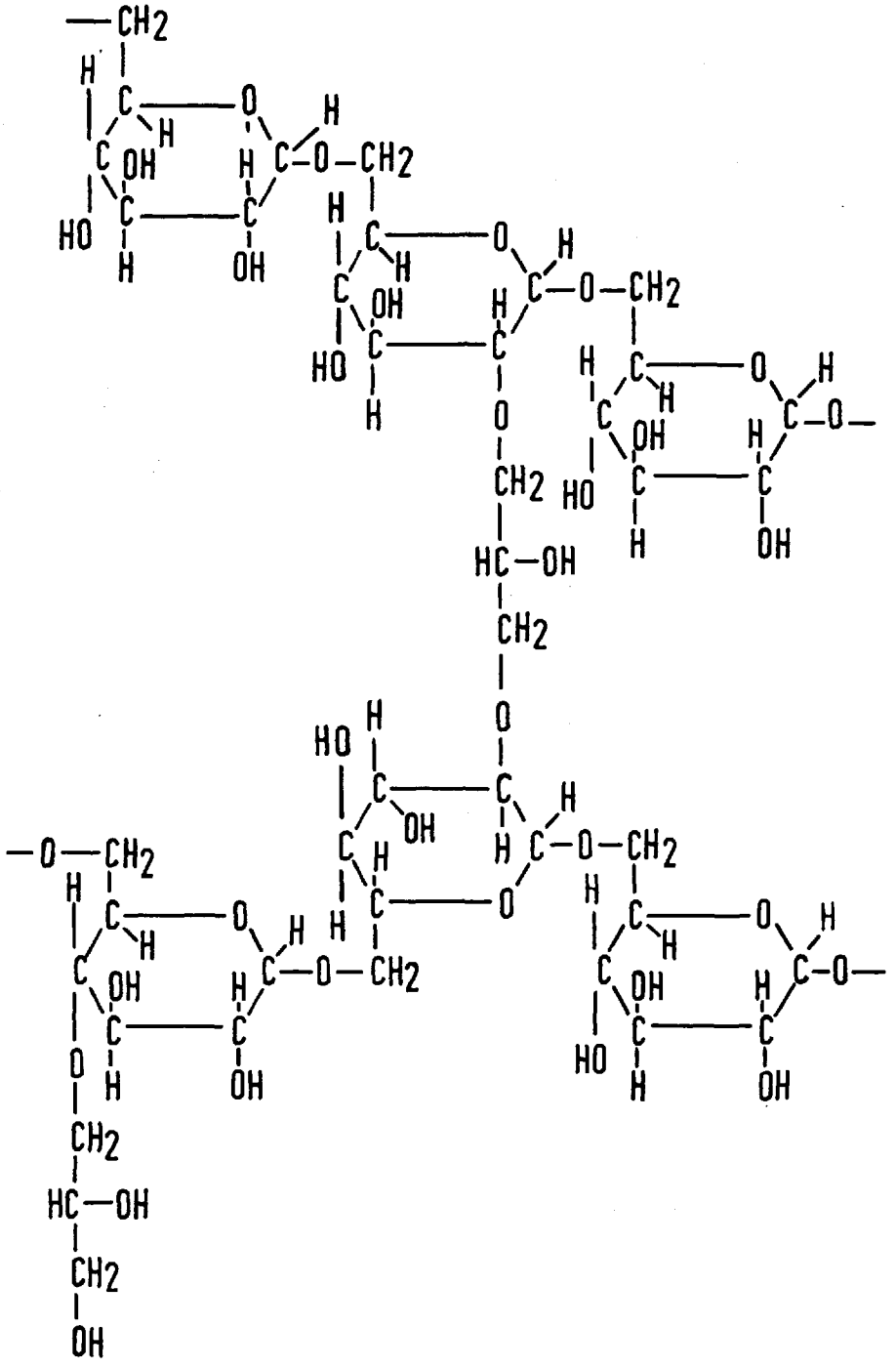


Figure VIII - 1. Structural formula showing the essential features of a dextran gel cross-linked with epichlorohydrin.

For large molecules not entering the gel when

$$V_e = V_o$$

K_D has a value of zero. Conversely, for molecules nearing complete entry into the hydrated gel particles where

$$V_e \cong V_o - V_i$$

K_D approaches unity. K_D values between unity and zero indicate that the solute has partially penetrated the gel. Values greater than unity indicate the solute has become adsorbed to the gel.

In practice imperfections in packing, longitudinal diffusion and local non-equilibrium prevent the attainment of ideal elution profiles. An expression has been derived (Glueckauf, 1955) for the behaviour of columns under non-ideal conditions in terms of the equivalent height of a theoretical plate (EHTP)

$$EHTP = \frac{l}{N} \qquad (4)$$

where l is the height of the column and N is the number of equivalent plates. The latter is calculated from the Gaussian-shaped elution profile.

$$N = 8\left(\frac{V_e}{\beta}\right)^2 \qquad (5)$$

where β is the width of the elution curve at the height C max/2.72 over the baseline.

EHTP is most affected by variations in gel particle size. The smaller the particle size the narrower the elution peak. For this

reason fine grade (200 mesh) Sephadex was used in the experiments reported in this chapter. The decrease in flow rate usually observed by using fine particle size was overcome by employing dextran gel in bead form.

Methods.

Purification by gel filtration: Approximately 100 mg of salt precipitated extract (Chapter VI), made up to 10 ml with 0.1M-acetate buffer, was diluted with an equal volume of buffer containing 0.2M-sodium chloride. The sample was applied to a 82 cm x 2.5 cm Sephadex Laboratory Column (Pharmacia) containing Sephadex G-100 (bead form) equilibrated with the latter solution and having a void volume of 129 ml. The column was developed with 0.1M-acetate buffer containing 0.2M-sodium chloride at a hydrostatic pressure of 20 cm of eluant. The flow rate was maintained at approximately 30 ml/hr. Fractions were collected with an LKB fraction collector using a 5 ml syphon and the extinction was read at 230 mμ in each tube.

Groups of ten consecutive tubes were pooled for purposes of assay. 3 ml doses from each pool were given undiluted to each of four assay rats by caudal vein infusion over a 1 hr. period as previously described in Chapter V. Control animals were given similar volumes of pure eluant.

Since previous experiments had indicated that activity did not penetrate 24/32 Visking membrane, an attempt was made to concentrate

the activity in the most active pooled fraction by dialysis. One 10 ml sample was dialyzed against 30 percent (W/V) "carbowax" (G. T. Gurr, Ltd.) until the volume was reduced to 1 ml. The retentate contained only 10 percent of the original total activity. A second 10 ml sample was dialyzed over night at 4° against 100 ml of 0.1M-hydrochloric acid. 44 percent of the thyroid activity remained in the dialysis residue.

Preliminary estimation of molecular weight by gel filtration:

An attempt was made to estimate the molecular weight of calcitonin by relating its elution volume to other peptides of known molecular weight (Table VIII - 1). A 82 x 2.54 cm Sephadex G-100 column equilibrated with 0.1M-acetate buffer, pH 4.6, containing 0.2M-sodium chloride was used. The equivalent height of a theoretical plate calculated for fraction V of bovine albumin (Sigma) on the basis of equations 4 and 5 was 0.42 cm.

Meticulous care of the column was necessary in order to assume accuracy and reproductibility. The following observances were found helpful:

1. Preparation of Sephadex G-100. Dry Sephadex was added slowly to a larger beaker of elution fluid with constant stirring to prevent clump formation. After sedimentation the fines were poured off and the gel resuspended in a volume of eluant approximately twice its volume. This process was continued 2-3 times daily for 2-3 days.

Table VIII - 1. Standards employed in molecular weight estimation of calcitonin using Sephadex G-100.

Substance	Supplier	Mol. Wt.	Method	Reference
Sodium chloride		23		
Angiotensin (Val-hypertensin II-asp-β-amide)	CIBA Laboratories Ltd.	1031	amino acid sequence	S.W.M.Hughes, personal communication
ACTH (β 1-24 corticotropin synthetic polypeptide)	CIBA Laboratories Ltd.	2933	amino acid sequence	Shepherd et al (1956)
Cytochrome C from horse heart, Type II	Sigma Chemical Co.	12.270	amino acid sequence	Margoliash (1962)
Ovalbumin	Sigma Chemical Co.	45,000	various	Warner (1960)
Bovine Albumin (Fraction V from bovine plasma)	Sigma Chemical Co.	67,000	various	Phelps and Putnam (1960)
Bovine albumin dimer	Present in bovine serum albumin	134,000	(2 x 67,000)	Hartley, Peterson & Sober (1962) Sogami and Foster (1962)

After the final decantation the slurry was resuspended.

2. Packing of column. The glass column was acid-washed before use and filled with eluant. The effluent tubing was allowed to fill and precautions were taken to rid the side walls of the column of air bubbles. Approximately 1/8th of the eluant was allowed to drain and the effluent tubing clamped. The slurry was slowly poured along the inner side of the column to the top. The column was not allowed to run until 3-5 cms of gel had sedimented to prevent excessive packing at the base. The outlet was opened and more slurry added every 5-20 min. until the desired height was obtained. This insured against trapping of air bubbles. Using a constant level reservoir eluant was run for two days continuously to provide proper packing.

3. Application of sample. 2 ml samples of buffer containing 20 mg of 1-3 standard proteins and polypeptides (Table VIII - 1) were applied to the column on each run. Addition of 5 mg of sucrose gave the samples sufficient density so that they could be layered under the eluant in an applicator cup with a 400 mesh nylon bottom resting on the top of the packed gel. The sample was applied slowly through microbore tubing by means of a syringe. With every second or third run cytochrome C was applied as a colour marker to observe the column dynamics to ensure even flow.

4. Collection of sample. After application of the sample a volume equal to half the sample plus the volume of the effluent tubing was

collected and discarded. Collection was made with a 5 ml syphon siliconized to minimize variations in volume. Flow rates between 25 and 30 ml per hr. were maintained throughout each column run. Even flow rates were facilitated by (1) running the eluant through the column several hours before applying the sample and (2) maintaining the reservoir level constant.

5. Readings. All tubes were read at 215 mμ in a Zeiss spectrophotometer except for cytochrome C which was read at 418 mμ. Runs were made in triplicate and V_e calculated by triangulation of the elution peak. The average elution volumes were used for the final plot.

6. Care of column. After every 2-3 runs the upper layer of gel was syphoned off and replaced with fresh material to the same level. The surface was always stirred and eluant was run for 6-8 hours to insure even re-packing.

Results.

Purification by gel filtration: The results are shown in Figure VIII - 2 and Table VIII - 2. Activity values of 23,000, 11,000 and 15,000 units per mg protein nitrogen were obtained in the most active fractions in three consecutive runs. All assays were done in triplicate and the average fall in plasma calcium used to assess activity. The most active material was found between tubes 61 and 82. Variations occurred

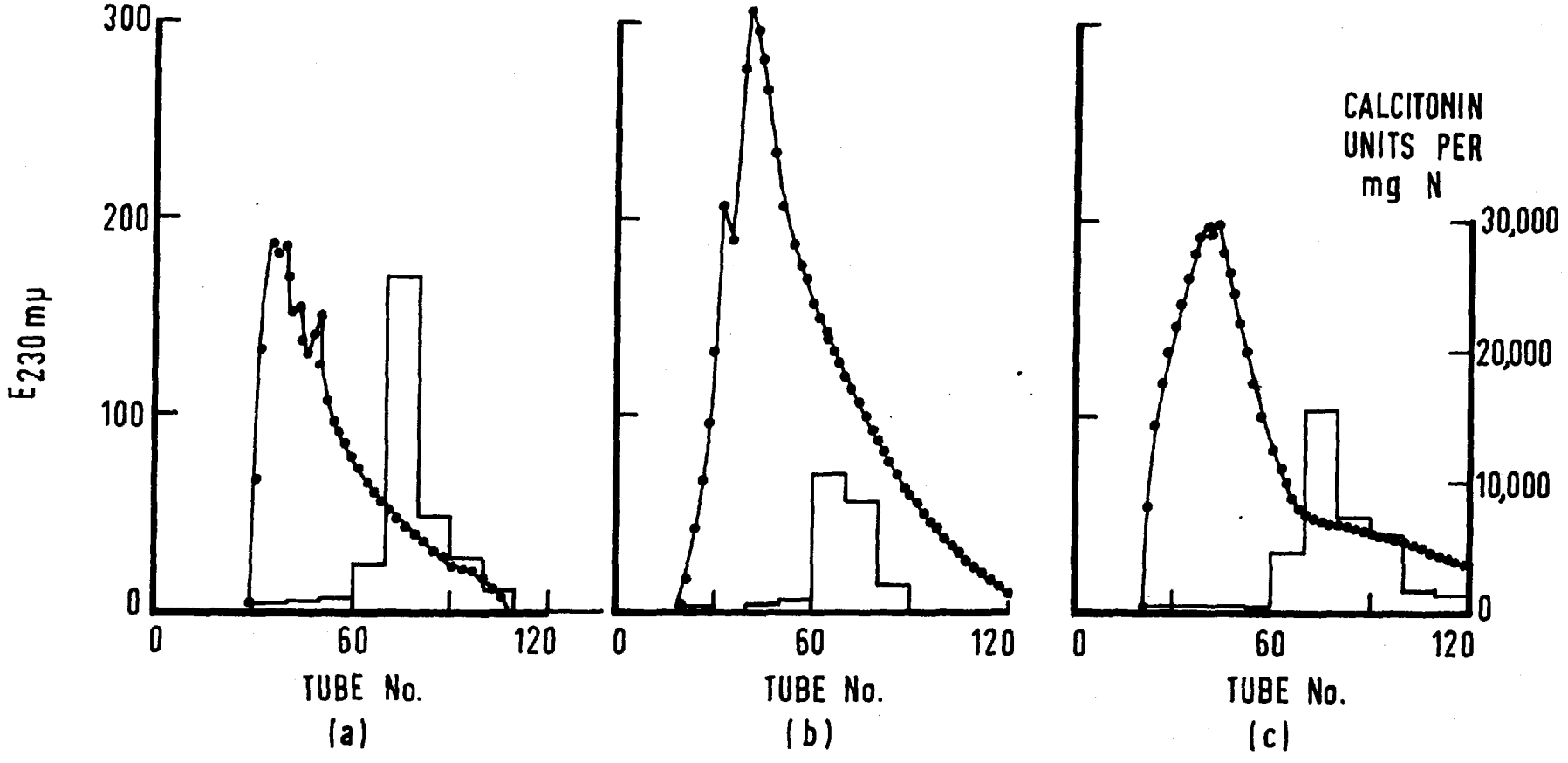


Figure VIII - 2. Gel filtration of calcitonin on Sephadex G-100. Salt precipitated extract (TE 56 and 60) resuspended in buffer was applied to the column in each of three consecutive runs. 0.1M-acetate, pH 4.6, containing 0.2M-sodium chloride was used as eluant and 5 ml fractions were collected. The activities reported are the average of assays done in triplicate. The most active fraction in each run contained 23,000, 11,000 and 15,000 units/mg protein nitrogen respectively.

Table VIII - 2. Partition of calcitonin activity on Sephadex G-100.

20 ml aliquots of 5 times concentrated salt precipitated extract (TE 56 and 60) taken up in 0.1M-acetate buffer, pH 4.6, were applied to a 2.5 x 82 cm Sephadex G-100, using 0.1M-acetate buffer, pH 4.6, containing 0.2M-NaCl as eluant. The calcitonin activities listed below are those obtained in pooled 5 ml fractions collected in three consecutive runs.

Run No. 1.		Run No. 2.		Run No. 3.	
Tubes	Calcitonin units/mg	Tubes	Calcitonin units/mg	Tubes	Calcitonin units/mg
30-39	144	21-30	255	23-32	330
40-49	237	31-40	0	33-42	334
50-59	523	41-50	197	43-52	188
60-69	3,822	51-60	567	53-62	168
70-79	23,148	61-70	10,929	63-72	4,534
80-89	12,346	71-80	8,185	73-82	15,199
90-99	3,931	81-90	1,776	83-92	7,112
100-109	1,495			93-102	5,766
110-119	1,282			103-112	1,836
				113-122	1,694

in the inactive region of the effluent profile with different batches of extract. These differences were likely due to variations in the concentration of large proteins.

Gel filtration resulted in a further 20-fold purification of the salt precipitated extract. The hormone was exceedingly potent. As little as 0.4 μg protein nitrogen produced a fall in plasma calcium of 10 percent in assay animals. Expressed in other terms 1 unit of calcitonin activity was contained in 0.04 μg protein nitrogen. Approximately 90 - 100 percent of the activity of the salt precipitated extract was recovered from the column.

Attempts to concentrate the most active fraction by dialysis failed to concentrate activity in the retentate. Since the same membrane had retained the activity of crude extract it appeared that purification had dissociated the active moeity from larger molecules. A similar phenomena has been described from oxytocin and vasopressin (Linder, Einquist and Porath, 1959; Ginsberg and Ireland, 1964).

Estimation of molecular weight: Recently a number of investigators have reported success in estimating molecular weight of proteins using gel filtration techniques (Andrews, 1964; Ackers, 1964; Whitaker, 1963; Andres, 1962; Hjerten, 1962; Flodin and Killander, 1962; Roskes and Thompson, 1963; Steere and Ackers, 1962). Within limits a linear correlation has been found between the logarithm of the molecular

weight and the ratio of the elution volume (V_e) to void volume (V_o).

$$K = \frac{MW}{V_e / V_o} \qquad (6)$$

The V_e/V_o ratio was found independent of column size and protein concentration, but for some proteins V_e/V_o was found dependent upon temperature (Whitaker, 1963). Parallel experiments carried out using both Sephadex G-75 and G-100 (Whitaker, 1963; Andrews, 1964; Andrews, 1965) have shown that Sephadex G-100 gives sharper elution profiles and better correlation between V_e/V_o and molecular weight because of smaller gel particle size.

The results of the studies designed to give a preliminary estimate of the molecular weight of calcitonin are shown in Figure VIII - 3 and Table VIII - 3. A near linear relationship was found for synthetic ACTH, cytochrome C, ovalbumin and bovine albumin when the elution volume was plotted against the logarithm of the molecular weight. The elution values for sodium-24 (as sodium chloride) and angiotensin did not bear the same relation. The volumes of eluant required to elute these substances fell below an extension of the linear plot for the larger peptides. These observations confirm the findings of Andrews (1964). This worker has shown that the linear relationship between molecular weight and V_e or V_e/V_o exists only for substances with molecular weights between 4000 and 150,000. Outside these values V_e/V_o plateaus.

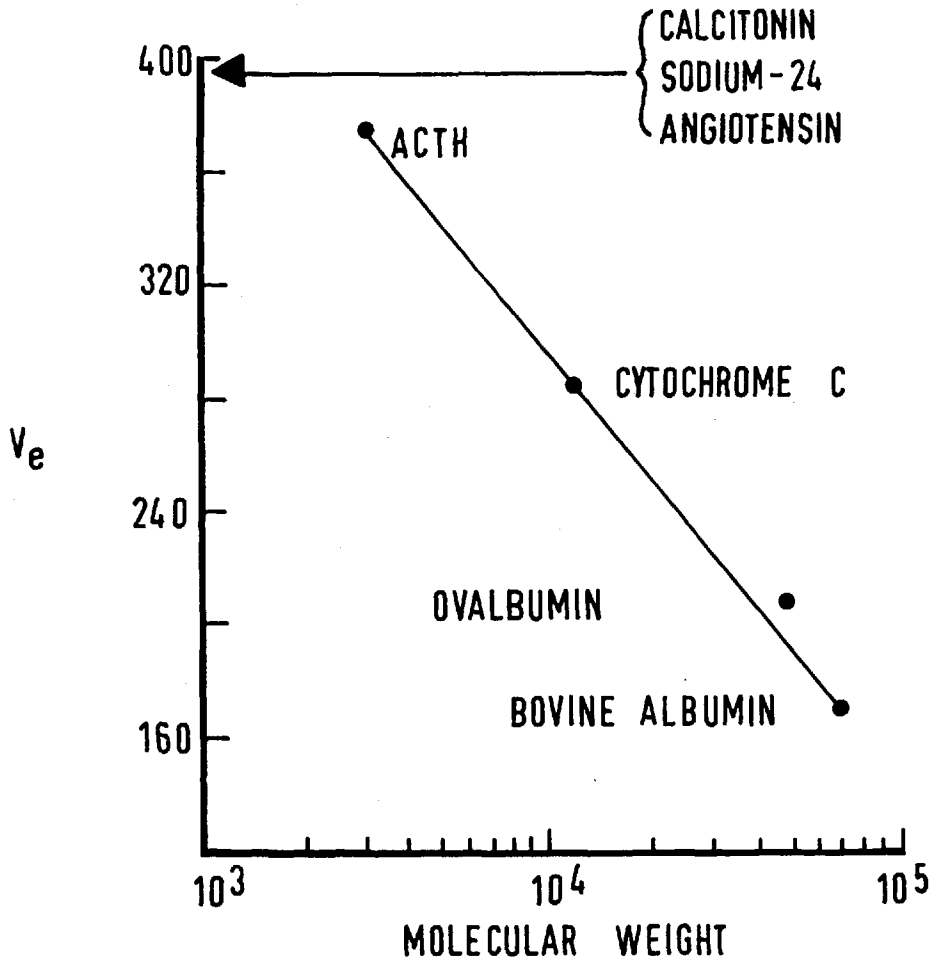


Figure VIII - 3. Estimation of the molecular weight of calcitonin. The Sephadex G-100 elution volume of calcitonin has been compared with the elution volumes of various peptides plotted against the log of their molecular weight. (See Table VIII - 1). The comparison indicates that calcitonin has a molecular weight less than 10,000 and, therefore, is a polypeptide. All runs were done at room temperature on a 82 x 2.5 cm column using 0.1M-sodium acetate - 0.2M-sodium chloride (pH 4.6) as eluant.

Table VIII- 3. Elution volumes obtained on a 82 x 2.5 cm column of Sephadex G-100. 0.1M-acetate buffer, pH 4.6, containing 0.2M-sodium chloride was used as effluent. All runs were done at room temperature. The values reported were obtained by triangulation of plotted elution peaks.

Substance	Elution Volume (ml)				Ave
	Run Number				
	1	2	3	4	
Bovine albumin dimer	112	114	118		114.7
Bovine albumin	172	174	168		171.3
Ovalbumin	207	209	203		206.3
Cytochrome C	282	284	283	282.5	282.9
ACTH				377	377
Calcitonin	392	394	388		391.3
Angiotensin				378	378
Sodium 24				387	387

The volume of eluant required to elute calcitonin fell outside the linear portion of the calculated curve. For this reason it was not possible to extrapolate a value for its molecular weight.

An attempt was made to estimate the distribution coefficient for calcitonin on Sephadex G-100 from equation (1), which, when rearranged appears as

$$K_D = \frac{V_e - V_o}{V_i} \qquad (7)$$

The elution volume, V_e, for calcitonin was known. The void volume V_o was established empirically by measuring the elution volume for Dextran Blue 2000 (Pharmacia). The volume imbibed by the gel V_i was estimated on the basis of the water regain value given by the manufacturer (Pharmacia). The calculated distribution coefficient was 1.072 which suggested that calcitonin may have interacted with the gel. Since V_i was only approximated and may have differed from the manufacturers value due to column packing and the buffer system used, the calculation of the distribution coefficient may not have been exact and any conclusions drawn from it may not be valid.

The elution volumes for calcitonin determined on these runs varied from each other by as much as 6 ml while the elution volumes of sodium and ACTH differed from each other by only 10 ml. Obviously the method used cannot be applied for estimating molecular weights of

peptides below 4000. Furthermore, low molecular weight polypeptides containing aromatic amino acids are retarded by adsorption to Sephadex (Carnegie, 1965). On the basis of this study it can only be concluded that calcitonin appears to behave on Sephadex G-100 as if it had a molecular weight approximating synthetic ACTH or somewhat less.

DISCUSSION

The purification protocol devised for calcitonin compares favourably with the two other methods published since the work in this chapter was done. Hirsch, Savery and Munson (1964) homogenized fresh pork thyroid glands in 0.1N-hydrochloric acid without defatting. Extraction was carried out in the cold. The supernatant from high speed centrifugation of the digest was eluted on Sephadex G-50 using 0.05M-sodium acetate, pH 3.8. More recently these workers have achieved further purification on carboxymethyl-Sephadex G-25 (Hirsch, Voelkel and Munson, 1964). Our protocol has two primary advantages: first, it is simpler, and second, larger scale preparation is possible with salt fractionation than with ultracentrifugation.

The second method (Arnaud, Tenenhouse and Rasmussen, 1965; Tenenhouse, Arnaud and Rasmussen, 1965) is essentially the same as that used in the purification of PTH (Rasmussen, Sze and Young, 1964). Acetone-chloroform dried pork thyroid was extracted with a solution of urea, hydrochloric acid and cysteine. Following precipitation with ether and removal of inactive material with acetone-sodium chloride,

activity was recovered with trichloroacetic acid. When applied to a Sephadex G-100 column the activity was recovered in the third peak and was of the same order of magnitude as our best preparations.

Recently further improvements have been made in our purification technique. Gudmundsson, MacIntyre and Soliman (in press) have eluted the salt-precipitated material on Sephadex G-100 using 0.1M-formic acid instead of acetate buffer and recovered activity from the most active fraction by precipitation with 5 percent (W/V) trichloroacetic acid. Residual trichloracetic acid was removed by ether extraction and a run on ion-exchange resin IRA 400. Final purification was performed by one of three methods: (1) three successive runs on Sephadex G-50 in 0.1M-acetic acid, (2) recycling on Sephadex G-50 in 0.1M-formic acid or (3) repetitive gel filtration on polyacrylamide. The final product was extremely active and appeared relatively homogeneous on urea starch-gel electrophoresis and thin-layer chromatography.

Chapter IX

Recent Progress in Calcitonin Research

Summary

The results of recent research on calcitonin are reviewed under the headings of (1) site and mode of action, (2) relationship to other endocrine systems and (3) studies in man. Animal experiments have shown that the hormone has two primary sites of action: bone and kidney. Calcitonin promotes the retention of calcium by bone and excretion of phosphate by the kidney. A parathyroid releasing factor has been suggested though not proven. The importance of the hormone to man has been established: calcium-lowering activity has been extracted from human thyroid and purified pig calcitonin has been shown to lower the serum calcium of patients with hypercalcaemia secondary to disseminated malignant disease. It is hoped that a radio-immunoassay will shortly be available to assess changes in hormonal levels in man.

Site and Mode of Action

Calcitonin acts by causing (1) bone retention of calcium and (2) renal phosphate excretion.

Effect on bone.

Much direct and indirect evidence now supports that calcitonin-induced falls in plasma calcium are due to calcium deposition in bone. Many sites of possible calcium retention have been eliminated. Of

the soft tissues that have been examined kidney, salivary glands, small intestine (Kenny, 1965), heart (Soliman, Robinson, Foster and MacIntyre, in press) and red blood cells (Foster, unpublished) have shown no increase in calcium following the injection of calcitonin extracts in rats. The possibility that the hormone acts by inhibiting the absorption of calcium from the gut has been excluded. Robinson (personal communication) found calcitonin causes no change in gastro-intestinal absorption of an oral dose of calcium in the rat. Aliapoulis and Munson (1965) have also shown that extracts were equally effective in lowering the plasma calcium in rats whose intestinal tracts had been removed as in intact controls. A renal mechanism of calcitonin induced hypercalcuria has likewise been excluded. Foster (unpublished) noted in dogs that urinary calcium excretion is diminished rather than increased when either endogenous calcitonin secretion is stimulated or following intravenous injections of extracts. Experiments in rats showing that nephrectomy does not diminish the plasma calcium-lowering effect of calcitonin have firmly established that the kidneys are not necessary for the calcitonin effect on calcium. (Gudmundsson, MacIntyre and Soliman, in press; Aliapoulis and Munson, 1965).

The present evidence for a direct effect on bone is five fold: (1) Small infusions of calcitonin promote the retention of calcium in the isolated blood-perfused cat tibia (MacIntyre and Parsons, in press).

(2) Short term in vitro incubations of femurs from fetal mice tagged in vitro with calcium-45 have shown that resorption in control systems or in systems containing PTH is inhibited in the presence of calcitonin (Friedman and Raisz, 1965).

(3) Mouse calvarii incubated in vitro in the presence of calcitonin appears more dense than bone from control cultures (Aliapoulis and Munson, 1965).

(4) Histological evidence of bone increase in embryonic mouse radii has been demonstrated when calcitonin was added to culture media (Gaillard, personal communication).

(5) Increased calcification in metaphyseal regions of long bones and vertebrae have been observed in rats chronically treated with calcitonin (Foster, Doyle and MacIntyre, unpublished).

A direct effect on bone is further supported by the studies of Martin (personal communication) who has shown a decrease in hydroxyproline excretion in rats infused with calcitonin and the calcium-45 kinetic experiments of Milhaud, Perault and Moukhtar (1965) which suggest that the hormone inhibits bone resorption.

In addition to its effect on calcium calcitonin also causes hypophosphatemia. Hirsch, Voelkel and Munson (1964) and Kenny and Heiskell (1965) have shown that falls in plasma phosphate as well as calcium occur in the rat in response to the hormone. The ratio

of the percent fall in phosphorus to the percent fall in calcium following administration is 1.6 (Gudmundsson, MacIntyre and Soliman, in press). Since a similar ratio of these elements is found in ossified tissue phosphate most probably is deposited in the bone together with calcium.

Effect on kidney.

Under certain conditions calcitonin may promote renal excretion of phosphate. Phosphaturia has been reported in intact rats given crude thyroid extracts (Kenny and Heiskell, 1965). However, this could not be interpreted as due to calcitonin since PTH is secreted in response to the induced hypocalcemia. Recently Robinson, Soliman, Martin and MacIntyre (personal communication) have investigated the effect of the hormone in parathyroidectomized rats. They have found calcitonin caused an immediate phosphaturia when infused intravenously.

Mode of action.

The mode of action has not been fully delineated. Clearly calcitonin does not act by simple inhibition of PTH. Both Soliman (Gudmundsson, MacIntyre and Soliman, in press) and Aliapoulis and Munson (1965) have demonstrated that the presence of the parathyroids is not essential for calcitonin to act. This has been confirmed by Welt et al (Gitelman, Kublj and Welt, 1965) and Tashjian (1965). The

hormone remained effective in animals after overdosage with vitamin D or during magnesium deficiency. The calcium-lowering effect was shown not to be dependent on vitamin D since hypocalcemia could be induced in rachitic rats. The possibility that the hormone might affect geneotropic control of messenger RNA production has been investigated by Soliman (Gudmundsson, MacIntyre and Soliman, in press) and Tashjian (1965). Both investigators found that actinomycin D injected prior to calcitonin did not block its action and suggest that concurrent synthesis of messenger RNA is not necessary for its effect.

Relation to Other Endocrine Systems

Parathyroids

Gittes and his co-workers (Gittes and Irvin, 1965; Gittes and Wells, 1965) have attempted to reconcile former disagreements over the origin of the calcium-lowering hormone. They have suggested that a substance from the parathyroids is at least partially responsible for the release of calcitonin from the thyroid. In support of their argument they have found the presence of the parathyroids in rats provides partial protection against hypercalcemia induced by exogenous PTH. In the presence of the thyroid and parathyroids ~~on thyroid alone~~ less rise in serum calcium was noted following PTH administration than in the absence of both glands.

With the parathyroids present alone, an intermediate response was seen.

New evidence has cast some doubt on the validity of these experiments. The studies of Gittes and Irvin have been repeated in intact and parathyroidectomized rats maintained on a high calcium low phosphate diet. The animals were challenged with intraperitoneal doses of calcium, instead of PTH (Foster, unpublished). The rise in plasma calcium was the same in both groups. These results clearly do not support a calcitonin releasing factor from the parathyroids. The difference observed by Gittes can only be explained by hypersensitivity of the parathyroidectomized animal to PTH.

Pituitary.

No relation of calcitonin secretion to pituitary function has yet been found. Studies with thyroid extracts suggest that calcitonin is not under pituitary control. Extracts prepared from thyroids of hypophysectomized rats have been found as effective in lowering the serum calcium in assay animals as thyroid extracts prepared from control animals (Milhaud and Moukhtar, 1965). Conversely, thyroid glands of rats treated with bovine TSH (thyroid stimulating hormone) were not noted to have increased calcitonin activity (Aliapoulis and Munson, 1965). In addition, the hormone is equally effective in lowering the plasma calcium in hypophysectomized rats as in intact controls (Baghdiantz, Blanquet, Croizet, Moura and Tayeau, 1965).

Studies in Man

Calcitonin has been shown to be present in human thyroid. Aliapoulis and Munson (1965) have reported that hypocalcemia activity has been extracted from thyroids obtained from six patients at operation as assayed in the rat. Similar studies carried out by Foster (unpublished) and Smith, Laljee and Dorrington (personal communication) have not confirmed the rat as a suitable assay animal. Smith and his colleagues have shown calcium-lowering activity in human thyroid extracts injected in the mouse. Maximum yields of calcitonin activity were obtained in extracts of fresh thyroid tissue; autopsy material showed decreasing levels of activity proportional to the interval between death and acetone drying. Marked calcium-lowering activity in both fresh and autopsy material has now been demonstrated using the monkey as an assay animal (Foster, Parsons and MacIntyre, unpublished; Milhaud, Moukhtar and Perault, 1965; Aliapoulis and Munson, 1965). These findings appear to confirm that species differences may be important in the response to the hormone (Tashjian and Munson, 1965)

Recently clinical studies have been carried out to assess the effect of animal calcitonin in man (Foster, Joplin, MacIntyre, Melvin and Slack, 1966). Highly purified preparations of pig calcitonin were given to three patients with hypercalcemia secondary to malignant disease. Serum calcium fell 0.6 - 0.8 m.Equiv./l and relative hypocalcemia persisted for 6 - 12 hr. These studies suggest that

calcitonin may be important in normal human physiology and that the hormone may have therapeutic potentialities.

Now that calcitonin has been successfully labelled with iodine-131 (Foster, Antcliff, DeBats and Wright, unpublished) it is hoped that a radio-immunoassay may be developed to assess the levels of the hormone in man.

Chapter X

The Significance of Calcitonin

Summary

Calcitonin has probably played an important role in evolutionary history. Undoubtedly it is involved in the process of bone remodelling and in the storage of phosphate reserves. Since the hormone is present in man, human physiology with respect to calcium metabolism must be revised. Five major pathological conditions require reexamination: thyroid disease, urinary tract lithiasis, cancer, cardiovascular disease and bone disease. In addition, calcitonin may prove of value in the prevention of the hypercalcemia of space travel.

Some basic considerations of the dynamics of calcium metabolism were reviewed in Part I of this thesis. Until recently it was generally accepted that endocrine control of calcium metabolism was mediated through changing rates of secretion of the parathyroid calcium-mobilizing hormone. It is now becoming increasingly evident that former concepts of calcium and phosphate metabolism must be revised and modified to take account of the physiological effects of calcitonin. This chapter is intended to review a few of these areas which require reexamination.

Evolutionary Importance of Bone and Calcitonin

Primitive forms of marine life survived relative to their ability to manipulate the environment to their advantage. A controlling mechanism to deposit calcium and phosphate would have served great value. The early development of calcified dermal armour for some marine species provided a form of protection but at the expense of restricting movement. Far more important was evolution of bone. The osseous skeleton provided for muscle attachment allowing rapid locomotion for larger forms of life. This facilitated movement to escape predators and enabled food-gathering over a greater area. Additionally, the evolution of the rib-cage made possible the intake and expulsion of water. This allowed the continuous ingestion of food and also supplied large volumes of water to aerate the blood supply via the gill apparatus. With the evolution of terrestial forms of life, the rib-cage and its attendant musculature was adapted to expanding the thoracic cage and lung to facilitate continuous respiratory exchange of oxygen and carbon dioxide.

Not only has bone served a structural function, but it has provided a store of much needed phosphate for marine life. The sea is normally low in phosphate (Berrill, 1955; Pautard, 1961; Urist, 1964). Its concentration is dependent upon the distribution of

plankton (Harvey, 1945) Since plankton growth is influenced by seasonal variations in temperature, winds and ocean currents, the supply of phosphate to marine life is irregular over much of the year. Phosphate is necessary for many high energy compounds. A hormonal mechanism which acts to conserve it in store against periods of deprivation would have great survival value.

Also of great evolutionary significance has been the ability of bone to undergo constant remodelling. This process allows bone growth to accommodate the growth of the rest of the organism. Mention has already been made of the continuous interchange of calcium ions between blood and bone (Part I). The control of remodelling requires exacting regulation. PTH participates by mobilising calcium from bone: calcitonin acts to cause its retention. Both processes are necessary for normal growth. Evidence suggestive of bone growth retardation has been found in growing animals chronically treated with calcitonin (Foster, Doyle and MacIntyre, unpublished). It would appear that interference with normal bone resorption interferes with growth. The entire problem of bone growth and remodelling requires review with kinetic assessment of the relative effects of PTH and calcitonin on bone accretion and resorption.

Need of Revision of Clinical Concepts

Now that calcitonin has been shown to be present in man human physiology with respect to calcium metabolism must be revised. Previous studies of patients with parathyroid abnormalities have failed to account for compensatory changes in the secretion of calcitonin. Hypercalcemic conditions, not due to deficient calcitonin production, stimulate the secretion of the hormone which, in turn, acts to reduce the calcemia and promote hypophosphatemia. Conversely, hypocalcemia decreases calcitonin production.

Likewise, former studies of phosphate metabolism also will have to be reevaluated. The reciprocal relationship of calcium and phosphate in plasma can no longer be attributed to simple changes in the equilibrium state with the exchangeable hydroxyapatite of bone. It was previously thought that a single chemico-physical relationship existed. A more likely alternative would appear to be that an increase in plasma calcium stimulates calcitonin secretion which produces the resulting hypophosphatemia. Similarly, hyperphosphatemia with low plasma calcium can be explained by a diminution in calcitonin secretion.

Former studies relative to parathyroid function must be reviewed for yet another reason. Many reports in the past have failed to differentiate parathyroid deficiency from thyro-parathyroid deficiency. In animal investigative work the effects of acute para-

thyroidectomy were frequently studied in animals which were also thyroidectomized. Because of the intimate relationship of both glands the thyroids were excised to ensure complete removal of the parathyroids. Since the iodothyronine thyroid hormones are relatively slow-acting no marked deficiency of these would be encountered in acute experiments. Complete lack of calcitonin, however, would result. Similarly studies in hypoparathyroid patients were often studied under inappropriate conditions. Most of the cases which have been reported were patients whose hypoparathyroidism had resulted from surgical removal of the thyroids. These patients were mostly maintained on oral thyroid replacement therapy. They were considered euthyroid on the basis of previously recognized tests for thyroid function. Since calcitonin is a peptide and is readily inactivated by gastric peptidases, they were, in fact, deficient in this hormone.

Among the many pathological conditions which now require reinvestigation, five general areas are of interest: thyroid disease, urinary tract lithiasis, cancer, cardiovascular disease and bone disease.

Patients with hyperthyroidism frequently have been reported to have hypercalcuria and hyperphosphaturia. If increased calcitonin secretion can be shown, the hyperphosphaturia may be attributed to

a direct effect. Hypercalcuria could be explained by a compensatory increased secretion of PTH. Less commonly, thyroid hypofunction is associated with hypercalcemia (Lowe, Bird and Thomas, 1961). This condition could be accounted for by a deficiency of calcitonin allowing PTH to act unopposed. The problem of non-toxic goitre is more complex. The common occurrence of thyroid nodules with parathyroid hyperfunction has long been known. Further studies may some day be able to show either that increased function of the parathyroids stimulates compensatory changes in the thyroid or the converse.

The possible relation of calcitonin to kidney stones is most intriguing. Hypercalcemia predisposes to stone formation. Approximately 4 percent of all stone formers are found to have parathyroid adenoma. Now that hyperparathyroidism is more diligently looked for, it is becoming increasingly apparent that such patients do not always have persistently elevated blood calciums. One might suggest that in cases of calcitonin deficiency the calcium homeostatic mechanisms would be less precise than normal. Without the rapid regulating effect of the thyroid hormone greater oscillations in plasma could be anticipated which might predispose to stone formation.

Calcitonin has already been shown to be effective in the treatment of hypercalcemia secondary to disseminated cancer. Hypercalcemia

associated with malignancy is caused by either increased local resorption of bone from skeletal lesions or by increased generalized bone resorption caused by secretion by the tumour of a PTH-like substance. The latter mechanism is rarely encountered. Calcitonin appears to act by preventing resorption. Should it be shown to have a direct effect locally on metastatic sites, it may prove of value in the control of metastatic bone disease.

The effect of the hormone on cardiovascular disease is unknown. Calcium is certainly involved in atherosclerosis. Calcification of atherosclerotic lesions predisposes to rupture of plaques and coronary occlusion. Bajusz and his coworkers (Bajusz, Jasmin and Mongeau, 1963) have shown calcification in damaged myocardium occurs more readily in parathyroidectomized animals. Calcification is prevented when the thyroid is also removed. These investigators have proposed that a substance from the thyroid is responsible for myocardial calcification. If calcitonin is the substance, isotopic ablation of the thyroid with subsequent thyroxine replacement might be of benefit to patients with massive myocardial infarcts, advanced atherosclerosis or vascular aneurysms. Calcitonin may also be of therapeutic use in the treatment of digitalis intoxication. Elevated plasma calcium levels predispose to toxicity. Calcitonin, by lowering plasma calcium, might rapidly prevent symptoms.

Finally, calcitonin is of obvious importance to bone disease. Diminished secretion may be responsible for osteoporosis. Since there are approximately 45 million post-menapausal cases with osteoporosis alone, calcitonin may be of widespread medical interest. The hormone could also be of value in space travel. Recent reports suggest that prolonged weightlessness results in hypercalcemia and loss of bone tissue. These changes might be prevented by administration of the hormone.

Undoubtedly calcitonin may find applications in other pathological conditions. This chapter is intended only to suggest promising areas for investigation. Regardless of further findings the physiological importance of calcitonin is now firmly established.

Chapter XI

Summary of Thesis

A review of the general aspects of calcium metabolism is presented in Part I of this thesis. The survey includes not only a discussion of the physiological and biochemical parameters which affect calcium absorption and excretion, internal calcium dynamics within the body, and the structure of bone, but also an historical review of parathyroid hormone research with special reference to assay systems, purification methods, studies of properties, and techniques used in investigating mechanisms of action. The main corpus of the thesis, contained in Part II, encompasses investigations done on the second major hormone controlling calcium homeostasis, calcitonin.

In 1962 a second parathyroid hormone was proposed with antagonistic effects to classical calcium mobilizing parathyroid hormone. This hormone remained unconfirmed until the present studies were undertaken. A technique was devised which enabled perfusion of the thyroid and parathyroid glands of animals with blood either high or low in calcium. High calcium perfusion of the thyroid and parathyroids together produced a fall in systemic plasma clacium in dogs.

Having confirmed the existence of a fast-acting, short-lived hormone secreted in response to a high calcium stimulus, experiments were carried out which suggested a thyroid rather than a parathyroid origin. Hypocalcemia was shown to occur following cauterization of the rat's thyroid. Plasma calcium-lowering activity was extracted from dog thyroid. Finally, high calcium perfusion of the dog thyroid alone after parathyroidectomy was shown to produce hypocalcemia. To resolve the question, studies were done in goats which showed that no fall in systemic plasma calcium occurred during hypercalcemic perfusion of the external parathyroid: a fall was found if the thyroid was included in the perfusion. Histochemical studies done on perfused dog thyroid showed changes in enzyme levels in the "mitochondrion-rich" cells following high calcium perfusion which suggested these cells secreted calcitonin.

The plasma calcium-lowering principle was extracted with hydrochloric acid from pork thyroid glands. Two bioassay techniques were developed. The fall in plasma calcium, following either a 1 hr. infusion of extract or 50 min. after a single intravenous dose, was measured in fasted rats and a dose response curve devised on the basis of an arbitrary unit. These assay systems facilitated further purification.

The crude acid extract was shown to be non-dialyzable and stable at pH 4.6 or below. Most of the inactive material could be precipitated with 1M-sodium chloride. These observations were made use of to produce a six fold purification. Crude extract was dialyzed against buffer at pH 4.6, adjusted to 1M-sodium chloride and the activity in the resulting supernatant precipitated with 2M-sodium chloride. The salt precipitated material was relatively stable to boiling at pH 4.6, but not in the presence of excess acid or alkali. Its peptide nature was demonstrated by inactivation by pepsin or trypsin. Salt precipitated calcitonin resisted inactivation by hydrogen peroxide oxidation and cysteine reduction, but lost activity when oxidized with performic acid. Incubation with 8M-urea failed to produce a demonstrable irreversible change.

A further 20-fold purification was achieved with gel filtration of the salt precipitated material on Sephadex G-100. The recovered activity appeared to penetrate dialysis membrane which previously contained the activity of crude extracts suggesting that the active moiety had become dissociated from larger carrier molecules. The molecular weight was estimated by comparing the elution of purified calcitonin on Sephadex G-100 with elution volumes of other peptides of known molecular weight. The results suggest that the hormone is of polypeptide dimensions.

The thesis concludes with a discussion on current research in progress and the significance of calcitonin.

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